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LOCALIZATION AND REGULATION OF PHOSPHOLIPASE D IN MITOGENIC SIGNALING

by

LIZHONG XU

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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Supervisory Committee

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Abstract

LOCALIZATION AND REGULATION OF PHOSPHOLIPASE D IN MITOGENIC SIGNALING

by

Lizhong Xu

Advisor: Professor David A. Foster

Phospholipase D (PLD), a cell membrane enzyme that catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid and choline, is activated by agonists that initiate cell signaling and by oncogenes that transform cells. But the mechanism is not clearly defined and the membrane localization of the mitogenic PLD is still debatable.

We found that the elevated PLD activity in NIH 3T3 cells transformed by activated oncogenic forms of Src, Ras, and Raf or in cells stimulated by epidermal growth factor (EGF) is largely restricted to the caveolin-enriched membrane microdomains (CEMMs). The apparent PLD substrate specificity in those cells for PC lacking polyunsaturated acyl groups is explained by the localization of PLD activity in the CEMMs where PC labeled with polyunsaturated fatty acids was excluded. Although both PLD1 and PLD2 were found in CEMMs, neither was particularly enriched in the CEMMs of the transformed relative to the parental cells. We suggest that PLD1 and PLD2 may work together in response to mitogenic signals initiated in the CEMMs where many signaling molecules co-localize.

We also found that PLD activity is elevated by the oncogenic H-Ras, but not K-Ras. Consistent with the previous report that Ral is required for PLD activity in H-Ras-transformed cells, we found that RalA was activated in H-Ras- but not in K-Ras-

transformed cells. In cells transformed by H-Ras, increased levels of ARF6 interacted with RalA. ARF6 but not ARF1 co-localized with RalA in CEMMs. Interestingly, ARF6 protein levels were elevated in H-Ras- but not K-Ras-transformed cells. A dominant-negative mutant of ARF6 inhibited mitogenic PLD activity. Activated mutants of either ARF6 or RalA were not sufficient to elevate PLD activity; however, expression of both activated RalA and activated ARF6 in NIH3T3 cells led to increased PLD activity. These data suggest a model whereby H-Ras stimulates the activation of both RalA and ARF6, which together lead to the elevation of PLD activity.

This work has underscored the mechanism of the regulation of PLD in the Ras pathway. New models of the dynamic regulation of PLD in signaling cascades and the localization of Ras and PLD on the membrane are provided.

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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
CRI~CRIV, conserved regions I~IV
DAG, diacylglycerol
DIGs, detergent-insoluble glycolipid-enriched complexes
DRMs, detergent-resistant membranes
EGF, epidermal growth factor
EGFR, epidermal growth factor receptor
ER, endoplasmic reticulum
fMLP, f-Met-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine
G protein(s), GTP binding protein(s)
GAP, GTPase-activating proteins
GDS, GDP dissociation stimulator
GEFs, guanine nucleotide exchange factors
GPCR, G protein-coupled receptors
GST, glutathione S-transferase
GTPγS, guanosine 5'-O-(3-thiotriphosphate)
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
PH, pleckstrin homology
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
PMA, phorbol 12-myristate 13-acetate
PS, phosphatidylserine
PTK, protein tyrosine kinase
PX domain, phox homology domain
TLC, thin layer chromatography
TPA, 12-O-tetradecanoylphorbol-13-acetate

Chapter I. View of the Regulation of Phospholipase D

Phospholipase D (PLD), a cell membrane enzyme, appears to be activated by a variety of agonists that initiate cell signaling and by oncogenes that transform cells. The mechanisms by which agonists and oncogenes activate PLD are not clearly defined; the membrane localization of PLD in response of mitogenic stimuli is still debatable. In this chapter I will discuss the regulation of PLD in detail and provide the rationale to study the localization and regulation of PLD mediated by oncogenes or growth factor stimuli.

1.1. Phospholipases

Phospholipases are an important class of lipolytic enzymes that catalyze the hydrolysis of the ester or phosphodiester bonds of glycerophospholipids (Fig. 1.1).

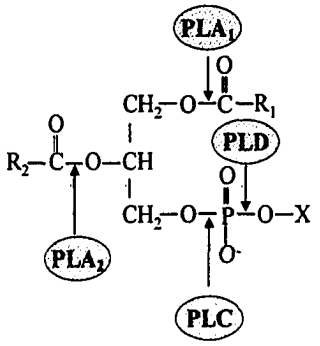


FIG. 1.1. Cleavage sites of phospholipases[1] A_1, A_2, C, and D (PLA_1, PLA_2, PLC, and PLD).
R_1 and R_2, fatty acyl chains; X, a base of head group.

These enzymes play important roles not only in phospholipid catabolism and turnover but also in cell signaling. For example, the enzyme phospholipase A_2 (PLA_2) can hydrolyze arachidonic acid-containing phospholipids to release arachidonic acid for

[1] Phospholipases B are a heterogeneous group of enzymes that share the ability to hydrolyze both sn-1 and sn-2 acyl ester bonds of glycerophospholipids and then display both PLA_1 (EC 3.1.1.32) and PLA_2 (EC 3.1.1.4) and lysophospholipase (EC 3.1.1.5) activity.

synthesis of prostaglandins. PLA_2 can also change phosphatidic acid (PA) to lysoPA, which is now recognized to be an important intercellular signal. Many cells respond to lysoPA through a membrane receptor that is coupled to G proteins (Exton, 1997b; Morris et al., 1997).

The enzyme Phospholipase C (PLC) (EC 3.1.4.3) can catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$, PIP_2] to generate inositol triphosphate (IP_3) and diacylglycerol (DAG) second messengers that control intracellular Ca^{2+} levels and protein kinase C (PKC) activity, respectively (Gomez-Cambronero and Keire, 1998; Morris et al., 1997). DAG can further be converted into PA by means of a DAG kinase (Fig. 1.2A).

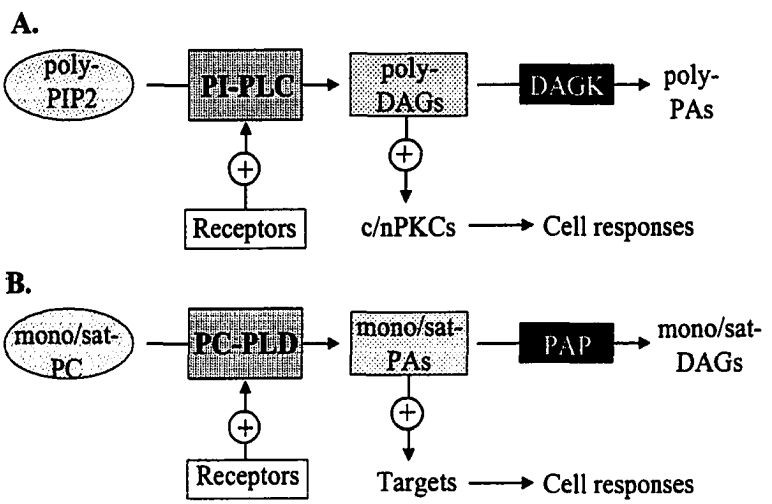


FIG. 1.2. Generation of diacylglycerol and phosphatidate by phospholipases. (A) Receptor-stimulated $PI(4,5)P_2$ hydrolysis by PI-PLC at the plasma membrane. (B) Receptor-stimulated PC hydrolysis by PC-PLD on membrane. It was proposed that polyDAGs and mono/satPAs are intracellular signaling molecules, which often operate in parallel when receptors regulate cell function, and that other DAGs and PAs have no signaling function (Hodgkin et al., 1998). c/nPKCs, conventional/novel protein kinases C; DAGK, DAG kinase; mono, mono-unsaturated; PAP, PA phosphohydrolase; poly, polyunsaturated; sat, saturated.

The enzyme phospholipase D (PLD, EC3.1.4.4) cleaves the distal phosphodiester bond of phospholipids, resulting in the formation of phosphatidic acid (PA) and a related

free base. In addition to the hydrolytic activity, PLD enzymes also catalyze a transphosphatidylation reaction in which the phosphatidyl group of the phospholipid is transferred to glycerol or a primary alcohol where glycerol or the primary alcohol acts as a nucleophilic acceptor instead of H_2O (Exton, 1997a; 1997b; Geng et al., 1998). PA-phosphohydrolase (PAP), or PLA_2, subsequently converts the main product of PLD reaction, PA, into DAG or lysoPA, respectively (Gomez-Cambronero and Keire, 1998; *Hodgkin et al., 1998) (Fig. 1.3).*

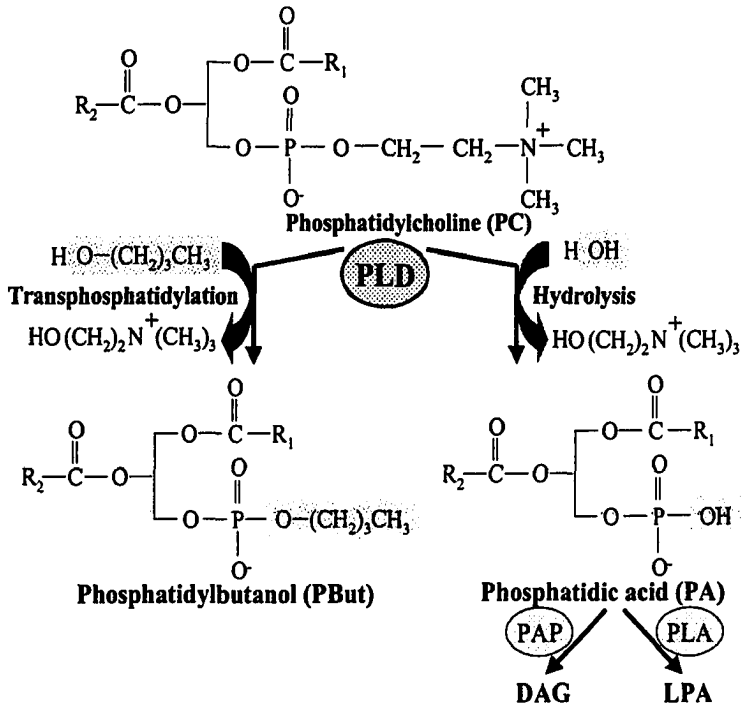


FIG. 1.3. PLD-catalyzed hydrolysis and transphosphatidylation reactions. PLD hydrolyzes the distal phosphodiester bond in phospholipids such as PC, using H_2O as an electron donor to generate PA and choline. PA can be subsequently converted into DAG or lysoPA (LPA) by PA phosphohydrolase (PAP) or PLA_2, respectively. In the presence of short-chain primary alcohols such as 1-butanol, PLD can catalyze the transphosphatidylation reaction to generate phosphatidylalcohol such as phosphatidylbutanol instead of PA.

The preferred substrates 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). PIP_2, a main substrate of PLC, is present at a very low concentration (<0.1% of total phospholipids) (Exton, 1997b). While the preferred substrate of PLD is phosphatidylcholine (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 acid-containing DAGs generated by PLC and monounsaturated/saturated fatty acid-containing PAs generated by PLD are intracellular signaling molecules (reviewed in Hodgkin et al., 1998) (Fig. 1.2).

1.2. Mammalian PLD isoforms and domain structure

PLD enzymes have been found in a wide variety of organisms. In addition to cloned phosphatidylcholine (PC)-specific PLD genes from bacteria (Hodgson et al., 1990), yeasts (Rose et al., 1995), and plants (Wang et al., 1994), two distinct mammalian PLDs have been cloned from human (Hammond et al., 1995; Lopez et al., 1998; Steed et al., 1998), mouse (Colley et al., 1997a; 1997b), and rat (Katayama et al., 1998; Kodaki and Yamashita, 1997; Park et al., 1997) species (see Table 1.1), with both having splice variants.

Table 1.1. A record of cloning mammalian PLD

Year*	Species	Isoforms	Authors
1995	Human	***PLD1***	Hammond *et al.*
1997	Mouse Rat	***PLD2***	Colley *et al.* (b) Kodaki and Yamashita; Park *et al.*
1997 1998	Mouse Rat	***PLD1***	Colley *et al.* (a) Katayama *et al.*
1998	Human	***PLD2***	Lopez *et al.* Steed *et al.*

* The indicated year is the year in which the paper was published.

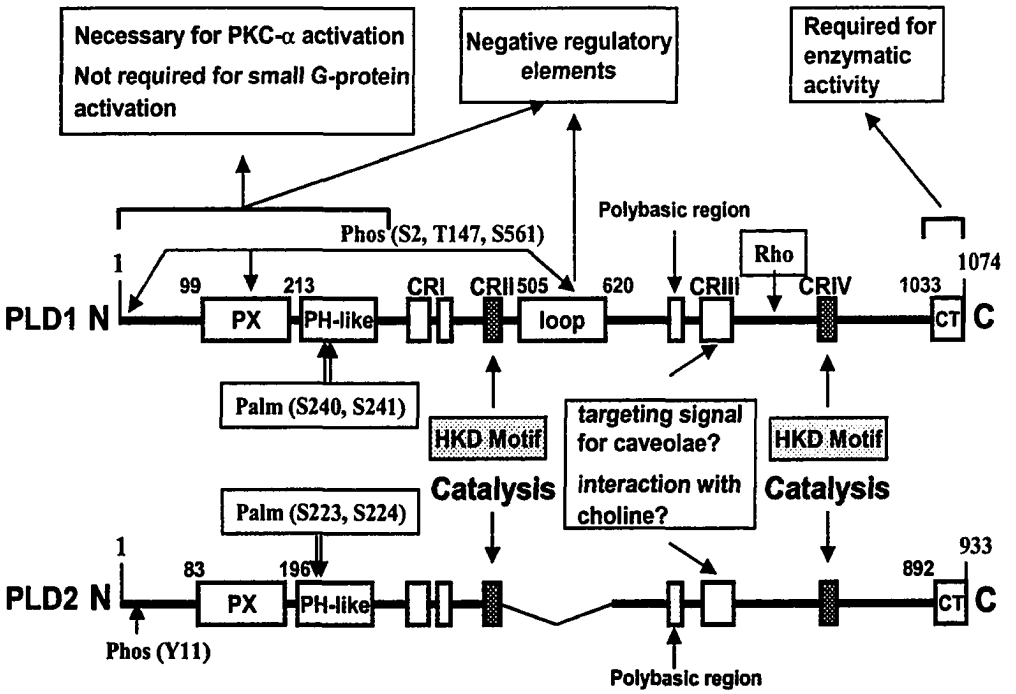


FIG. 1.4. Conserved and unique features for mammalian PLD1 and PLD2. The PLD amino acid sequences encode regions that are either unique to PLD1 (loop region) or that are conserved among mammalian PLDs and some or all PLDs from non-mammalian species (other boxed regions). Possible functions that have 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 and PLD2 are ~50% identical. 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. 1.4). The N-terminal regions and the "loop" sequence are believed to be negative regulatory elements (see below). 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), and any change of these residues including deletion, mutation, or addition can cause loss of

PLD activity. CRII and CRIV are catalytic domains, or transphosphatidylation domain. 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). The presence of one or more HKD motif defines a PLD superfamily. This includes PLD and non-PLD phosphatidyl-transferases (e.g. cardiolipin synthase and phosphatidylserine synthase), as well as certain poxvirus envelope proteins and several endonucleases (reviewed in Exton, 2002b; Liscovitch et al., 2000). 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).

Both mammalian PLD1 and PLD2 absolutely require of phosphatidylinositol 4,5-bisphosphate (PIP_2) for activity (Brown et al., 1995; Colley et al., 1997b; Hammond et al., 1995). The PIP_2-binding site does not only lie inside the putative weak PH-like domain since deletion of this domain does not change the PIP_2 responsiveness for PLD activity (Sung et al., 1999a; 1999b). Recently, a second PIP_2-binding domain was identified between CRII and CRIII (Sciorra et al., 1999; 2002) (see §1.3.3.5).

The basal activity of PLD1 *in vitro* is low. Protein kinase Cα (PKCα), ADP-ribosylation factor (ARF) family GTP binding proteins (G proteins), and Rho family G proteins (such as RhoA, Rac1 and Cdc42) can activate PLD1 *in vitro* in a synergistic manner (Hammond et al., 1995; 1997; Sung et al., 1999b). Ral also associates with PLD1, but it does not by itself activate PLD1 in the absence of ARF (Luo et al., 1998).

Deletion of the N-terminal regions on PLD1 resulted in increased PLD basal activity; these regions are required for PKCα activation but not for activation by ARF1 or RhoA (Park et al., 1998; Sung et al., 1999b). However, co-immunoprecipitation assay showed that this PLD deletion mutant can still bind to PKCα (Sung et al., 1999b). Co-precipitation assay also showed that a recombinant GST-fused PLD1 peptide (319-582) without N-terminus can also bind PKCα (Min and Exton, 1998). These results suggested that PKC interaction sites are present in both N- and C-terminal sequences of PLD1. Deletion of the "loop" region from PLD1 increases PLD basal activity and insertion of this region into PLD2 decreases PLD activity, suggesting that the "loop" region serves as negative regulatory region (Sung et al., 1999a; 1999b).

PLD2 is constitutively active *in vitro*. The activity is unaffected by G proteins or PKCα (Colley et al., 1997b) and only mildly stimulated by ARF (Lopez et al., 1998). Interestingly, deletion of 308 amino acids from the N-terminus of PLD2 eliminates most of the basal activity but the resulting truncated protein becomes responsive to ARF proteins (Sung et al., 1999a). Although ARF proteins have no impact upon PLD2 activity *in vitro*, ARF proteins have been implicated in regulating PLD2 *in vivo* (Kim et al., 2003; Lopez et al., 1998), suggesting that ARF may regulate both PLD1 and PLD2 in intact cells. In addition, PKCα is constitutively associated with PLD2 (Slaaby et al., 2000). PKCα was also implicated to be involved in the regulation of PLD2 activity, since over-expressed PLD2 in mast cells is also subject to stimulation by PMA and antigen (O'Luanaigh et al., 2002). It has also been reported that PLD2 can be regulated by unsaturated fatty acids, including oleate (18:1), linoleate (18:2), and arachidonate (20:4) (Kim et al., 1999b; Sarri et al., 2003).

Other types of eukaryotic PLD, such as phosphoinositide-specific PLDs, phosphatidylserine (PS)/phosphatidylethanolamine (PE)-hydrolyzing PLD, and Lyso-PLD, etc. are still elusive and not yet cloned (reviewed in Liscovitch et al., 2000). GPI anchor-specific PLD (GPI-PLD) has been cloned but has no relationship with the PC-hydrolyzing PLD genes (Scallon et al., 1991).

The subject of my dissertation focuses on the mammalian PC-hydrolyzing PLD. The regulation of PLD will be discussed in details in the following section and relevant models will be proposed in Chapter V.

1.3. Regulation of PLD

1.3.1. Two ways to regulate PLD: enzyme availability and enzyme activity

In general, PLD, just as other enzymes, can be regulated in two ways: control of enzyme availability and control of enzyme activity. The availability of an enzyme depends on both its rate of synthesis and its rate of degradation in a cell. It is not very clear yet how PLD is regulated in this way. Both PLD1 and PLD2 are expressed to various extents in almost every tissue and cell type studied. Some studies showed that the expression of PLD in mammalian cells changes during differentiation (reviewed in Liscovitch et al., 2000; Meier et al., 1999), neuron development (Peng and Rhodes, 2000; Zhao et al., 1998), or in response to forebrain ischemia (Lee et al., 2000b), cyclic AMP (Yoshimura et al., 1996), ceramide-induced apoptosis (Yoshimura et al., 1997), nerve growth factor (Gibbs and Meier, 2000; Hayakawa et al., 1999), and phorbol ester (Jin et al., 2002). Recent study showed that in primary astrocytes, PLDs are very stable, with the half-life of 2-3 days for PLD2 and 4-6 days for PLD1 (Jin et al., 2002). So far, most

studies have focused on the regulation of PLD enzymatic activity and thus will be discussed here in detail.

1.3.2. Regulation by extracellular agonists and intracellular oncogenes

PLD activation can be initiated in different cell types by a variety of agonists (Exton, 1998; 1999; Gomez-Cambronero and Keire, 1998; Rizzo and Romero, 2002). These agonists can be grouped in the following 3+1 classes: (1) agents acting through protein tyrosine kinase (PTK) receptors (e.g. EGF, b-FGF, insulin, NGF, and PDGF, etc.), (2) agonists acting through PTK-associated receptors[2] that activate non-receptor tyrosine kinases (e.g. prolactin, growth hormone, antigens, etc.), and (3) ligands acting through trimeric G protein-coupled receptors (GPCR) (e.g. acetylcholine, angiotensin, bradykinin, carbachol, bombesin, chemostactic peptides [such as f-Met-Leu-Phe (fMLP)], interleukin 8, endothelin, epinephrine, norepinephrine, glutamate, lysoPA, sphingosine 1-phosphate, and vasopressin, etc.). In addition, other agents (+1), such as Ca^{2+} ionophores, phorbol esters [e.g. phorbol 12-myristate 13-acetate, 12-O-tetradecanoylphorbol-13-acetate (PMA, TPA)] and H_2O_2 are also potent stimulants of PLD.

PLD activation is also elevated in response to the oncogenes v-Src (Song et al., 1991), v-Ras (Carnero et al., 1994; Jiang et al., 1995a; 1995b), v-Fps (Jiang et al., 1994), and v-Raf (Frankel et al., 1999). It is intriguing that the oncogene-regulated PLD activity is transformation-independent, since the temperature-sensitive v-Src and inducible v-Raf

---

[2] PTK-associated receptors lack a catalytic domain, but work through associated PTK, such as Src or Janus families of nonreceptor PTKs. Prolactin, growth hormone, some antigens that bind to immuno-receptors, and some growth factors may activate PLD through these pathways.

activate PLD very quickly after the oncoproteins are induced (Frankel et al., 1999; Song and Foster, 1993). PLD, however, is critical for the transformed phenotype induced by v-Src and v-Ras (Aguirre-Ghiso et al., 1999; Urano et al., 1996) as well as protein tyrosine kinases c-Src and EGF receptor (Joseph et al., 2001; Lu et al., 2000).

Although the linkage between the stimulation of PLD and the extracellular signals has been studied for more than a decade, the mechanism is still not completely defined yet. The study of the regulation of PLD by oncogenes, however, has helped to uncover the veil of the mechanism (see Chapter V). As discussed in this Chapter, many studies demonstrated that several classes of proteins are involved, such as PKC, G proteins Ral, ARF, and Rho. Interestingly, as discussed in Chapter V, these proteins are downstream effectors of the PLC/PKC and Ras/phosphatidylinositol 3-kinase (PI3-Kinase, PI3K) pathways, by which the signals of those agonists are relayed. In fact, the activation of PLD by H-Ras through ARF6 and Ral is one of the main subjects in this dissertation (see Chapter IV). In Chapter V, I will discuss the possible mechanism of how the agonists or oncoproteins, via the PLC/PKC and Ras/PI3-Kinase pathways, activate PKC, Ral, ARF, and Rho, and subsequently activate PLD. In the rest of this section, I will discuss the regulation of PLD through these proteins, as well as by inositol phospholipid, post-translational modification, and other factors, such as cytoskeleton proteins and caveolin.

1.3.3. Regulation by interaction with proteins or lipid: PKC, Ral, ARF, Rho, and PIP_2

The interaction of PLD with proteins such as PKC, Ral, ARF, and Rho, or with lipids such as PIP_2 can regulate PLD activity (reviewed by Cockcroft, 2001; Exton, 1999;

2002a; 2002b; Powner and Wakelam, 2002; Rizzo and Romero, 2002). The recent important progress on these factors regulating PLD activity will be summarized below.

1.3.3.1. 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; 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 fourth subfamily member of PKCs or a distinct family called protein kinase D (PKD), which does not respond to either DAG or calcium but require PS as a cofactor (reviewed by Toker, 1998).

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, 2002b), and I will discuss it later. PKCs in the novel family, such as PKCδ and PKCε, have been shown to play an inhibitory role in the regulation of PLD activity. For example, downregulation of PKC isoforms by TPA increases PLD activity in c-Src-transfected cells, and inhibition of PKCδ by rottlerin or overexpression of dominant-negative PKCδ increases the PLD activity as high as EGF does (Hornia et al., 1999). Overexpression of PKCε also inhibits

PMA- and PDGF-induced PLD activity (Kiss et al., 1999). On the other hand, there were also some controversial reports about PKCδ in the regulation of PLD. For example, PKCδ has been shown to activate 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). In addition, the same group that reported the activation of PLD by PKCδ also reported that the dominant-negative PKCδ potentiates both basal and EGF-induced PLD activity (Han et al., 2002b). Recently, PKC-ι in the atypical subfamily has also been shown to activate PLD2 (Mwanjewe et al., 2001), and it was suggested that PKCι is downstream of PLD1, but upstream of PLD2. In support of these results, PKCζ, with close sequence homology to PKCι, is downstream of PLD (Guizzetti and Costa, 2000), and PA was shown to activate PKCζ *in vitro* (Limatola et al., 1994; 1997). Another group also showed that primary butanol or PLD1 antisense oligonucleotides inhibited translocation of PKCδ, -ε, and -ζ, but had no effect on the upstream association of PKCα or ARF6 with PLD1 (Melendez et al., 2001). Interesting, it has also been shown that PLCζ mediates norepinephrine-induced PLD activation and PLCζ appears to be upstream of PLD activity (Parmentier et al., 2003).

PKCα is involved in mediating PLD activation by the 3+1 classes of agonists as mentioned in §1.3.2. These include: (1) EGF (Hornia et al., 1999; Yeo and Exton, 1995), b-FGF (Yeo and Exton, 1995), PDGF (del Peso et al., 1997; Eldar et al., 1993; Yeo and Exton, 1995; Yeo et al., 1994), and insulin (Slaaby et al., 2000); (2) the antigen that cross-links IgE receptors (FcεRI) (Powner et al., 2002) or IgG receptors (FcγRI) (Melendez et al., 2001); (3) bombesin (Yeo and Exton, 1995), thrombin (Pachter et al.,

1992), and carbachol (Boyano-Adanez et al., 1997; Zhang et al., 1999); and (+1) phorbol ester (Balboa et al., 1994; Kotter et al., 2000; Zhang et al., 1999) and H_2O_2 (Lee et al., 2000a; Min et al., 2001; 1998a).

Although PKCα is constitutively associated with PLD2 (Slaaby et al., 2000), 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 (Conricode et al., 1992; 1994; Hammond et al., 1997; Melendez et al., 2001; Min et al., 1998b; Sciorra et al., 2001; Singer et al., 1996), since ATP or kinase activity is not required; 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; 1999c). 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-β-cyclodextrin dramatically reduces the phosphorylation of PLD in the light membranes, but does not affect the PLD1 activity (Kim et al., 2000). It appeared that PKC has dual roles to regulate PLD1: through interaction and by phosphorylation (Oka et al., 2002). Recent report showed that both the regulatory and catalytic domains of PKCα are required in 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.

1.3.3.2. Regulation by Ral

Ral, ARF and Rho are all members of small G proteins in the Ras superfamily (reviewed in Takai et al., 2001). Small G proteins are referred to monomeric GTP-binding proteins with molecular masses of 20-40 kDa. Small G proteins have two interconvertible forms: GDP-bound inactive and GTP bound active forms. G proteins become activated upon interaction with specific guanine nucleotide exchange factors (GEFs), which promote GDP release from the G proteins, allowing GTP to bind the empty nucleotide binding sites. The conformational change upon GTP-binding allows the G proteins to interact with the downstream effector(s). The GTP-bound form of G proteins is inactivated by GTPase-activating proteins (GAP), which activate their intrinsic GTPase activity to hydrolyze GTP to GDP, and then release the bound downstream effectors.

The Ral gene, a Ras related gene was isolated by the use of a synthetic probe and named Ral for Ras-like (Chardin and Tavitian, 1986). Two Ral genes, RalA and RalB, have been identified and the expressed proteins are 85% identical. Ral may be activated by Ral-specific guanine nucleotide exchange factors (Ral-GEFs), including Ral-GDP dissociation stimulator (Ral-GDS[3]) and two putative Ral-GEFs—RGL/RSB2 and RGL2/RLF for Ral-GDS-like factors (reviewed in Feig et al., 1996). Ral-GDS is one of

[3] In most cases, Ral-GEF is referred to as Ral-GDS, and for clarity I will use Ral-GDS in this dissertation for Ral-GEF.

at least three classes of downstream targets of Ras proteins: Raf-kinase, PI3-Kinase and Ral-GDS. Ral has been implicated in regulation of vesicle trafficking, cytoskeleton organization, gene expression, and cell transformation (reviewed in Takai et al., 2001). Ral proteins become activated in response to ligands such as EGF, which can activate Ras and as a consequence, they mediate as least some of the cellular actions of Ras (Wolthuis et al., 1998b). Ral can influence a variety of cellular processes by interacting with different downstream targets, such as PLD, Ral-BP1, filamin, an actin cross-linking protein (Takai et al., 2001), and the exocyst complex (Brymora et al., 2001; Moskalenko et al., 2002; Mott et al., 2003; Novick and Guo, 2002; Polzin et al., 2002).

Foster and colleagues first reported the regulation of PLD through Ral (Jiang et al., 1995b). It was demonstrated that the activation of PLD by v-Src is mediated by Ras (Jiang et al., 1995a), and Ral is linked to the Src signaling pathway through the activation of Ral-GDS by Ras (Jiang et al., 1995b). RalA directly associates with PLD1 in GTP-independent manner (Jiang et al., 1995b; Luo et al., 1998). Even though the Ras-mediated activation of PLD in v-Src-transformed cells can result in the stimulation of Ral-GDS and RalA, activated RalA itself does not stimulate PLD activity (Jiang et al., 1995b; Luo et al., 1998) in the absence of additional factors such as ARF, PKCα, or Rho family GTPase. However, RalA is required for the activation of PLD in v-Ras, v-Src (Jiang et al., 1995b; Luo et al., 1998), and v-Raf transformed cells (Frankel et al., 1999). The PLD1 precipitated with the GST-RalA fusion protein from v-Src and v-Ras transformed cell lysates is active and contains ARF proteins (Luo et al., 1998; 1997). Thus, the activation of PLD by Ras is through another mechanism, probably through ARF or another regulator(s) (Jiang et al., 1995b; Luo et al., 1998). Therefore, it was

proposed that RalA/PLD1/ARF form a complex and RalA regulates PLD activity by localization of PLD to membrane (Luo et al., 1998) (Fig. 1.5). Another group also reported that both RalA and ARF stimulate PLD activity *in vitro* by direct interaction with PLD1 that was immunopurified from bovine brain (Kim et al., 1998).

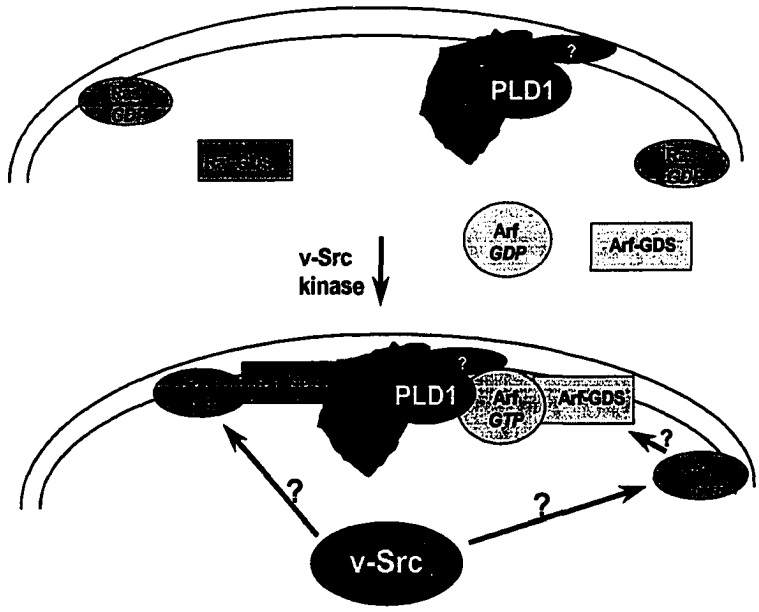


FIG. 1.5. Model of the activation of PLD by v-Src via Ras, RalA, and Arf. It was proposed that in response to the tyrosine kinase activity of v-Src, Ras goes from the inactive GDP form (Ras-GDP) to the active GTP form (Ras-GTP). The activated Ras associates with Ral-GDS, resulting in the recruitment of RalA-PLD1 complex and GDP-GTP exchange on RalA. However, because activated RalA is not sufficient to activate PLD1, whereas activated Ras is sufficient for PLD1 activation, it was proposed that Ras stimulates GDP-GTP exchange on ARF via another mechanism involving an Arf-GDS, leading to an association between RalA and Arf that is dependent upon the RalA amino terminus. An as yet unknown factor was postulated to facilitate the interaction between RalA and activated Arf-GTP. Adapted from (Luo et al., 1998).

Ral is not only involved in the activation of PLD in cells transformed by v-Src (Jiang et al., 1995b), v-Ras (Luo et al., 1998), and v-Raf (Frankel et al., 1999), but also in response to the agonists, including (1) EGF (Lu et al., 2000; Voβ et al., 1999) and insulin (Voβ et al., 1999), (2) growth hormone (Zhu et al., 2002), and (+1) phorbol ester (Schmidt et al., 1998; Voβ et al., 1999). In all these cases, the Ras pathway has been

implicated to activate Ral. How Ras activates PLD through the Ral pathway would be still unclear without further knowledge of ARF.

1.3.3.3. Regulation by ARF

ARF proteins are monomeric G proteins of the Ras superfamily. ARF was originally identified as a protein cofactor required for the efficient modification of the α-subunit of Gs, the heterotrimeric G protein that stimulates adenylate cyclase by cholera toxin (Schleifer et al., 1982). ARF was later shown to be involved in protein trafficking in the Golgi apparatus (Moss and Vaughan, 1993). The activation of PLD by ARF was first recognized by the groups of Sternweis and Cockcroft (Brown et al., 1993; Cockcroft et al., 1994). 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).

In vivo, ARF proteins have been shown in mediating PLD activation by a number of agonists. These include: (1) insulin (Karnam et al., 1997; Shome et al., 1997), EGF (Kim et al., 2003), and PDGF (Shome et al., 1998); (2) agents cross-linking of the IgE receptors (FcεRI) (Powner et al., 2002; Way et al., 2000) or IgG receptors (FcγRI) (Melendez et al., 2001); (3) angiotensin II (Shome et al., 2000), endothelin-1 (Shome et al., 2000), fMLP (Fensome et al., 1998; Houle et al., 1995; Kaldi et al., 2002), ligands binding VPAC receptors (McCulloch et al., 2001), and agents stimulating m3 muscarinic receptors (Rumenapp et al., 1995); and (+1) phorbol ester (Kotter et al., 2000; Shome et al., 1998) and NaF (Bourgoin et al., 1996).

PLD is stimulated by ARF 1, 3, 5, and 6 *in vitro*, and the myristoylated ARFs are more effective than the non-myristoylated forms (Brown et al., 1995; Massenburg et al., 1994). Whereas all ARF family proteins seem to be able to activate PLD *in vitro* (Colley

et al., 1997a; Hammond et al., 1997; Massenburg et al., 1994; Sung et al., 1999b), little is known about the members in class II of the ARF family, although ARF4 was recently implicated in the EGFR-dependent PLD2 activation (Kim et al., 2003). Class I ARFs have been implicated primarily in vesicle transport in the endoplasmic reticulum (ER) and Golgi (Moss and Vaughan, 1998). Although many studies showed that PLD1 can be activated by ARF1 *in vitro*, there is less information about the role of ARF1 in activation of PLD1 *in vivo*. Class III (ARF6) has been implicated in receptor endocytosis (D'Souza-Schorey et al., 1995), vesicle recycling at the plasma membrane (D'Souza-Schorey et al., 1998), exocytosis (Caumont et al., 1998; 2000; Chavrier and Goud, 1999; Jones et al., 1999b; Vitale et al., 2002), and regulation of actin cytoskeleton (Boshans et al., 2000; D'Souza-Schorey et al., 1997; Franco et al., 1999; Frank et al., 1998; Radhakrishna et al., 1999; Radhakrishna and Donaldson, 1997; Song et al., 1998, and reviewed in Stamnes, 2002; Turner and Brown, 2001). PLD activity, similarly, has been implicated in receptor endocytosis (Shen et al., 2001), exocytosis (Caumont et al., 1998; Fensome et al., 1996; Humeau et al., 2001; Vitale et al., 2001) and regulation of actin cytoskeleton (Boshans et al., 2000; D'Souza-Schorey et al., 1997; Radhakrishna et al., 1996; Santy and Casanova, 2001; Schafer et al., 2000). PLD1 has also been reported to colocalize 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 FcγRI (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; Jenkins et al., 1994; Moritz et al., 1992; Moss and Vaughan, 1998), which may further activate PLD. Thus, there is a correlation between the function of ARF6 and mitogenic PLD activity. However, it is not clear how

ARF6 is related to PLD activity in signaling cascades. The work in Chapter IV will shed light on the pathway of H-Ras-mediated PLD1 activation by the synergistic action of both RalA and ARF6, and more about regulation of PLD by ARF will be discussed there.

1.3.3.4. Regulation by Rho family G proteins

Rho family G proteins or Rho/Rac/Cdc42 proteins have been shown to regulate both cytoskeletal reorganization and gene expression (see Takai et al., 2001 for a review). Rho family proteins have been shown to mediate PLD activation by the 3+1 classes of agonists. These include (1) insulin (Karnam et al., 1997) and PDGF (Malcolm et al., 1996); (2) the antigen that cross-links IgE receptors (FcεRI) (Ojio et al., 1996; Powner et al., 2002); (3) lysoPA (Malcolm et al., 1996), sphingosine 1-phosphate (Meacci et al., 2002; 2001; 1999), bradykinin (Meacci et al., 1999), endothelin I (Malcolm et al., 1996), carbachol (Zhang et al., 1999), fMLP (Fensome et al., 1998), and glutarmate receptor agonists (Kanumilli et al., 2002); and (+1) phorbol ester (Kotter et al., 2000; Malcolm et al., 1996; Meacci et al., 1999; Ojio et al., 1996) and the Ca^{2+}-mobilizing agent thapsigargin (Cissel et al., 1998).

As discussed above, Rho, PKCα, and ARF activate PLD1 in a synergistic manner *in vitro*. Rho is required for PLD activity in v-Raf transformed cells (Frankel et al., 1999). The interaction site for RhoA on PLD1 has been mapped to the C-terminus (reviewed in Exton, 2002a), but PLD1 activation by Rho is modest when compared with the activation by ARF (Rizzo and Romero, 2002). RhoA can activate PLD directly by binding with PLD1 or indirectly through the activation of phosphatidylinositide 5-kinase to generate PIP_2, a cofactor for both PLD1 and PLD2 activity, or through other undefined pathway(s) (reviewed in Exton, 2002b).

1.3.3.5. Regulation by PIP_2

PIP_2 has been implicated in many cellular functions, such as production of second messengers, membrane targeting, enzyme activation of PLD, cytoskeleton reorganization, and membrane trafficking (see McLaughlin et al., 2002 for a review). As discussed above, PIP_2 is essential for PLD activity. The consensus of PH domain in the N-terminal region was believed to be important for PIP_2 binding, but deletion of this domain does not affect the activation of PLD1 or PLD2 by PIP_2 *in vitro* (Sung et al., 1999a; 1999b), suggesting that this domain is unlikely to be responsible for PIP_2-sensitive catalysis. Mutation studies identified a polybasic region between CRII and CRIII as the second PIP_2 binding motif, responsive for PLD catalytic activation by PIP_2 but not for PLD membrane localization (Sciorra et al., 1999). Although the PH domain is a selective and major PIP_2 binding regulatory domain (Hodgkin et al., 2000), a recent study (Sciorra et al., 2002) showed that this domain has lower affinity but sufficient selectivity, and it functions in concert with the less selective but highly affinitive, polybasic motif to target PLD to PIP_2-rich membranes. Therefore, PIP_2 have a dual role in PLD regulation: membrane targeting mediated by PH domain and stimulation of catalysis mediated by the polybasic motif (Sciorra et al., 2002).

Interestingly, the enzyme PI(4)P5-Kinase, which generates $PI(4,5)P_2$ from PI(4)P, can be activated by PLD's activators, such as ARF (Fensome et al., 1996; Honda et al., 1999; Jones et al., 2000; O'Luanaigh et al., 2002; Skippen et al., 2002; Way et al., 2000) and Rho (Chong et al., 1994; Ren et al., 1996; Tolias et al., 1998; Weernink et al., 2000) as well as PLD's enzymatic product PA (Arneson et al., 1999; Honda et al., 1999; Jenkins et al., 1994; Jones et al., 2000; O'Luanaigh et al., 2002; Siddhanta et al., 2000;

Skippen et al., 2002). This enzyme also binds to both PLD1 and PLD2 (Divecha et al., 2000).

1.3.4. Post-translational regulation of PLD: Phosphorylation and acylation

PLD can be regulated by post-translational modification, such as phosphorylation and fatty acylation.

1.3.4.1. Phosphorylation of PLD

Serine/threonine phosphorylation of PLD

As discussed above, PKCα not only can interact with PLD1 to activate it, but it can also phosphorylate PLD1 at ser/thr residues and inactivate it (Hu and Exton, 2003). However, it is still not clear whether the phosphorylation on these sites may play any other role(s) for PLD regulation.

It was also found that PLD1 is phosphorylated only on threonine but not serine residues in human-airway epithelial cells when stimulated by sphingosine 1-phosphate, and the data suggested a mechanism involving PKCδ and Src (Ghelli et al., 2002). However, the significance of this study is not known.

It was also reported that when the rat pheochromocytoma PC12 cells were treated with PMA or bradykinin, PKCδ was translocated from cytosol to the plasma membrane where PLD2 is localized, and PKCδ then associated and phosphorylated PLD2 (Han et al., 2002a) for activation. However, the same group reported later (Han et al., 2002b) that PKCδ plays a negative role on EGF-induced PLD activity in COS-7 cells, since dominant-negative PKCδ potentiates both basal and EGF-induced PLD activity, which is consistent with the previous reports by Foster's group showing that PKCδ inhibits PLD

activity (Hornia et al., 1999). The role of PLD phosphorylation by PKCδ still remains elusive.

Tyrosine phosphorylation of PLD

Agents that increase reactive oxygen species, such as H_2O_2 in the presence of vanadate (peroxyvanadate) can induce tyrosine phosphorylation on PLD1 (Marcil et al., 1997; Min et al., 2001; 1998a). 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 tyrosine phosphorylate 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). The activation of PLD by H_2O_2 and EGF involves PKC activation (Min et al., 2001; 1998a). Very recently, it was 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).

In summary, although PLD1 and PLD2 can be phosphorylated on Ser, Thr, and Tyr residues *in vivo*, the significance of phosphorylation in the regulation of PLD activity and intracellular localization needs to be clarified.

1.3.4.2. Fatty acylation

It was shown that PLD1 can be fatty acylated when using [^{3}H] labeled palmitate (Manifava et al., 1999). Interestingly, this modification is undetectable in a catalytically inactive mutant of PLD1, and also, removal of palmitate by hydroxylamine to break the thioester bond renders PLD1 catalytically inactive. The fatty acylation sites were mapped at cysteine residues 240 and 241 in the PH-like domain (Sugars et al., 1999). Mutation of these residues reduced PLD activity *in vivo* but not *in vitro*; the localization was also changed from intracellular membranes and punctate structures to the plasma membrane and at a lesser extent to the cytosol (Sugars et al., 1999). The same mutant was also found to weaken the PLD1 association with membrane as was demonstrated by more stringent biochemical methods (Xie et al., 2001). Recently, it was further suggested that palmitoylation on the PH domain of PLD1 leads to strong membrane binding (Sugars et al., 2002) and palmitoylation is required for the localization, phosphorylation, and activation of PLD1 to the caveolin-enriched light membrane (Han et al., 2002b). Rat PLD2, having the same conserved sequences, was found to be palmitoylated on Cys223 and Cys224 in COS7 cells, and this palmitoylation was also implicated for membrane association (Xie et al., 2002).

1.3.5. Regulation by other factors

It has also been reported that cytoskeleton-associated proteins regulate PLD. These proteins include actin (Lee et al., 2001), actin-binding proteins, such as gelsolin

(Steed et al., 1996), fodrin (Lukowski et al., 1996) and α-actinin (Park et al., 2000), synaptojanin (Chung et al., 1997), and clathrin-assembly protein 3 (AP3) (Lee et al., 1997). PLD is also involved in altering f-actin and β-integrin point contact distribution (Aguirre Ghiso et al., 1997) and actin stress fiber formation (Kam and Exton, 2001). Although actin and most of these actin-binding proteins have inhibitory effects on PLD activity, it was reported that stimulation of human PLD1 by GTPγS in a cell-free system results in the association of PLD activity with detergent-insoluble actin cytoskeleton, which contains PLD1, Rho, actin, and many actin-binding proteins (Iyer and Kusner, 1999). It was found that PLD1 binds both monomeric G-actin, as well as polymerized actin filaments; G-actin inhibits both basal and stimulated PLD activity, but the polymerized F-actin produces the opposite effect (Kusner et al., 2002). Thus, it was proposed that actin exerts bi-directional modulation of PLD activity, and PLD can be activated on the actin cytoskeleton in a polymerization-dependent manner (Kusner et al., 2002). This mechanism seems to be conserved from bacteria to humans (Kusner et al., 2003).

Both PLD1 and PLD2 can interact with caveolin-1 (Czarny et al., 2000; 1999; Kim et al., 1999a). Whereas PLD1 is very sensitive to the inhibition by caveolin scaffolding peptide (IC50≈0.5) (Kim et al., 1999a), the PLD activity in the detergent-insoluble light membrane is stimulated by low concentrations of caveolin peptide [1-10 μM] but is inhibited at higher concentrations (Czarny et al., 1999). It appeared that caveolin stimulates PLD activity indirectly, since high protein levels from the light membrane is required to achieve the stimulation by caveolin, and immunopurified PLD2 can only be inhibited but not activated by caveolin peptide (Czarny et al., 2000).

In summary, PLD regulation is a complex and highly regulated process. Extracellular agonists, intracellular factors, as well as translational modification can regulate PLD. Although many signaling pathways are involved in the regulation of PLD, the link from extracellular agonists to intracellular factors has not been clearly identified. The detailed structural change of PLD upon activation is still unknown. How the posttranslational modification is related to PLD catalytic activity is still elusive. The work in this dissertation will shed light on the mechanism by which the extracellular signals that are relayed through the Ras pathway can be regulated by both Ral and ARF6 to activate PLD. A new view of PLD regulation is presented in Chapter V.

1.4. Subcellular Localization of PLD

So far, there is no general agreement about the intracellular localization of PLD. Early studies demonstrated the presence of PLD or PLD-like activities in many cellular compartments, including cytosol, cytoskeleton, nucleus, nuclear envelope, endoplasmic reticulum, Golgi apparatus, secretory vesicles, and plasma membrane (reviewed in Liscovitch et al., 1999). However, the localization of PLD in oncogene-transformed cells had not been identified before this dissertation work was started.

It was reported that PLD activity is highly enriched in caveolin-enriched Triton-insoluble membrane domains and the activity appears to be due to PLD2 but not PLD1 since PLD1 is excluded from the low density Triton-insoluble membrane domains (Czarny et al., 1999). The presence of PLD1 in caveolin-enriched membrane fractions was later reported in 3Y1 rat fibroblasts, by using a detergent-free, high pH sodium carbonate method (Kim et al., 1999a).

We were the first group that used the non-detergent, non-sodium carbonate method to study the membrane localization of PLD. By using this method, we found that the mitogenic PLD activity is mostly restricted to the caveolin-enriched light membrane microdomains in the oncogene-transformed cells or in response to growth factor stimulus. We reported this discovery in different conferences (Xu et al., 1998a; 1998b; 1999) and published it later (Xu et al., 2000). As presented in Chapter III, our studies indicate that PLD2 is localized almost exclusively in the light membrane fractions that co-fractionate with caveolin, and PLD1 is also present in the light membrane fractions, but primarily associated with heavier membrane fractions. These results are in agreement with most studies which indicate that PLD1 is found throughout the cell, but primarily in perinuclear, Golgi, and heavy membrane fractions; whereas PLD2 is localized to the plasma membrane or caveolin-enriched light membrane microdomains (Colley et al., 1997b; Czarny et al., 2000; Freyberg et al., 2001). Our discovery about the presence of PLD1 in caveolin-enriched light membrane fractions, along with others' (Han et al., 2002b; Kim et al., 1999a) indicates the possibility that PLD1 can be physically regulated on the plasma membrane.

The proposed heterodimerization of PLD1 and PLD2 (Kam and Exton, 2002) is also consistent with the localization of PLD1 and PLD2 in the plasma membrane and perinuclear regions.

Distinguishing characteristics of PLD1 and PLD2 are summarized in Table 1.

Table 1.2. Characteristics of PLD isoforms

	PLD1	PLD2
PIP_2-dependent	+	+
RalA-associated	+	-
Activated by:		
ARF family GTPases	+	?
Rho family GTPases	+	-
PKC α	+	?
Fatty acids	-	+
Molecular Weight	120 kDa	95 kDA
Predominant cellular location:	Perinuclear, Golgi	Plasma membrane, rafts

1.5. Mitogenic signaling of PLD

As discussed above, PLD activity is elevated in response to the oncogenic stimuli of several signaling oncogenes. PLD activity is also elevated in response to a variety of agonists, such as peptide growth factors, growth hormone, and chemokines, indicating that PLD likely plays an important role in mitogenic signaling.

PLD has been implicated in a variety of cellular processes including intracellular vesicle trafficking, endocytosis, exocytosis, mitogenesis, superoxide production, actin cytoskeleton reorganization (reviewed in Exton, 2002a), as well as tumor metastasis and cell motility (Aguirre Ghiso et al., 1997; Imamura et al., 1993; Pai et al., 1994). Recently, it was reported that a polymorphism of the PLD2 gene is associated with the prevalence of colorectal cancer (Yamada et al., 2003). It is not clear, however, how PLD participates in these cellular events.

How PLD functions through downstream targets have been difficult to establish. Although DAG and LPA can be involved, it is widely believed that PA, the main direct product of PLD action, is the real player for intracellular function (Hodgkin et al., 1998).

First, PA is required for the activation of PI(4)P 5-Kinase (Jenkins et al., 1994; Moritz et al., 1992), which converts PI(4)P to $PI(4,5)P_2$, a critical cofactor for both PLD isoforms. The increase production of PIP_2 mediated by PA, may serve as a positive feedback mechanism to further activate PLD.

Second, PA can also directly bind to Raf-1 (Ghosh et al., 1996) at a 36 amino acid stretch in the kinase domain of Raf and facilitates the recruitment of Raf1 to the plasma membrane (Rizzo et al., 1999; 2000), where it is proposed to participate in activation of the MAP kinase pathway (reviewed in Rizzo and Romero, 2002).

Third, PA is required for activation of a protein kinase mTOR, the mammalian target of rapamycin (Fang et al., 2001). mTOR controls both cell cycle progression and cell growth by regulating translation, transcription, membrane trafficking, and protein degradation (Kuruvilla and Schreiber, 1999; Schmelzle and Hall, 2000). PA was shown to interact with mTOR competitively with rapamycin (Fang et al., 2001). Consistent with this hypothesis, elevated PLD activity confers resistance to rapamycin (Chen et al., 2003), which further implicates mTOR as a downstream target of PLD.

Moreover, more and more studies supported a role of PLD and its product PA in vesicle transportation and signaling. PLD has been implicated in vesicle budding and trafficking in Golgi membranes (reviewed in Siddhanta and Shields, 1998), exocytosis in neutrophils and mast cells (reviewed in Cockcroft et al., 2002; Jones et al., 1999a), as well as receptor-mediated endocytosis (Shen et al., 2001). It is not clear how PLD and its

product PA might influence the membrane, but it is likely that association between PA and divalent cations such as Ca^{2+} results in clustering of PA and membrane curvature, which could initiate invagination of a vesicle (Rizzo and Romero, 2002). Interestingly, blocking PA production not only prevented endocytosis but also inhibited the activation of MAP kinase, but not MEK, the kinase that phosphorylates MAP kinase (Shen et al., 2001). This is consistent with the results by Vieira et al., showing that EGF activation of MAP kinase depends on the endocytosis of EGF receptor (Vieira et al., 1996). A reasonable explanation is that blocking PLD will not block the pathway to MEK, but PLD plays more than one role in the activation of MAP kinase—activation of MEK by activation of Raf and endocytosis of the receptor to where MAP kinase can be phosphorylated (Fig. 1.6).

There are also other targets for PA, such as NADPH oxidase, protein tyrosine phosphatase, PLC isoforms, cAMP-specific phosphodiesterase, and PKC isoforms, etc. (reviewed in Exton, 2002a).

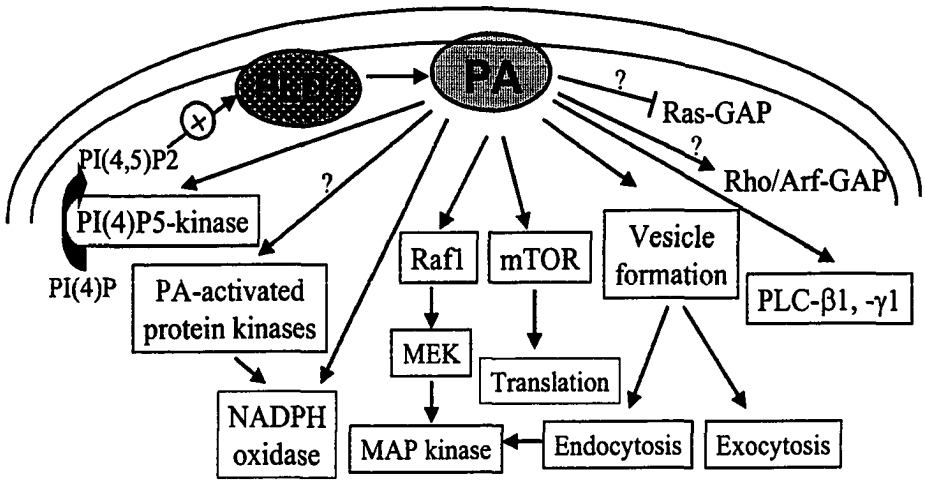


FIG. 1.6. Potential cellular targets of PA. See text for details.

1.6. Rationale of this work

PLD activity is critical for the transformed phenotype induced by oncoproteins v-Src and v-Ras (Aguirre-Ghiso et al., 1999; Urano et al., 1996) as well as the EGF receptor (Lu et al., 2000). As discussed above, the mechanism of regulation of PLD by extracellular agonists and oncogenic signals has not been clearly identified. The membrane compartmentalization of PLD along with certain molecules that regulate PLD should be investigated. To uncover these will help understand the mechanism of PLD regulation.

In Chapter II, the methods that we used for this dissertation study is presented.

Chapter III focuses on the membrane localization of PLD. There has been an unsolved question: why PLD in v-Src transformed cells seems not to be able to utilize polyunsaturated fatty acid-labeled phosphatidylcholine *in vivo* (Song and Foster, 1993; Song et al., 1991). Does the PLD activated by v-Src have a substrate preference for PC containing no polyunsaturated fatty acids or is the PC with polyunsaturated fatty acids was excluded to where the PLD activated by v-Src? To answer these questions, I demonstrated that PLD activity is mostly restricted in the caveolin-enriched light membrane microdomains (CEMMs) where the PC with polyunsaturated fatty acids is excluded. This work also supports the idea that mitogenic signaling of PLD is initiated from cell membranes, especially the lipid rafts.

We found that both PLD1 and PLD2 are present in the CEMMs, even though PLD2 is almost exclusively localized there (see Chapter III). Since PLD activity is mostly found in the CEMMs, the PLD activity cannot be explained only by localization of PLDs. We found that RalA and ARF6, but not ARF1, are localized to the CEMMs.

We also found that H-Ras but not K-Ras can activate PLD. We then used the Ras system to study the regulation of PLD. We demonstrated that H-ras but not K-Ras can activate both RalA and ARF6. RalA and ARF6 interact with each other in H-Ras transformed cells or inresponse to EGF stimulus. Both RalA and ARF6 are required for PLD activity, but neither alone can activate PLD in mouse fibroblast NIH 3T3 cells. Thus, we suggested H-Ras but not K-ras activates PLD through the synergistic action of both ARF6 and RalA. This work is shown in Chapter IV.

Understanding the Ras pathway to Ral and ARF6 has provided an important link between extracellular signals and PLD activation. This has shed light on the mechanisms. Thus, this work, along with others' helps shape new connections between signaling cascades. A new view in the regulation of PLD through these signaling networks will be discussed in Chapter V. Having carefully reviewed the current literature as well as our results about the localization and regulation of PLD, we provide a new model about the localization and regulation of Ras and PLD in Chapter V.

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-, v-Src-, and v-Raf-transformed NIH 3T3 cells, NIH 3T3 cells overexpressing activated RalA (Q72L), and 3Y1 rat fibroblasts overexpressing the EGF receptor 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 phosphatidylinostol-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 βII, 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 *Eco*RI 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 (22-25 krpm 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 (see Appendix, Table A.1 for the refractive index). 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 (fraction 13) was sonicated in 1 ml 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, 1μM leupeptin, 0.15 μM aprotinin, 1 μM protease inhibitor E-64)], incubated at 4^0C for 15 min by gently rocking, sonicated for 20 seconds on ice, and centrifuged at 12,000Xg at 4^0C for 10 min. The supernatant was precleared with Protein G Sepharose 4 Fast Flow beads

(Amersham Pharmacia Biotech), and 500 μg of the precleared proteins was adjusted to 500 μl 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 μl of Protein G Sepharose 4 Fast Flow bead slurry, collected by centrifugation at 12,000Xg 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 100^0C 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 chemilluminescent detection system (Pierce).

2.7. Assay of PLD Activity

2.7.1. *in vitro* PLD assay

A liposome-based *in vitro* PLD assay based largely on strategies used by Brown et al. (Brown et al., 1993) was used to assay PLD activity in cell homogenate or the fractions. PLD activity was determined by examining the ability to convert [^{3}H]-PC in prepared liposomes to phosphatidylethanol in the presence of exogenously provided

ethanol. Liposomes were prepared by mixing chloroform solutions of phospholipids containing 1 μCi / reaction of [^{3}H]-PC (42 Ci/mM) and drying under a stream of nitrogen followed by resuspension with sonication for 10 min at room temperature in lysis buffer (25 mM HEPES, pH 7.2; 100 mM KCl; 10 mM NaCl; 0.5 mM EGTA; 0.5 mM EDTA; 1 mM DTT). The liposomes consist of phosphatidylethanolamine / phosphatidylinositol-4,5-bisphosphate / PC in a molar ratio of 16/1.4/1 with PC suspended to a final concentration of 8.6 μM. The reaction buffer consisted of lysis buffer plus 5 mM $MgCl_2$, 0.16 mM $CaCl_2$ and 1% ethanol in a total volume of 0.1 ml. The reaction mixtures were incubated for 45 min at 37 °C. The reaction was stopped by adding 400 μl of 10% trichloroacetic acid and 200 μl of 10 mg/ml bovine serum albumin. Precipitated lipids and proteins were removed by centrifugation at 3000xg for 10 min at 4^{0}C. An aliquot of the supernatant containing the released choline was removed and analyzed by liquid scintillation spectroscopy.

2.7.2. *in vivo* PLD activity

in vivo 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.

2.8. Characterization of phospholipid metabolites by thin layer chromatography (TLC)

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).

2.9. 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 glutathione-agarose 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).

Chapter III. Mitogenic PLD Activity is Restricted to Caveolin-Enriched Membrane Microdomains

PLD activity is elevated in response to oncogenic stimuli or a variety of agonists, indicating that PLD likely plays an important role in mitogenic signaling. It was demonstrated previously that the PLD activity induced by v-Src could not be detected in cells that were radiolabeled with the polyunsaturated fatty acid arachidonate, in spite of arachidonate being incorporated efficiently into phosphatidylcholine (PC), the substrate for PLD. We tested whether PLD localizes to the regions where the PC with a polyunsaturated fatty acyl chain is excluded or PLD has substrate specificity. We found that the elevated PLD activity in NIH 3T3 cells transformed by activated oncogenic forms of Src, Ras, and Raf is largely restricted to the caveolin-enriched membrane microdomains (CEMMs), where many proteins that mediate mitogenic signaling are localized. Likewise, the PLD activity stimulated by EGF is also restricted to the CEMMs. Although both PLD1 and PLD2 were found in CEMMs, neither was particularly enriched in the CEMMs of the transformed relative to the parental cells, indicating that it is the specific activity of PLD that is increased in the CEMMs. An apparent PLD substrate specificity in transformed cells for PC lacking arachidonate acyl groups is also explained by the localization of activity in the CEMMs where [^{3}H]-arachidonate-labeled PC was excluded. These data indicated that mitogenic signaling through PLD is initiated in CEMMs where many signaling molecules co-localize.

3.1. Introduction

Phospholipase D activity is elevated in response to many extracellular signals (Exton, 1998) indicating that PLD plays an important role in the transduction of

intracellular signals. PLD activity is also elevated in response to the oncoproteins v-Src (Song et al., 1991), v-Ras (Carnero et al., 1994; Jiang et al., 1995a; 1995b), v-Fps (Jiang et al., 1994), and v-Raf (Frankel et al., 1999). PLD is also critical for the transformed phenotype induced by Src and Ras (Aguirre-Ghiso et al., 1999; Urano et al., 1996) as well as the EGF receptor (Lu et al., 2000).

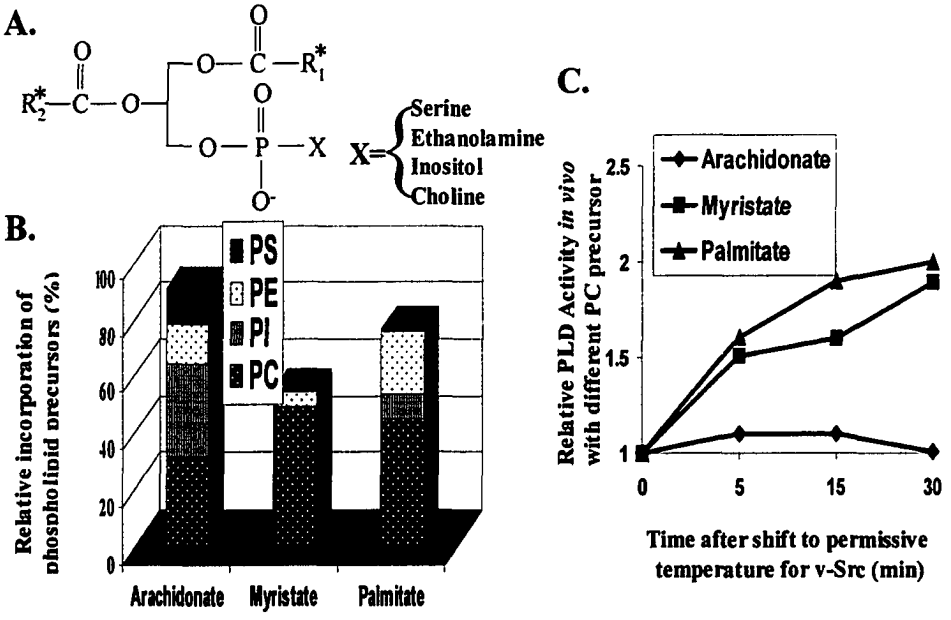


FIG. 3.1. The apparent substrate specificity for saturated fatty acid-labeled PC in v-Src- induced PLD activity *in vivo*.
(A) Label of different phospholipids (with different head group X) by using different [^{3}H]-labeled precursors to the R* group. (B) All the used precursors, including arachidonate, are efficiently incorporated into PC by TLC assay. (C) PLD activity induced by v-Src could not be detected in cells that were radiolabeled with the polyunsaturated fatty acid arachidonic acid. Adapted from Song and Foster, 1993; Song et al., 1991.

It was demonstrated previously by Foster's group that the PLD activity induced by v-Src could not be detected in cells that were radiolabeled with the polyunsaturated fatty acid arachidonic acid (20:4) (Song and Foster, 1993; Song et al., 1991). This exclusion occurred in spite of arachidonic acid being incorporated efficiently into PC (Song and Foster, 1993), the substrate for PLD. PLD activity in v-Src-transformed cells was only detected when radiolabeled saturated fatty acids like palmitate (16:0) or

myristate (14:0) were used to label membrane phospholipids (Fig. 3.1). This suggested that either: 1) the PLD activated by v-Src had a substrate preference for PC containing saturated fatty acids or 2) that PC with saturated fatty acids was localized to where the PLD activated by v-Src (Fig. 3.2).

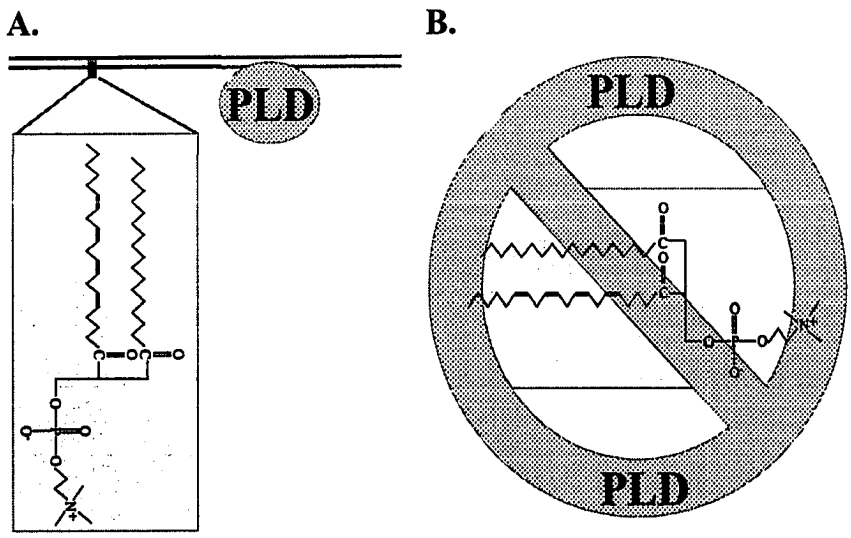


FIG. 3.2. Two hypotheses to explain the apparent substrate specificity of PLD. (A) PLD does not co-localize with arachidonate labeled PC? This hypothesis can be tested by cellular localization assay. (B) PLD cannot cleave the PC with polyunsaturated acyl chain? This hypothesis can be test in test tube by adding the arachidonate-labeled PC with PLD.

The Src family of tyrosine protein kinases localize to the plasma membrane by virtue of being doubly acylated with two saturated fatty acids in the N-terminus (Resh, 1999). v-Src has been reported to localize to caveolae or caveolin-enriched membrane microdomains (CEMMs) (Lisanti et al., 1995).

Caveolae are plasma membrane invaginations that form in the presence of caveolin (Anderson, 1998). The main structural proteins of caveolae are the caveolins. It has been proposed that caveolins form a scaffold onto which many classes of signaling molecules can assemble to generate "preassembled signaling complexes" (Okamoto et al., 1998). These proteins include Gα subunit, H-Ras, Src family tyrosine kinases,

endothelial nitric-oxide synthase (eNOS), EGFR and related receptor tyrosine kinases, PDGF receptor, PI3-kinase, erk2, Raf-1, MEK-1, and PKC isoforms (reviewed in Okamoto et al., 1998). Caveolae are rich in glycosphingolipids, cholesterol, and phospholipids enriched with saturated fatty acids, and it has been proposed that the strong van der Waals interactions between the closely packed saturated fatty acids and cholesterol create membrane microdomains that float in the plasma membrane much like a "raft" (Harder and Simons, 1997; Simons and Ikonen, 1997). These lipid rafts contain many of the proteins that facilitate the transduction of intracellular signals and have been proposed to serve as recruitment and assembly sites for intracellular signaling complexes (Okamoto et al., 1998).

Since v-Src localizes to these CEMMs, the observed preference of the v-Src-induced PLD for PC with saturated fatty acids could be due to localization of these PC species to these membrane microdomains that are rich in phospholipids with higher percentages of saturated fatty acids. In this report, we have investigated whether the PLD activity elevated in response to the oncogenic signals of v-Src, v-Ras, and v-Raf is restricted to the CEMMs, where there is enrichment for phospholipids with saturated fatty acids such as the PC utilized as substrate by the v-Src-induced PLD.

3.2. Results

3.2.1. Elevated levels of PLD activity in transformed cells is restricted to the CEMMs

PLD activity is elevated in cells transformed by several signal transducing oncogenes including v-Src (Song et al., 1991), v-Ras (Carnero et al., 1994; Jiang et al., 1995a; 1995b), and v-Raf (Frankel et al., 1999). The level of induction of PLD activity in these cells was generally rather small (2- to 3-fold) relative to the level of other

responses induced by mitogenic signals. Since PLD activity has been implicated in other cellular functions such as protein trafficking (Roth and Sternweis, 1997), it is possible that the low level of PLD activity induced in response to mitogenic signals is due to a high background PLD activity involved in other cellular activities. Many proteins implicated in mitogenic signaling including Src, Ras and Raf have been reported to localize to CEMMs (Anderson, 1998; Okamoto et al., 1998). We therefore wanted to examine PLD activity in different membrane fractions from both normal and transformed cells. NIH 3T3 cells and NIH 3T3 cells transformed by v-Src, v-Ras and v-Raf were harvested and membranes isolated, broken up by sonication and centrifuged over a discontinuous sucrose gradient to separate the light membrane CEMM fraction from other membranes. The distribution of proteins known to localize to caveolae (caveolin 1), the plasma membrane (Na^+,K^+-ATPase βII), the trans-Golgi network (TGN38) and the endoplasmic reticulum (BiP) was examined in NIH 3T3 cells. As shown in Fig. 3.3A, caveolin 1 was present predominantly in fractions 4 and 5, which contains about 5~6% of the total proteins (not shown) and represents the light membrane fraction containing the CEMMs. BiP, Na^+,K^+-ATPase, and TGN38, as expected, were found in heavier membrane fractions (Fig. 3.3A), indicating that the fractionation procedure was able to separate the CEMMs from the heavier membrane fractions. Similar data were obtained with the transformed cells except that, as described previously (Lisanti et al., 1995), caveolin 1 levels were substantially reduced (data not shown). We also investigated the distribution of v-Src, v-Ras and v-Raf in the gradient and as shown in Fig. 3.3B, all three oncoproteins could be found in the light membrane fraction. v-Src

and H-Ras were found almost exclusively in the light membrane fraction, whereas Raf was detected all fractions throughout the gradient.

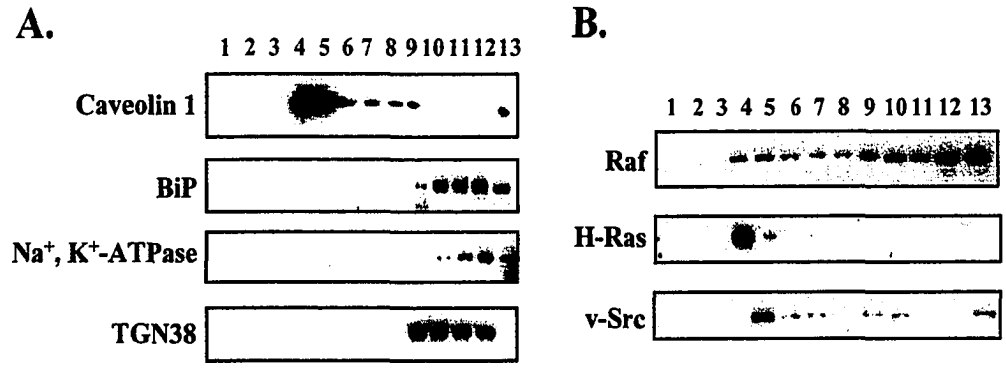


FIG. 3.3. Distribution of proteins in light and heavy membrane fractions. (A) NIH 3T3 cells were disrupted by Dounce and Polytron homogenization and then sonicated as described in Chapter II. The membrane fragments were then run over a discontinuous gradient of 5%, 35% and 45% sucrose. 12 fractions and a pellet were recovered and subjected to Western blot analysis using antibodies to caveolin 1, the endoplasmic reticulum protein BiP, the plasma membrane protein Na^+,K^+-ATPase βII, and the trans Golgi network protein TGN38. The amount of material loaded onto the gels was normalized by volume from each of the fractions. The figure shown is a representative of three independent experiments. (B) NIH 3T3 cells transformed by either v-Src, v-Ras or v-Raf were subjected to the same fractionation procedure as in A, and then subjected to Western blot analysis using antibodies raised against Src, Ras, and Raf as shown.

To examine the PLD activity in isolated membranes, we needed to use an *in vitro* PLD assay. We showed previously (Luo et al., 1998), that the elevated PLD activity observed in cells transformed by v-Src and v-Ras was still observed in broken cell lysates. To confirm this observation and to establish that this was also the case in v-Raf-transformed cells, both *in vivo* and *in vitro* PLD assays were compared in the transformed and parental NIH 3T3 cells. As shown in Fig 3.4A, the similar increase in PLD activity was observed using either the *in vivo* transphosphatidylation reaction or the *in vitro* choline release assay. These data indicate that the elevated PLD activity seen in the transformed cells is maintained in this broken cell system.

We next examined the PLD activity in the parental and transformed NIH 3T3 cells in fractions obtained from the sucrose gradient. Although elevated PLD activity relative to the NIH 3T3 cells could be detected in the intermediate density fractions (fractions 6 - 8), the most significant increase in PLD activity was seen in the light membrane fractions 4 and 5 (Fig. 3.4B). As much as an 8-fold increase in PLD activity in the cells transformed by v-Raf was seen in fraction 4 whereas only 2- to 3-fold increase was seen in the intermediate fractions. Similar differences were observed for the v-Src- and v-Ras-transformed cells relative to the parental NIH 3T3 cells.

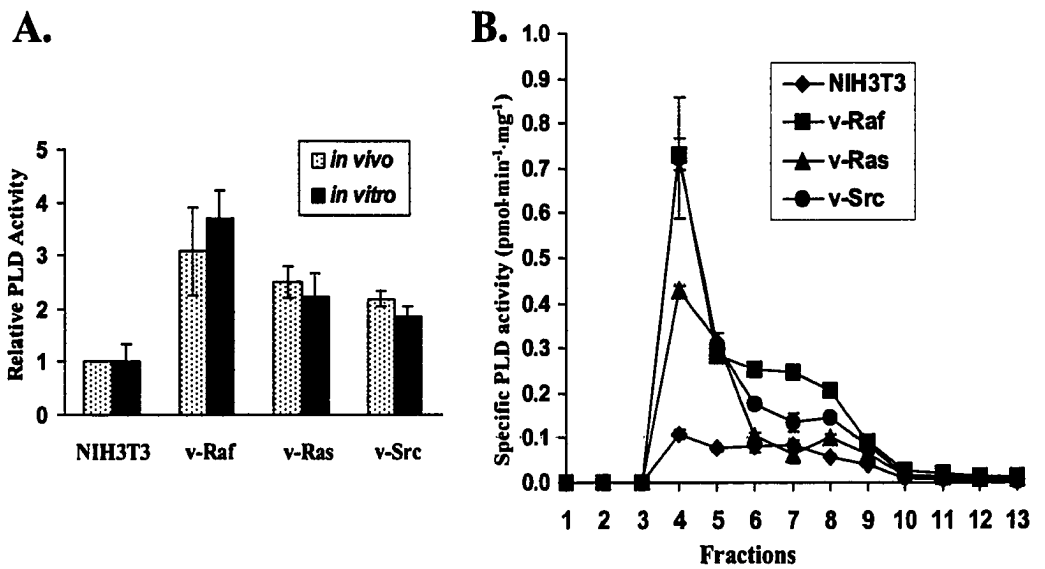


FIG. 3.4. Elevated levels of PLD activity in transformed cells are restricted to the CEMMs.

(A) PLD activity in NIH 3T3 cells and NIH 3T3 cells transformed by v-Src, v-Ras or v-Raf was determined by transphosphatidylation (*in vivo*) or choline release (*in vitro*) as described in Chapter II. Error bars represent standard deviations for duplicate experiments repeated three times (*in vivo*) or four times (*in vitro*). (B) NIH 3T3 cells and NIH 3T3 cells transformed by v-Src, v-Ras or v-Raf were subjected to the fractionation procedure described in Fig. 3.3A. Fractions were recovered and the PLD activity in the different fractions was determined by adding liposomes containing [^{3}H]-choline-labeled PC and measuring choline release as described in Chapter II. PLD activity in each fraction was normalized for total protein in each fraction. Error bars represent the range for duplicate results from a representative of two independent experiments.

3.2.2. Increased PLD activity is not due to increased levels of either PLD1 or PLD2

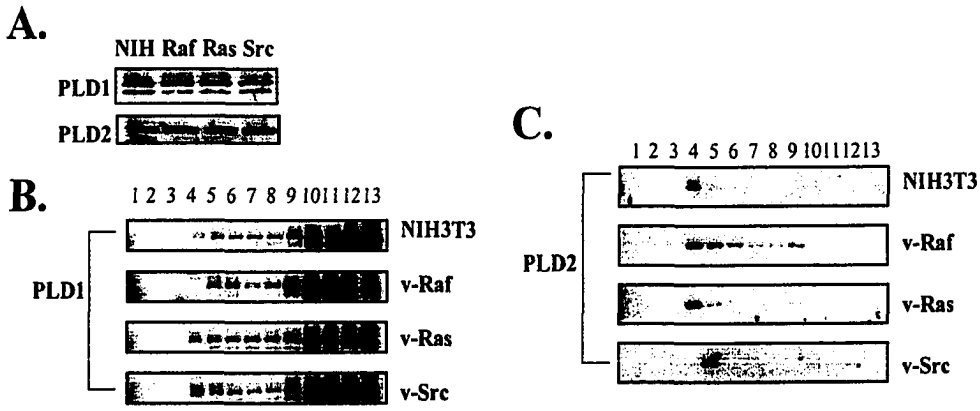


FIG. 3.5. Increased PLD activity is not due to increased levels of PLD1 or PLD2. Lysates from NIH 3T3 cells and NIH 3T3 cells transformed by either v-Src (Src), v-Ras (Ras) or v-Raf (Raf) were subjected to Western blot analysis using antibodies raised against either PLD1 or PLD2 (A). The parental and transformed NIH 3T3 cells were then subjected to fractionation procedures described in Fig. 3.3A and the recovered fractions were subjected to Western blot analysis using antibodies raised against either PLD1 (B) or PLD2 (C). The data shown are representative experiments of three (for PLD1) or two (for PLD2) independent experiments.

We next investigated the levels of PLD1 and PLD2 in the transformed and parental NIH 3T3 cells. In Fig. 3.5A, it is shown that there was no significant difference in the levels of either PLD1 or PLD2 in the parental or transformed cells that could account for elevated PLD activity in the transformed cells. Both PLD1 and PLD2 have been reported to be present in CEMMs (Czarny et al., 1999; Kim et al., 1999a; Liscovitch et al., 1999). We therefore examined the levels of PLD1 and PLD2 in the CEMM fractions of the NIH 3T3 and transformed NIH 3T3 cells. Both PLD1 (Fig. 3.5B) and PLD2 (Fig. 3.5C) could be detected in the CEMM fraction. PLD1 was present in both the heavy and light membrane fractions whereas PLD2 was largely restricted to the light membrane fraction. There did not appear to be any dramatic changes in the distribution of either PLD1 or PLD2 in the transformed cells relative to the parental NIH 3T3 cells

that could account for the increased PLD activity observed in the light membrane fractions. These data suggest that the increased PLD activity in the transformed cells is due to a change in the activity of PLD rather than to an increase in either PLD1 or PLD2 protein.

3.2.3. EGF-induced PLD activity is predominantly in CEMMs

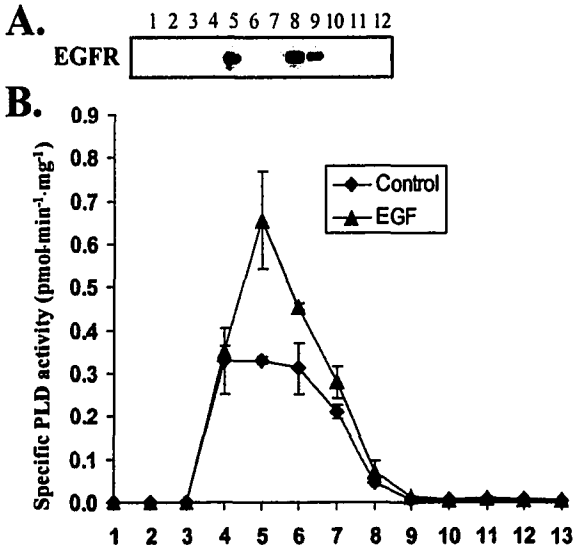


FIG. 3.6. EGF-induced PLD activity is predominantly in CEMMs.
(A) 3Y1 cells overexpressing the EGF receptor were subjected to the fractionation procedures used in Figs. 3.3A and the presence of the EGF receptor in the different fractions was determined by Western blot analysis. (B) The 3Y1 cells overexpressing the EGF receptor were treated with EGF (100 ng/ml, 4 min), subjected to fractionation and the PLD activity in the different fractions was determined as in Fig. 3.4B. The PLD activity in each fraction was normalized for total protein in each fraction. Error bars represent the range for duplicate results from a representative of two independent experiments.

EGF causes an increase in PLD activity (Hornia et al., 1999; Song et al., 1994; Yeo and Exton, 1995). The EGF receptor has also been reported to be present in CEMMs and upon EGF treatment, the receptor leaves the CEMMs and internalized (Mineo et al., 1996). We examined the distribution of the EGF receptor in 3Y1 rat fibroblasts that overexpress the EGF receptor (Hornia et al., 1999). As shown in Fig. 3.6A, the EGF receptor was present in the light membrane fraction 5. However, there

was also a substantial amount of the receptor in the intermediate density fractions 8 and 9 (Fig. 3.6A). We next examined the PLD activity in the different fractions of the 3Y1 cells overexpressing the EGF receptor, and as shown in Fig. 3.6B, the most significant increase in PLD activity was observed in the light membrane fraction 5. Interestingly, there was very little increase seen in the intermediate fractions 8 and 9 where there was also substantial EGF receptor. This population of EGF receptor likely represents internalized EGF receptor. These data indicate that the PLD activity elevated in response to EGF occurs in the CEMMs where EGF receptor is present prior to internalization.

3.2.4. Substrate specificity for PC lacking arachidonate is explained by exclusion from CEMMs

We previously reported that the PLD activity elevated in transformed cells, as measured by transphosphatidylation, could not be detected when PC was prelabeled with the poly-unsaturated fatty acid arachidonic acid (Song and Foster, 1993; Song et al., 1991). In contrast, PLD activity elevated in response to TPA could readily be detected when substrate PC was prelabeled [^{3}H]-arachidonic acid. This result could have been due to exclusion of PC containing the polyunsaturated arachidonic acid from the light membrane fraction, which is enriched with membrane lipids containing saturated fatty acids (Harder and Simons, 1997; Simons and Ikonen, 1997). We therefore investigated the distribution of PC prelabeled with either the polyunsaturated arachidonate, or the saturated fatty acids myristate and stearate (18:0). We first examined the PLD activity in either whole cells or in isolated CEMMs (fractions 4 and 5). As shown in Fig. 3.7A, the PLD activity in whole cells, prelabeled with [^{3}H]-arachidonic acid, ranged from 20% and 30% of that observed in cells labeled with [^{3}H]-myristic acid. In contrast, there was virtually no PLD activity detected in the CEMM fraction when cells were labeled with

[^{3}H]-arachidonic acid (Fig. 3.7B). We next examined the level of [^{3}H]-arachidonate-, [^{3}H]-myristate-, and [^{3}H]-stearate-labeled PC in whole cells and the CEMM fraction. As shown in Fig. 3.7C, the PC detected in whole cells prelabeled with [^{3}H]-arachidonic acid was approximately 30% of that observed in cells labeled with either [^{3}H]-myristic or [^{3}H]-stearic acid. In contrast, virtually no PC could be detected in the CEMM fraction when cells were labeled with [^{3}H]-arachidonic acid (Fig. 3.7D). These data suggest that the apparent substrate specificity of mitogenic PLD activity for PC lacking arachidonic acid (Song and Foster, 1993) was due to exclusion of arachidonate-containing PC from the membrane microdomain where the mitogenic PLD is activated.

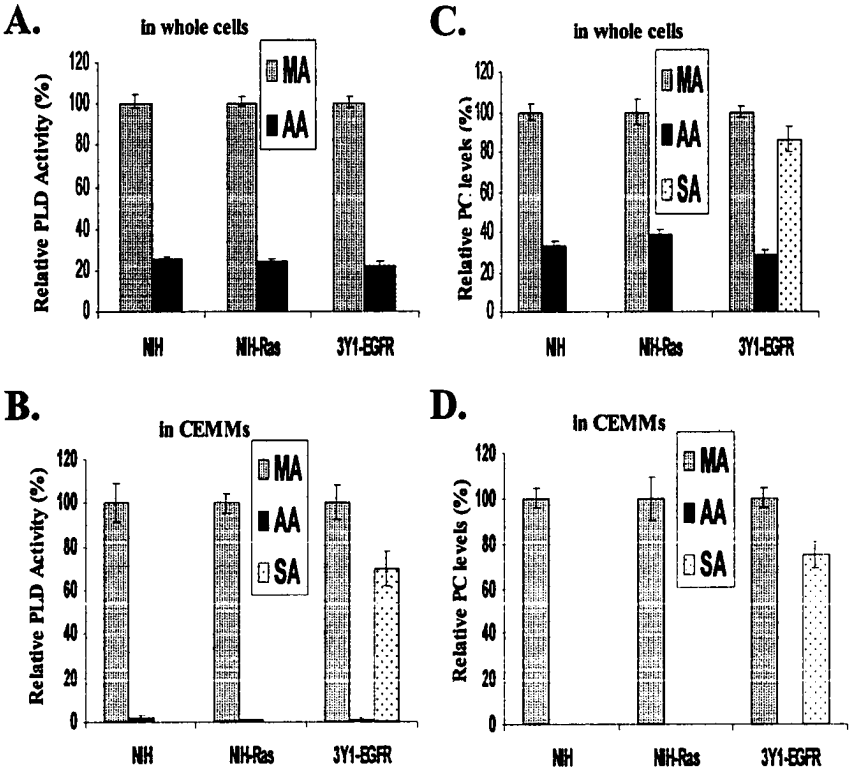


FIG. 3.7. PC prelabeled with [^{3}H]-arachidonic acid is excluded from CEMMs. NIH 3T3 (NIH), Ras-transformed NIH 3T3 (NIH-Ras), and 3Y1-EGFR cells were prelabeled with either [^{3}H]-myristic acid (MA), [^{3}H]-arachidonic acid (AA), or [^{3}H]-stearic acid (SA). PLD activity was then determined in whole cells (A) or in the CEMM fraction (fractions 4 and 5) of isolated membranes (B) by measuring the production of phosphatidylbutanol by TLC as described in Materials and Methods. The PLD activity in [^{3}H]-arachidonic acid- and [^{3}H]-stearic acid-labeled cells was normalized to the PLD activity in the [^{3}H]-myristic acid-labeled cells, which was assigned a value of 100% for

each cell line. Relative PC levels in whole cells (C) and in the CEMM fraction (D) was determined scanning of autoradiographs from TLC plates where PC had been resolved. The PC from the [^{3}H]-arachidonic acid- and [^{3}H]-stearic acid-labeled cells was normalized to the PC in the [^{3}H]-myristic acid-labeled cells, which was assigned a value of 100% for each cell line. The data represent the average of three independent experiments ± standard deviation. Note that SA in NIH and NIH-Ras in B, C and D was not tested. These data were kindly provided by Troy Joseph and Annika Bryant.

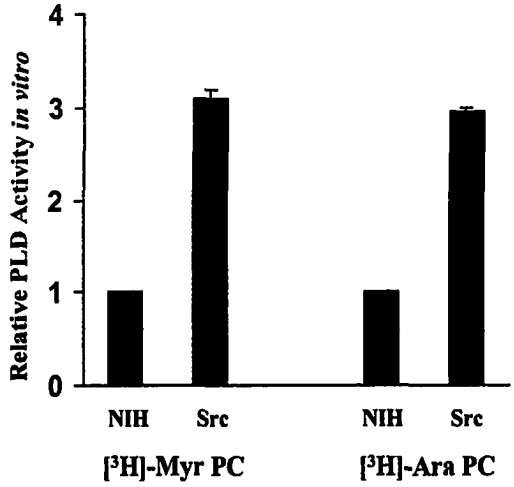


FIG. 3.8. v-Src-induced increase in PLD activity has no substrate specificity *in vitro*. [^{3}H]-PC was isolated from cells labeled with either [^{3}H]-arachidonic acid or [^{3}H]-myristic acid as described in Materials and Methods. The [^{3}H]-arachidonic acid- and [^{3}H]-myristic acid-labeled PC was then used to prepare [^{3}H]- PC-labeled liposomes ([^{3}H]-Myr PC or [^{3}H]-Ara PC as shown). Membranes from either parental or v-Src-transformed NIH 3T3 cells were added and PLD activity, as determined by transphosphatidylation in the presence of 1% butanol, was determined as described previously (Jiang et al., 1995b; Luo et al., 1997). The data represent the range of two independent experiments.

To demonstrate that there was no inherent substrate specificity of the PLD activated by mitogenically activated PLD, we examined PLD activity in membranes from parental and v-Src-transformed NIH 3T3 cells using substrate PC in generated liposomes. Substrate PC was generated by labeling v-Src-transformed NIH 3T3 cells with either [^{3}H]-myristate or [^{3}H]-arachidonate and then recovering TLC-resolved PC. Membranes from either parental or v-Src-transformed NIH 3T3 cells were then mixed with liposomes containing the recovered [^{3}H]-myristate- or [^{3}H]-arachidonate-labeled PC. As shown in Fig. 3.8, the increased PLD activity in the membranes from the v-Src-transformed cells

was observed using either [^{3}H]-myristate- or [^{3}H]-arachidonate-labeled PC. These data indicate the PLD activated in response to v-Src does not have any intrinsic substrate specificity that would exclude PC containing the polyunsaturated arachidonic acid. The data further support the hypothesis that the PLD elevated in response to mitogenic signals takes place in CEMMs, which are enriched with phospholipids containing saturated fatty acids.

3.3. Discussion

In this report we have shown that the elevated PLD activity in transformed cells is largely restricted to CEMMs where many signaling molecules aggregate to form signaling complexes (Okamoto et al., 1998). As reported previously (Czarny et al., 1999; Kim et al., 1999a), both PLD1 and PLD2 could be detected in the CEMM fraction, however there was no dramatic increase in either PLD1 or PLD2 in these fractions that would account for the increased activity. The apparent substrate specificity of mitogenically stimulated PLD for PC lacking arachidonic acid (Song and Foster, 1993) could also be explained by exclusion of arachidonate-containing PC from the CEMMs. These data provide evidence that the PLD activity elevated in response to mitogenic signals takes place in CEMMs, where many of the proteins involved in the initiation of mitogenic signals are localized.

The restriction of elevated PLD activity in the transformed cells to the light membrane CEMM fraction explains our previous observation that elevated PLD activity in transformed cells was not observed when cells were radiolabeled with the polyunsaturated fatty acid arachidonic acid (Song and Foster, 1993; Song et al., 1991). Data presented here demonstrates that arachidonate-labeled PC was absent in the light

membrane fraction. Moreover, the elevated PLD activity in v-Src-transformed cells had no substrate preference in an *in vitro* PLD assay. Thus, the apparent substrate specificity for PC lacking arachidonic acid observed previously for mitogenically stimulate PLD activity is likely due to the localization of this PLD activity to the CEMMs that are enriched with phospholipids containing saturated fatty acids.

The PLD activity elevated in response to Src, Ras, Raf and EGF is dependent upon the small GTPase RalA (Frankel et al., 1999; Hornia et al., 1999; Jiang et al., 1995b; Lu et al., 2000; Voβ et al., 1999). We previously demonstrated that PLD1, but not PLD2, could be precipitated by immobilized GST-RalA fusion protein (Jiang et al., 1995b; Luo et al., 1997). We also demonstrated that the PLD associated with RalA was responsive to Arf (Luo et al., 1997), which is also a characteristic of PLD1 (Hammond et al., 1995; 1997). These data indicated that the PLD activity mediated by RalA was the Arf-responsive PLD1. The data presented here show that while PLD1 is present in the CEMMs, only a small percentage of the total PLD1 is present in this fraction. In contrast, almost all of the PLD2 is in light membrane fractions. Thus, while we believe that the Arf-responsive, RalA-associated PLD1 is likely involved in mitogenically stimulated PLD activity, an additional role for PLD2, which is apparently localized almost exclusively to the CEMM fraction can not be ruled out. It is possible that PLD1 and PLD2 work together in response to the mitogenic signals initiated in the CEMMs.

Chapter IV. Elevated PLD Activity in H-Ras- but Not K-Ras-transformed Cells by the Synergistic Action of RalA and ARF6

Phospholipase D activity is elevated in response to the oncogenic stimulus of H-Ras, but not K-Ras. PLD activity in H-Ras-transformed cells is dependent upon RalA, and consistent with a lack of elevated PLD activity in K-Ras-transformed cells, RalA was not activated in K-Ras-transformed cells. Although H-Ras-induced PLD activity is dependent upon RalA, an activated mutant of RalA is not sufficient to elevate PLD activity. It was reported previously that RalA interacts with PLD activating ARF (ADP-ribosylation factor) proteins. In cells transformed by H-Ras, we found increased co-precipitation of ARF6 with RalA. Moreover, ARF6 co-localized with RalA in light membrane fractions. Interestingly, ARF6 protein levels were elevated in H-Ras- but not K-Ras-transformed cells. A dominant negative mutant of ARF6 inhibited PLD activity in H-Ras-transformed NIH 3T3 cells. Activated mutants of either ARF6 or RalA were not sufficient to elevate PLD activity in NIH 3T3 cells; however, expression of both activated RalA and activated ARF6 in NIH 3T3 cells led to increased PLD activity. These data suggest a model whereby H-Ras stimulates the activation of both RalA and ARF6, which together lead to the elevation of PLD activity.

4.1. Introduction

PLD activity is elevated in response to many oncogenic signals including those generated by v-Src (Song et al., 1991), v-Ras (Carnero et al., 1994; Jiang et al., 1995a; 1995b), v-Fps (Jiang et al., 1994), and v-Raf (Frankel et al., 1999). The activation of

PLD by v-Src, v-Ras, and v-Raf is dependent upon the small GTPase RalA (Frankel et al., 1999; Jiang et al., 1995b), which interacts directly with PLD1 (Luo et al., 1997). RalA has also been shown to be required for the transformed phenotype induced by v-Src, v-Ras, and v-Raf (Aguirre-Ghiso et al., 1999; Urano et al., 1996). Additionally, RalA, PLD1, or PLD2 could cooperate with either c-Src or the EGF receptor to transform rat fibroblasts (Joseph et al., 2001; Lu et al., 2000). Elevated expression of either PLD1 or PLD2 was able to overcome a cell cycle block induced by high intensity Raf signaling (Joseph et al., 2002). RalA has also been implicated in the activation of PLD by phorbol esters (Schmidt et al., 1998) and by growth factor receptor tyrosine kinases (Lu et al., 2000; Voβ et al., 1999). These studies suggest an important role for the RalA and PLD in mitogenic and oncogenic signaling. However, while RalA is necessary for activation PLD by oncoproteins and growth factors, activated RalA was not sufficient for elevating PLD activity (Jiang et al., 1995b). Active RalA/PLD complexes also contain ARF GTPases (Luo et al., 1998), which activate PLD1 (Brown et al., 1993; Cockcroft et al., 1994; Hammond et al., 1995; 1997). The presence of ARF in active RalA/PLD1 complexes (Luo et al., 1998) also implicated ARF in mitogenic signaling. These data suggested that the activation of PLD in response to mitogenic signals requires multiple signals involving the activation of both RalA and ARF G proteins.

The v-Ras that was used in Chapter III and implicated to activate PLD is v-H-Ras (Jiang et al., 1995b; Xu et al., 2000). The 21 kD transforming proteins of the Harvey (H) and Kirsten (K) murine sarcoma viruses are called v-H-Ras and v-K-Ras, which are oncogenic mutation of normal cellular Ras (c-H-Ras and c-K-Ras). The neuroblastoma (N)-Ras has not been found in any retrovirus yet (Adjei, 2001). The three main

potentially oncogenic Ras genes in human cells, H-Ras, N-Ras, and K-Ras, share a high degree of sequence homology (> 90%) (Prior and Hancock, 2001). K-Ras has two alternatively spliced forms: K-Ras 4A and K-Ras 4B. K-Ras 4B isoform is ubiquitously expressed and is usually referred to as K-Ras. The main difference between the Ras isoforms lies on the hypervariable regions of the C-termini (see Fig. 4.1) (Adjei, 2001; Prior and Hancock, 2001). They all carry the "CAAX" sequence at the C-termini, where "C" represents cysteine, "A" represents an aliphatic amino acid (Leucine, isoleucine, or Valine), and "X" is methionine, serine, leucine or glutamine. The "CAAX" motif is farnesylated by the enzyme farnesyl transferase (FT), which transfers a farnesyl group (F) from farnesyl-pyrophosphate (FPP) to the cysteine residue (C), and the "AAX" was cleaved followed by methylation at the farnesylated cysteine (Adjei, 2001). It is believed that the membrane anchorage of H-Ras, N-Ras, and K-Ras are through farnesylation on the C-termini for all Ras isoforms, as well as palmitoylation on one or two cysteines near the C-termini for H-Ras, N-Ras, and K-Ras 4A, but polybasic motif for K-ras 4B (Downward, 2003).

The activation of PLD by Ras has been somewhat controversial. While it has been reported that activated Ras leads to elevated PLD activity (Carnero et al., 1994; Jiang et al., 1995a; 1995b; Lucas et al., 2000), it has also been reported that activated Ras does not elevate PLD activity (Alam et al., 1995). Most, if not all studies showing that Ras activates PLD activity were performed with H-Ras, while the study showing that Ras did not activate PLD activity employed K-Ras[4]. These data suggest that H-Ras activates signals not activated by K-Ras. In this chapter, we have investigated the differential

[4] K-Ras 4B is ubiquitously expressed and usually referred to as K-Ras. I use K-Ras 4B as K-Ras for the purpose of clarity in the rest of this dissertation.

activation of RalA and PLD by H-Ras and K-Ras, and describe the synergistic activation of PLD by ARF6 and RalA. A model is proposed for the activation of PLD by H-Ras whereby a RalA/PLD complex is activated leading to the recruitment of the PLD activator ARF6 into an active RalA/PLD/ARF6 complex.

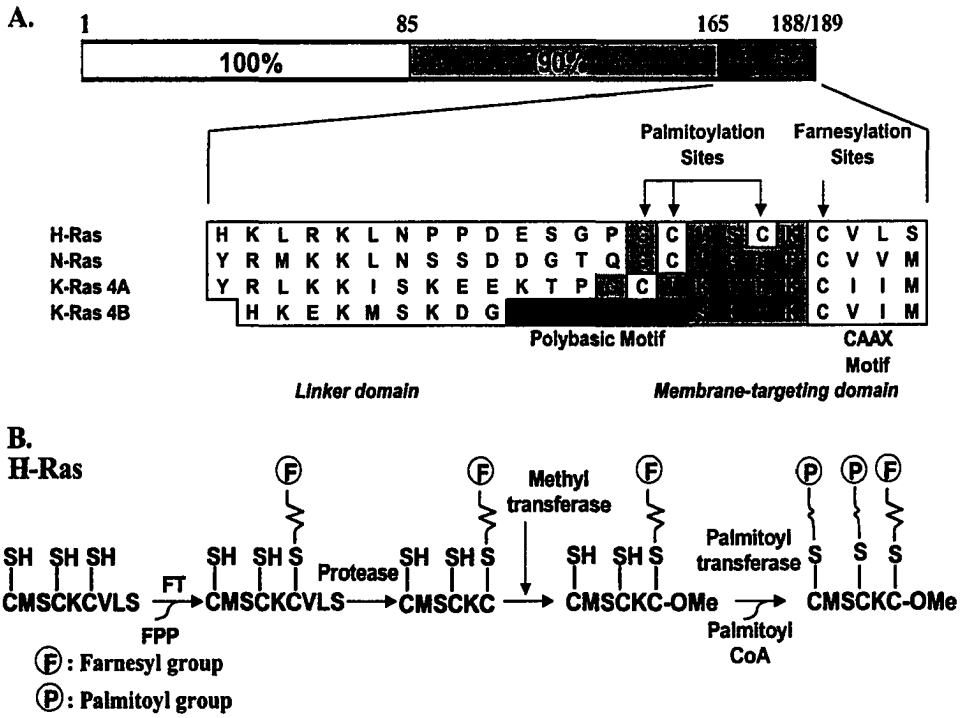


FIG. 4.1. Comparison of Ras hypervariable regions.
(A) Ras hypervariable regions. The diagram shows the degree of sequence conservation between isoforms along the Ras proteins. All of the effector, exchange factor and nucleotide-binding sites are found in the N-terminal conserved domains. The hypervariable region (HVR) is the only region of significant divergence between the Ras proteins and exhibits <10-15% sequence identity between any two isoforms. The HVR can be divided into two domains: the membrane-targeting domain and the linker domain. The membrane-targeting domain comprises the C-terminal CAAX motif, common to all Ras proteins, plus second signal sequences: cysteine palmitoylation sites in H-Ras, N-Ras and K-Ras4A or a polybasic motif in K-Ras4B. Note that in the text K-Ras refers to K-Ras4B. (B) Simplified scheme of the post-translational processing of H-Ras. Farnesyl transferase (FT) transfers a farnesyl group (F) from farnesyl-pyrophosphate (FPP) to the thiol group of the cysteine residue of the CAAX motif (CVLS in the case of H-Ras). The terminal tri-peptide is cleaved by a specific endoprotease in the endoplasmic reticulum. The methyl donor for the reaction catalyzed by a prenyl protein-specific methyl transferase is S-adenosylmethionine. Palmitoylation of C-terminal cysteine residues occurs before membrane localization. CoA=coenzyme A. Adapted from Adjei, 2001; Prior and Hancock, 2001; Voice et al., 1999.

4.2. Results

4.2.1. PLD activity is elevated in H-Ras-, but not K-Ras-transformed cells

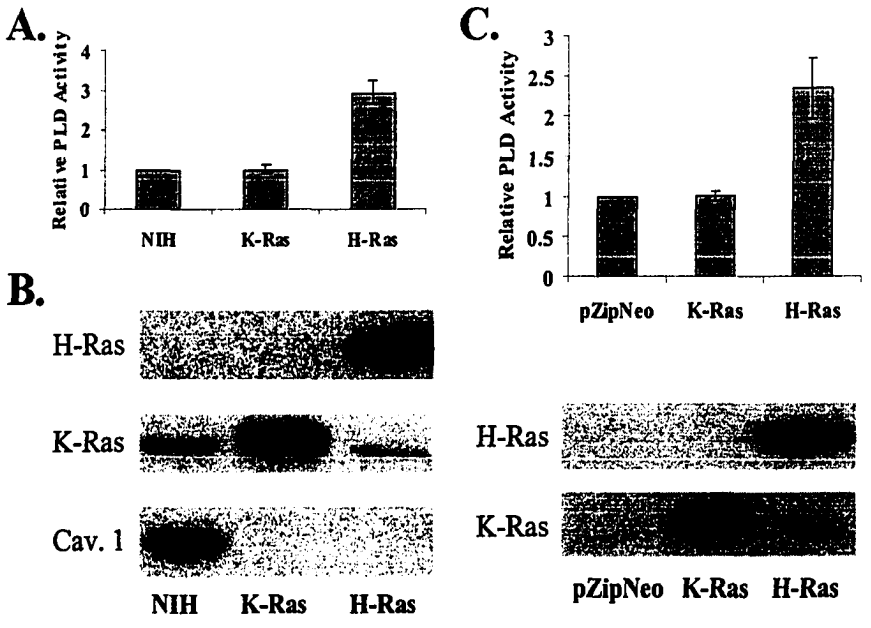


FIG. 4.2. PLD activity is elevated in H-Ras-, but not K-Ras-transformed cells. (A) NIH 3T3 cells and NIH 3T3 cells transformed by H-Ras and K-Ras were placed in media containing 0.5% serum for 1 day. PLD activity was then determined by the transphosphatidylation reaction in the presence of 0.8% butanol as described in Chapter II. The PLD activity was normalized to the PLD activity in the parental NIH 3T3 cells, which was given a value of one. Error bars represent the standard deviation for 3 independent experiments performed in duplicate. (B) Cell lysates from the parental, H-Ras and K-Ras-transformed NIH 3T3 cells were subjected to Western blot analysis using antibodies specific for H-Ras, K-Ras and caveolin 1 as indicated. (C) NIH 3T3 cells were transiently transfected with vectors that express H-Ras, K-Ras, and the parental pZIP-NeoSV(X)1 empty vector. 24 h after transfection, the cells were placed in fresh media containing 0.5% serum and after an additional 24 h, PLD activity was determined as in (A) and normalized to the PLD activity in the NIH 3T3 cells that were transfected with the empty vector pZIP-NeoSV(X)1, which was given values of one. Ras protein levels were determined by Western blot analysis as shown.

PLD activity was examined in NIH 3T3 cells transformed by either H-Ras or K-Ras. As shown in Fig. 4.2A, PLD activity was found to be elevated in the H-Ras-, but not the K-Ras-transformed cells. To ensure that the lack of PLD activity in the K-Ras

transformed cells was not due to a lack of expression of the K-Ras protein, we examined the level of Ras proteins in the H-Ras- and K-Ras-transformed cells. As shown in Fig. 4.2B, there were elevated levels of Ras proteins in both the H-Ras- and K-Ras-transformed cells. Also shown in Fig. 4.2B are the levels of caveolin 1, which is lost in transformed cells (Engelman et al., 1999; Lisanti et al., 1995), and consistent with previous reports, caveolin 1 expression was lost in both H-Ras- and K-Ras-transformed cells. The K-Ras-transformed cells were also transformed as determined by morphology and the ability to form colonies in soft agar (data not shown). This experiment has been repeated with 6 independent clones of H-Ras- and K-Ras-transformed cells as well as in pooled clones from H-Ras and K-Ras-transfected NIH 3T3 cells (data not shown), indicating that the effects are not due to clonal variation in the H-Ras- and K-Ras-transformed NIH 3T3 cells. To further establish this, we examined the PLD activity in NIH 3T3 cells transiently transfected with plasmids expressing H-Ras and K-Ras. As shown in Fig. 4.2C, PLD activity was elevated in the H-Ras-, but not the K-Ras-transfected cells. Comparable levels of H-Ras and K-Ras proteins were expressed in the transfected cells (Fig. 4.2C). These data reveal an apparent difference in the ability of H-Ras and K-Ras to activate PLD activity.

4.2.2. Differential activation of RalA in H-Ras and K-Ras

The S28N dominant negative RalA mutant (defective for GDP/GTP exchange) inhibits PLD activation by H-Ras (Jiang et al., 1995b). We therefore investigated whether there was a differential ability of H-Ras and K-Ras to activate RalA. Upon activation, RalA binds GTP and associates with the downstream effector molecule Ral-BP1 (Cantor et al., 1995). We took advantage of this by using the Ral-binding domain

(Ral-BD) of Ral-BP1 fused to GST (GST-Ral-BD) to detect activated GTP-bound RalA as described previously (Lu et al., 2000). Cell lysates from parental, H-Ras-, and K-Ras-transformed NIH 3T3 cells were treated with GST-Ral-BD immobilized on glutathione-agarose beads. The GST-Ral-BD was recovered by centrifugation and the pellets were subjected to Western blot analysis using an antibody raised against RalA. As shown in Fig. 4.3, there was 2.5-fold more RalA detected in GST-Ral-BD precipitates from H-Ras-transformed cells than in the untransformed and K-Ras-transformed cells. The level of RalA detected in the Ral-BD precipitate from the K-Ras-transformed cells was equivalent to the level detected in the parental NIH 3T3 cells. These data suggest that the reduced PLD activity is likely due to the reduced ability of K-Ras to activate RalA, and further confirm the different biological effects of H-Ras and K-Ras.

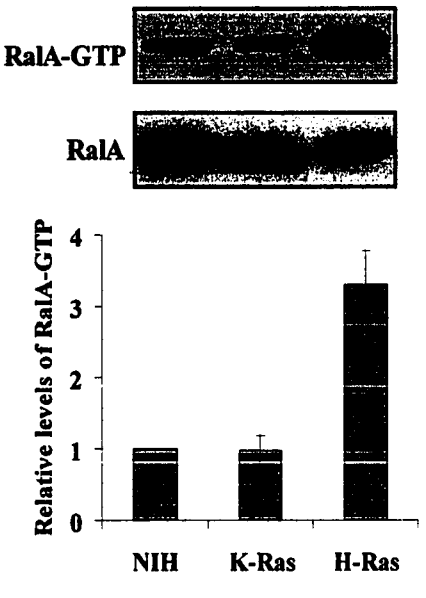


FIG. 4.3. RalA activation in H-Ras- and K-Ras-transformed cells.
NIH 3T3 cells and NIH 3T3 cell transformed by either H-Ras or K-Ras were lysed and then treated with immobilized GST-Ral-BD as described in Chapter II. GST-Ral-BD was recovered by centrifugation and the precipitate was subjected to Western blot analysis using an antibody raised against RalA. Total RalA protein levels in the cell lysates were also examined by Western blot analysis of the untreated lysates as shown. The figure shown is a representative of four independent experiments. The relative level of activated RalA was determined by densitometric analysis of the Ral-BD precipitates for four independent experiments normalized to the control NIH 3T3 cells, which was given a value of one. Error bars represent the standard error for the four experiments.

4.2.3. Increased co-precipitation of ARF6, but not ARF1, with RalA in H-Ras-transformed cells

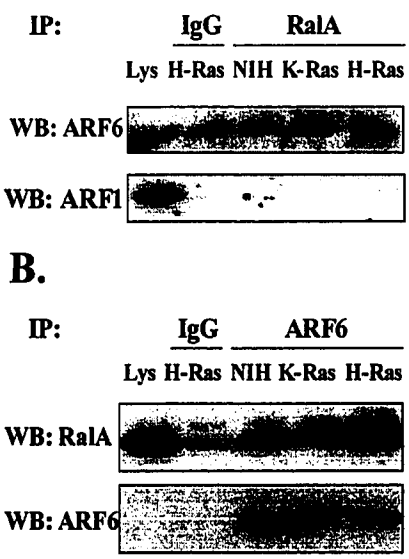


FIG. 4.4. Increased co-precipitation of ARF6, but not ARF1, with RalA in H-Ras-transformed cells. (A) RalA was immunoprecipitated from lysates of parental and H-Ras- and K-Ras-transformed NIH 3T3 cells using a mouse monoclonal RalA antibody. The RalA immunoprecipitates (RalA) were then subjected to Western blot analysis using antibodies raised against either ARF1 or ARF6 (rabbit IgGs). Non-immune IgG control (IgG) is shown, as is a portion of whole cell lysate (4%) (Lys) that was not subjected to immunoprecipitation. (B) A reciprocal experiment is shown where lysates of parental, H-Ras-, and K-Ras-transformed NIH 3T3 cells were immuno-precipitated with anti-ARF6 (rabbit IgG) in antigen excess and subjected to Western blot analysis using an antibody to RalA (mouse IgG). The membrane was stripped and reprobed with anti-ARF6 antibody to check levels of ARF6 protein immunoprecipitated. The figures shown are representative of at least three independent experiments.

RalA co-precipitates with the PLD activating ARF GTPases (Luo et al., 1998). Both ARF1 and ARF6 have been implicated in the regulation of PLD activity (Exton, 2000). The ability of ARF proteins to co-precipitate with RalA from H-Ras and K-Ras-transformed NIH 3T3 cells was therefore examined. RalA was immunoprecipitated from lysates of H-Ras- and K-Ras-transformed NIH 3T3 cells and then subjected to Western blot analysis using antibodies raised against ARF1 and ARF6. As shown in Fig. 4.4A, elevated levels of ARF6 were detected in the RalA immunoprecipitates from the H-Ras, but not K-Ras cell lysates. ARF1 was barely detectable in RalA immunoprecipitates even though ARF1 band in the lysate was stronger than that of ARF6 (Fig. 4.4A), suggesting that RalA binds much less percentage of ARF1 than that of ARF6. After longer exposure, ARF1 was detected and there were not elevated levels of ARF1 in RalA

immunoprecipitates from either H-Ras- or K-Ras-transformed cells relative to the parental NIH 3T3 cells (data not shown). Reciprocally, an ARF6 antibody co-immunoprecipitated elevated levels of RalA from H-Ras-transformed cell lysates relative to K-Ras-transformed cell lysates, in spite of equivalent levels of immuoprecipitated ARF6 (Fig. 4.4B). These data indicate that H-Ras stimulates the association between RalA and ARF6 and that ARF6 contributes to the elevation of PLD activity in H-Ras-transformed cells.

4.2.4. ARF6 co-localizes with RalA and is elevated in H-Ras-transformed cells

PLD activity elevated in response to mitogenic stimulation has been reported to be restricted largely to light membrane fractions (Xu et al., 2000). Therefore the sub-cellular distribution of RalA, ARF6 and ARF1 was examined. As shown in Fig. 4.5, RalA was detected in both light and heavy membrane fractions, however the majority of RalA was found in the light membrane fractions from H-Ras-transformed cells. ARF6 also found predominantly in the light membrane fraction. In contrast, ARF1 localized exclusively with the heavier fractions representing heavy membranes and soluble proteins. Thus, consistent with the increased association observed between RalA and ARF6 in H-Ras-transformed cells, ARF6 and RalA co-localized with the same light membrane fraction where the PLD activity elevated in response to mitogenic stimulation was also found (Xu et al., 2000). This is also the same membrane fraction to which both H-Ras and caveolin localize as described previously (Xu et al., 2000).

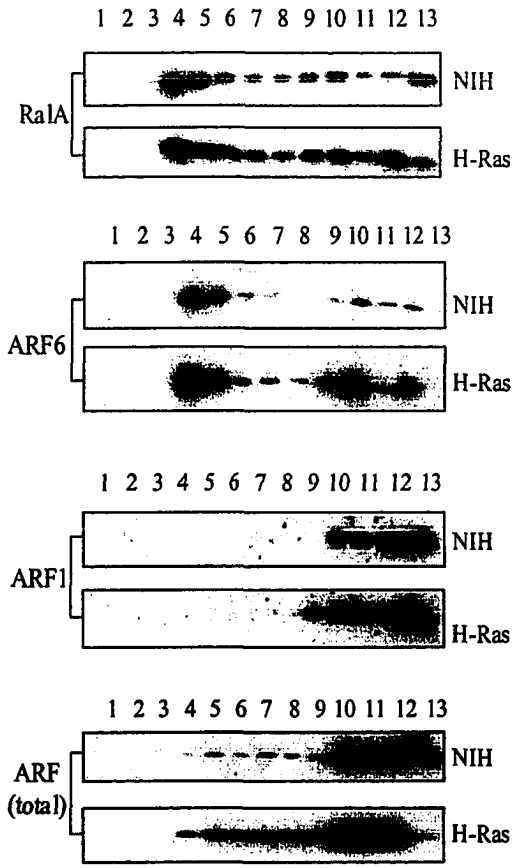
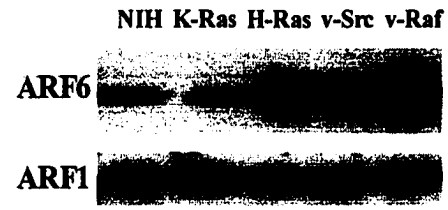


FIG. 4.5. RalA co-localizes with ARF6, but not ARF1.
Parental and H-Ras-transformed NIH 3T3 cells were disrupted by Dounce and Polytron homogenization and then sonication as described in Chapter II. The membrane fragments were then run over a discontinuous gradient of 5%, 35% and 45% sucrose. 12 fractions and a pellet were recovered and subjected to Western blot analysis using antibodies to RalA, ARF1, ARF6 and total ARF (monoclonal antibody 1D9). The amount of material loaded onto the gels was normalized by volume from each of the fractions. The figure shown is a representative of three independent experiments.



	NIH	K-Ras	H-Ras	v-Src	v-Raf
Relative ARF6	1	1.07	3.27	2.17	3.58
S.D.	0	0.28	0.96	0.57	1.28
n	6	6	7	4	4

FIG. 4.6. ARF6 is elevated in H-Ras- but not K-Ras-transformed cells.
Homogenates from NIH 3T3 cells and NIH 3T3 cells transformed by H-Ras, K-Ras, v-Src, or v-Raf were prepared as described in Chapter II and subjected to Western blot analysis using antibodies for ARF1 and ARF6. The data shown is a representative of at least 4 independent experiments. The data from several (n) experiments were quantified by densitometry of Western blots. The level of ARF6 protein relative that in the parental NIH 3T3 cells (Relative ARF6) was then determined with standard deviations (S.D.) as shown.

Interestingly, there appeared to be elevated levels of ARF6 in the H-Ras-transformed cells relative to the parental NIH 3T3 cells. The level of ARF proteins was therefore examined in the H-Ras and K-Ras transformed cells. As shown in Fig. 4.6,

there were elevated levels of ARF6 protein found in the H-Ras-transformed cells. Elevated levels of ARF6 were also observed in cells transformed by v-Src and v-Raf (Fig. 4.6). We did not detect elevated levels of ARF1 in any of the cell lines examined (Fig.4.6). These data suggest that Src, Raf and H-Ras oncoproteins stimulate elevated ARF6 expression. The lack of elevated ARF6 in the K-Ras-transformed cells further distinguish between the biological effects of H-Ras and K-Ras.

4.2.5. Activated ARF6 elevates PLD activity in NIH 3T3 cells expressing activated RalA

While there is a RalA requirement for the activation of PLD by H-Ras (Jiang et al., 1995b), an activated form of RalA (Q72L) is not sufficient to activate PLD activity by itself (Jiang et al., 1995b). We therefore examined whether a combination of activated RalA and ARF could elevate PLD activity in NIH 3T3 cells. NIH 3T3 cells or NIH 3T3 cells that express activated RalA (Q72L) were transiently transfected with vectors that express either activated ARF1 (Q71L) or activated ARF6 (Q67L), or the empty vector pcDNA3. As shown in Fig. 4.7A, neither activated ARF1, nor activated ARF6 was able to increase PLD activity in the NIH 3T3 cells. However, in the NIH 3T3 cells expressing activated RalA (Q72L), activated ARF6 (Q67L) stimulated a 2.5 fold increase in PLD activity, whereas activated ARF1 (Q71L) had no effect upon PLD activity in these cells (Fig. 4.7A). Expression of Ral and ARF protein levels in the transfected cells was determined by Western blot and is also shown in Fig. 4.7A. We also examined the effect of co-transfecting an activated RalA expression vector with activated ARF1 and ARF6 expression vectors and, as expected, only the combination of activated RalA and

activated ARF6 led to increased PLD activity (Fig. 4.7B). These data indicate a synergistic effect of RalA and ARF6 in the activation of PLD.

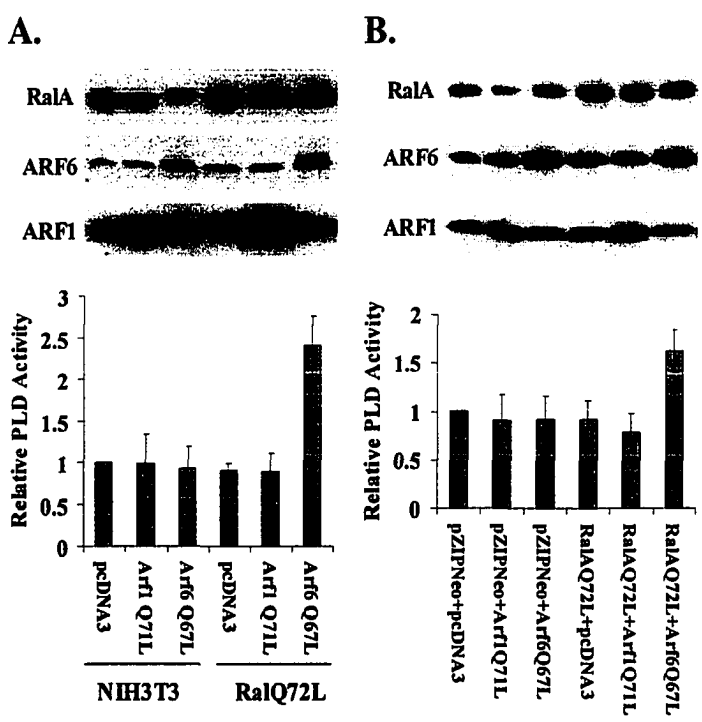


FIG. 4.7. Activated ARF6 elevates PLD activity in NIH 3T3 cells expressing activated RalA. (A) NIH 3T3 cells and NIH 3T3 cells overexpressing an activated RalA mutant (Q72L) (Jiang et al., 1995b) were transiently transfected with vectors that express activated mutants of ARF1 (Q71L) and ARF6 (Q67L) and the parental pcDNA3 empty vector. 24 h after transfection, the cells were placed in fresh media containing 0.5% serum and after an additional 24 h, PLD activity was determined as in Fig. 4.2A and normalized to the PLD activity in the NIH 3T3 cells that were transfected with the empty vector, which was given a value of one. The levels of RalA, ARF1 and ARF6 were determined by Western blot analysis as shown. (B) NIH 3T3 cells were transiently transfected with either pZIP-NeoSV(X)1 empty vector or activated RalA (Q72L) mutant, along with pcDNA3 empty vector or with vectors expressing either the activated mutant of ARF1 (Q71L), or ARF6 (Q67L), as indicated. PLD activity was determined as in (A) and normalized to the PLD activity in the NIH 3T3 cells transfected with the empty vector controls. RalA and ARF protein levels were determined as in (A). Error bars for A and B represent the standard deviation for 3 independent experiments performed in duplicate.

4.2.6. H-Ras-induced PLD activity is dependent upon ARF6

The data in Fig. 4.7 indicate that the combination of activated RalA and activated ARF6 is sufficient to activate PLD activity, but do not indicate whether RalA and ARF6 are necessary for PLD activation by H-Ras. However, RalA was reported previously to be necessary for H-Ras-activation of PLD activity (Jiang et al., 1995b). To investigate whether ARF6 is necessary for the activation of PLD activity by H-Ras, we introduced a

dominant negative mutant of ARF6 (T27N) into both parental and H-Ras-transformed NIH 3T3 cells. As shown in Fig. 4.8, the T27N ARF6 mutant had no effect upon the PLD activity in the NIH 3T3 cells, but reduced the elevated PLD activity in the H-Ras-transformed cells almost to the level seen in the parental NIH 3T3 cells. A dominant negative ARF1 mutant (T31N) had no significant effect upon the PLD activity in the H-Ras transformed cells. These data indicate that H-Ras-induced PLD activity is dependent upon ARF6.

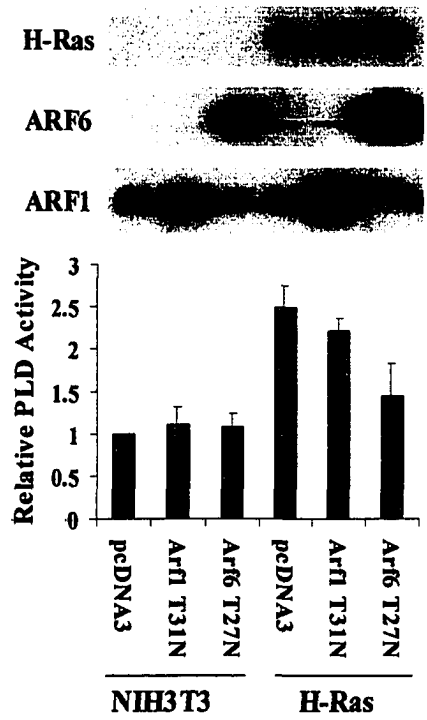


FIG. 4.8. H-Ras-induced PLD activity is dependent on ARF6.
Parental and H-Ras-transformed NIH 3T3 cells were transiently transfected with vectors that express dominant negative mutants for ARF1 (T31N) and ARF6 (T27N) as described in Chapter II. As a control, these cells were transfected with pcDNA3 empty vector. 48 h after transfection, PLD activity was determined as in Fig. 4.7. The PLD activity was normalized to the PLD activity in the empty vector control for the NIH 3T3 cells, which was given a value of one. Ras and ARF protein levels were determined as in Fig. 4.7. Error bars represent the standard deviation for 3 independent experiments performed in duplicate.

4.2.7. ARF6 mediates EGF induced PLD activity

EGF-induced PLD activity, which is dependent upon RalA (Lu et al., 2000; Shen et al., 2001), has been shown to be restricted to light membrane fractions (Xu et al., 2000). EGF also stimulates GTP loading on ARF6 (Boshans et al., 2000). Therefore the dependence of EGF-induced PLD activity on ARF6 was examined. For these experiments, 3Y1 cells overexpressing the EGF receptor (EGFR cells) (Hornia et al.,

1999; Lu et al., 2000; Shen et al., 2001; Xu et al., 2000) were used. These cells were stably transfected with an empty vector and the vector expressing the dominant negative ARF6 (T27N). Several clones were pooled and then examined for the ability of EGF to induce PLD activity. In Fig. 4.9A, it is shown that the dominant negative mutant ARF6 inhibited EGF-induced PLD activity.

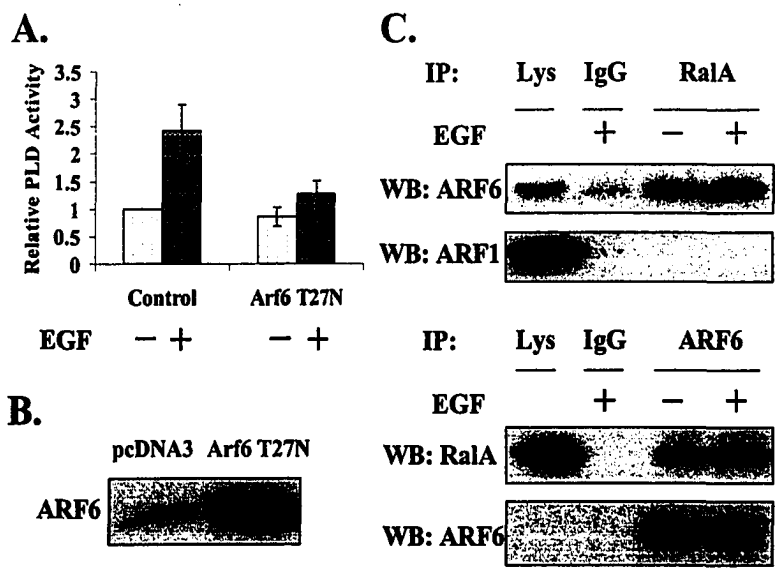


FIG. 4.9. ARF6 mediates EGF induced PLD activity.
(A) EGFR cells were stably transfected with pcDNA3 (empty vector) and the vector expression the dominant negative mutant ARF6 (T27N). Cells were selected in the presence of G418 for 2 weeks and clones were pooled and used for experiments. EGF (100 ng/ml) was added as indicated and the PLD activity was determined after 15 min. The PLD activity was normalized to the PLD activity in the empty vector control cells without EGF, which was given a value of one. Error bars represent the standard deviation for 3 independent experiments performed in duplicate. The level of ARF6 proteins in the transfected cells was confirmed by Western blot analysis (B). (C) Lysates from the EGFR cells that were either untreated or treated with EGF (100 ng/ml) for 15 min were lysed and then immunoprecipitated with an anti-RalA antibody. Immuno-precipitates were then subjected to Western blot analysis with antibodies to either ARF1 or ARF6 as in Fig. 4.4. A reciprocal experiment is shown where cell lysates were immunoprecipitated with anti-ARF6 antibody and subjected to Western blot analysis using an antibody to RalA. The membrane was stripped and reprobed with anti-ARF6. The figure shown is a representative of at least three independent experiments.

We also examined whether EGF could induce the association between ARF6 and RalA seen in the H-Ras-transformed cells. As shown in Fig. 4.9C, EGF increased the

amount of ARF6 in RalA immunoprecipitates and the amount of RalA in ARF6 immunoprecipitates. These data indicate that the role of ARF6 in regulating PLD activity is not an indirect effect of transformation. And since the stimulated increase in association could be detected within 15 min, the data also demonstrate that the effect ARF6 is not due to the elevated ARF6 seen in transformed cells.

4.3. Discussion

4.3.1. Working model for activation of PLD1 in H-Ras-transformed cells

In this report, we have demonstrated that H-Ras but not K-Ras activates PLD activity. Similarly, H-Ras, but not K-Ras, led to elevated levels of ARF6 protein. H-Ras but not K-Ras activated RalA, which interacts directly with PLD1 (Jiang et al., 1995b; Luo et al., 1998; 1997). Neither activated RalA nor activated ARF6 were able to elevate PLD activity by itself; however, a combination of activated RalA and activated ARF6 was able to elevate PLD activity in NIH 3T3 cells. Since the activation of PLD activity in H-Ras-transformed NIH 3T3 cells was dependent upon both ARF6 and RalA, the data presented here suggest a model whereby H-Ras activates PLD activity through the synergistic activation of both RalA and ARF6. In Fig. 4.10, we present a working model for the activation of PLD activity by H-Ras. It is proposed that H-Ras activates two parallel pathways leading to the activation of a guanine nucleotide dissociation stimulator (GDS) for RalA (Urano et al., 1996) and an as yet unspecified ARF-GDS. The activation of Ral-GDS by H-Ras activates RalA, which is already in a complex with PLD1 (Jiang et al., 1995b; Luo et al., 1998; 1997). The activation of RalA is proposed to recruit activated ARF6 into the RalA-PLD1 complex, or alternatively, activated ARF6 recruits

the activated RalA/PLD1 complex. Activated ARF6, now in a RalA/PLD1/ARF6 complex, would activate PLD1.

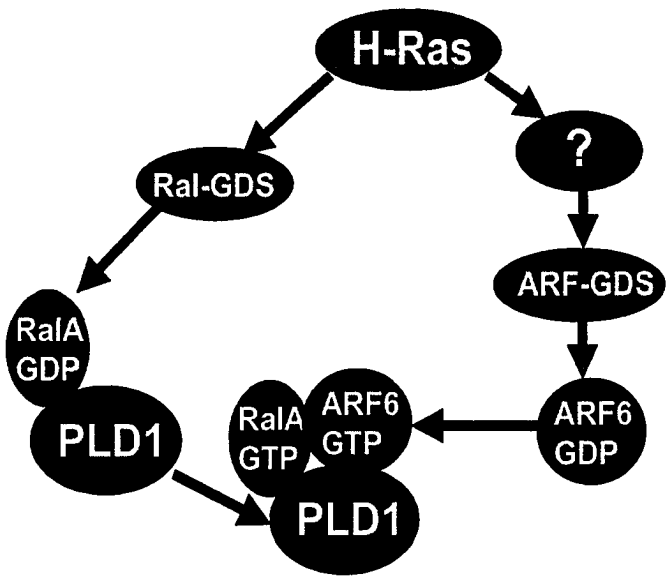


FIG. 4.10. Working model for activation of PLD1 by the synergistic action of RalA and ARF6 in H-Ras transformed cells.
It is proposed that H-Ras activates parallel pathways leading to the activation of Ral-GDS and an as yet unspecified ARF-GDS. Activation of Ral-GDS activates RalA, which is already in a complex with PLD1. The activation of RalA is proposed to recruit activated ARF6 into the RalA-PLD1 complex, or alternatively activated ARF6 recruits the activated RalA/PLD1 into a RalA/PLD1/RalA complex. The activated ARF6 then activates PLD1.

4.3.2. Correlation between elevated PLD activity and ARF6

As reviewed in Chapter I, there is a correlation between the function of ARF6 and mitogenic PLD activity: both ARF6 and PLD are involved in receptor endocytosis, exocytosis, regulation of actin cytoskeleton, and the activation of PI(4)P5-kinase (§1.3.3.3). The data presented here provide evidence that ARF6 is a regulator of the PLD activity elevated in response to mitogenic stimulation, which like ARF6 is localized primarily in light membrane or "lipid raft" fractions (Xu et al., 2000).

RalA, which interacts directly with PLD1 (Luo et al., 1997), was also localized in the light membrane fraction and was co-immunoprecipitated with ARF6. It was

previously demonstrated that ARF1 and RalA could synergistically activate PLD1 *in vitro* (Kim et al., 1998). In the *in vivo* studies presented here, ARF1 was unable to stimulate PLD activity in cells with activated RalA, and a dominant negative ARF1 was unable to inhibit H-Ras-induced PLD activity. These data likely reflect the different distribution of ARF1 and ARF6 in cells where ARF6 localizes to the plasma membrane and lipid rafts where the PLD activity elevated in response to mitogenic stimulation is localized (Xu et al., 2000). Whereas ARF1 is able to activate PLD1 *in vitro*, ARF1 would not have access to RalA/PLD1 complexes in intact cells because of the differential localization. In this regard, the recent report that antigen stimulation of RBL-2H3 cells leads to the co-localization of PLD1 and ARF6 at the plasma membrane (Powner et al., 2002) further suggests that ARF6 is a physiological regulator of PLD1.

4.3.3. PLD1 is likely the primary target of RalA and ARF6

ARF proteins activate PLD1, but not PLD2 *in vitro* (Colley et al., 1997b; Exton, 2000; Hammond et al., 1997). RalA, which we have shown to co-precipitate with ARF6, interacts directly with PLD1 (Luo et al., 1998). These data implicate PLD1 as being responsible for the elevated PLD activity in cells expressing activated forms of RalA and ARF6. However, the elevated PLD activity in H-Ras-transformed cells is largely restricted to light membrane fractions where there is very little PLD1 and lots of PLD2 (Xu et al., 2000). In this regard, it is of interest that both PLD1 and PLD2 are able to cooperate with either EGF receptor or c-Src overexpression to transform rat fibroblasts (Joseph et al., 2001; Lu et al., 2000). In addition, both PLD1 and PLD2 were required for ligand-induced endocytosis of the EGF receptor (Shen et al., 2001). These data suggest that PLD1 and PLD2 may be working together to generate phosphatidic acid and the

downstream effects of PLD signaling. A recent report by Mwanjewe et al. (Mwanjewe et al., 2001) suggested that the activation of PLD2 was dependent upon the activation of PLD1. Thus, the activation of PLD1 through RalA and ARF6 may also lead to the activation of PLD2 in the light membrane fractions where both PLD1 and PLD2 are present (Czarny et al., 1999; Kim et al., 1999a; Liscovitch et al., 1999; Xu et al., 2000). While it is not clear how PLD1 might lead to the activation of PLD2, the data presented here suggest that the PLD activated through the synergistic action of RalA and ARF6 is PLD1, since RalA associates with PLD1 and ARF proteins activate PLD1.

4.3.4. The activation of ARF6 by H-Ras

While it has been demonstrated that H-Ras activates RalA by activating Ral-GDS through a GTP-dependent direct interaction (Urano et al., 1996), how H-Ras activates ARF6 is not clear. We reported previously that the elevated PLD activity in H-Ras-transformed cells was partially sensitive to brefeldin A (BFA) (Luo et al., 1998). However, the known GDS proteins for ARF6-ARNO, EFA6, and ARF-GEP_{100} have all been reported to be insensitive to BFA (Chavrier and Goud, 1999; Franco et al., 1999; Someya et al., 2001). Others have reported that PLD activity stimulated by the mitogenic stimulus of PDGF and insulin are also sensitive to BFA (Rizzo et al., 1999; Shome et al., 1998; 2000; 1997). Thus, at present, we must conclude that either: A) GDS proteins for ARF6 can display differential sensitivities to BFA in different contexts; or B) that there is another ARF6-GDS that has yet to be identified that is sensitive to BFA. In support of hypothesis A, PMA-induced PLD activity has been reported to be both BFA sensitive (Shome et al., 1998) and BFA insensitive (Guillemain and Exton, 1997). Moreover, all ARF-GDS proteins characterized to date have the *sec7* domain, which is critical for

interacting with BFA (Chavrier and Goud, 1999; Peyroche et al., 1999; Robineau et al., 2000). In this regard, the concentration of BFA used in different experimental systems could be important. A wide range of BFA concentrations have been used to block different BFA-sensitive functions. Thus, at present, it is not clear which ARF-GDS is activating ARF6 in H-Ras-transformed cells, nor is it apparent how H-Ras might activate the ARF6-GDS. This parallel signaling pathway activated by H-Ras that contributes to the activation of PLD activity might reveal another distinct signaling pathway initiated by activated Ras GTPases.

PDGF-induced PLD activity was reported to be dependent upon Ras (Lucas et al., 2000), and was also blocked by dominant negative mutants of both ARF1 and ARF6 (Shome et al., 1998). This data would appear to be in conflict with the data reported here where we showed that the elevated PLD activity in H-Ras-transformed cells was inhibited by a dominant negative ARF6, but not a dominant negative ARF1. This difference could indicate that the activation of PLD by PDGF involves a more elaborate mechanism, or that in cells transformed by H-Ras, there is a preferential utilization of ARF6 over ARF1 that does not occur in the non-transformed cells treated with PDGF. In the cells we have used, ARF1 is not present in the light membrane fraction where the PLD activity elevated in response to mitogenic stimulation is localized (Xu et al., 2000). This apparent difference in the ARF1 dependence suggests that under some circumstances that ARF1 may localize differently and therefore regulate the PLD activity elevated in response to mitogenic signaling. The data may also reflect differences in BFA sensitivity since activation of ARF1 is sensitive to BFA in some circumstances (Moss and Vaughan, 1998; Robineau et al., 2000). In this regard, PDGF-induced PLD

activity was completely inhibited by BFA (Shome et al., 1998), whereas the elevated PLD activity in H-Ras-transformed cells was only partially inhibited by BFA (Luo et al., 1998).

Santy and Casanova (Santy and Casanova, 2001) demonstrated that ARNO, via the activation of ARF6, can lead to elevated PLD activity in Madin-Darby canine kidney epithelial cells. These data suggested that that activation of RalA was not needed to elevate PLD activity in these cells. This observation could reflect differences between the epithelial cells used in this study and the fibroblasts we have used in our studies. The data from the epithelial cell study could also indicate that ARNO can do more than activate ARF6. Although this study indicated that ARNO did not activate ARF1, Rac1 was activated in these cells and Rac1 has been implicated as a regulator of PLD1 (Bowman et al., 1993; Hammond et al., 1995). Thus, the regulation of ARF and PLD activity may be different in different cell types involving different GTPases.

4.3.5. Differential PLD activation by H-Ras and K-Ras

The differential activation of PLD by H-Ras and K-Ras suggests that H-Ras and K-Ras may have different downstream targets. Differential effects of H-Ras and K-Ras have been reported previously. H-Ras activates phosphatidylinositol-3-kinase more efficiently than K-Ras (Yan et al., 1998), whereas K-Ras activates Raf1 (Voice et al., 1999; Yan et al., 1998) and Rac1 (Yan et al., 1998) more efficiently than H-Ras. Interestingly, H-Ras induced apoptosis more efficiently than K-Ras (Joneson and Bar-Sagi, 1999; Yan et al., 1998). H-Ras was more efficient at focus formation, and K-Ras was more efficient in inducing anchorage independent growth (Voice et al., 1999). K-Ras was also shown to be more efficient in inducing cell migration than H-Ras (Voice et

al., 1999). While several differences in the biological effects of H-Ras and K-Ras have been documented, it is not clear how the different effects of H-Ras and K-Ras are generated. It was recently reported that H-Ras and K-Ras are differentially sensitive to mutants of caveolin (Roy et al., 1999) and fractionate with different membrane microdomains (Prior et al., 2001). It has also been demonstrated that H-Ras and K-Ras take different paths to the plasma membrane (Apolloni et al., 2000; Choy et al., 1999), which might explain the reported differential membrane location of H-Ras and K-Ras (Prior et al., 2001). In the fractionation study, it was reported that H-Ras, but not K-Ras, associates with light membrane fractions (Prior et al., 2001). In contrast, other studies indicated that both H-Ras and K-Ras fractionate with light membrane fractions (Fensome et al., 1996). This differential fractionation behavior of H-Ras and K-Ras was generated using a high pH method of fractionation and it has been argued that this method could lead to artifactual dissociation of K-Ras from the light membrane fraction (White and Anderson, 2001). K-Ras associates with membrane due to both prenylation and a stretch of lysines in the C-terminus of K-Ras. Since the pK for lysine is about 10.5, these lysines would be largely uncharged at pH 11 and therefore the forces holding K-Ras on the membrane would be reduced. In contrast, Ha-Ras, which is both prenylated and palmitoylated, would not be similarly affected by the elevated pH. We have performed fractionation studies on membranes from both H-Ras- and K-Ras-transformed cells using both high and neutral pH strategies. Our results showed that at neutral pH, both H-and K-Ras fractionated with the light membrane fraction as reported by Furuchi et al., (Furuchi and Anderson, 1998), and using the high pH method, only H-Ras fractionated with the light membrane fraction as reported by Prior et al. (Prior et al., 2001) (see also

Appendix, Figs. A.1 and A.2). However, PLD activity is elevated in H-Ras-, and not in K-Ras-transformed cells, and the PLD activity In H-Ras-transformed cells is restricted to the light membrane fraction. Therefore, it would appear that H-Ras and K-Ras have some kind of differential location within light membranes or perhaps in distinct light membrane microdomains. This could reflect the different pathways taken to the plasma membrane (Apolloni et al., 2000; Choy et al., 1999). Thus, while there is still much to be learned as to how H-Ras and K-Ras activate different downstream signaling machinery, the data provided here provide further evidence for different signaling pathways mediated by the two GTPases.

The differential activation of PLD by H-Ras and K-Ras may also be of significance in tumor progression. Activating mutations to K-Ras are common in many human cancers, whereas activating mutations to H-Ras are relatively rare and restricted to select tumor types (Bos, 1989). Since the mutations that can activate either H-Ras or K-Ras are similar and since activated forms of both H-Ras and K-Ras transform cells in culture, it is likely that activating mutations to H-Ras, which in theory, are just as likely to occur, are selected against. If true, then it is possible that the additional function of PLD activation by H-Ras leads to apoptosis. Consistent with this hypothesis, Walsh and Bar-Sagi (Walsh and Bar-Sagi, 2001) have shown that H-Ras induces apoptosis more efficiently than K-Ras. Elevated expression of either PLD1 or PLD2 is able to cooperate with an overexpressed tyrosine kinase to transform rat fibroblasts (Joseph et al., 2001; Lu et al., 2000) and PLD activity has been reported to be elevated in some human cancers (Noh et al., 2000; Zhao et al., 2000). These data indicate that PLD activity can play a role in mitogenic signaling. However, in response to inappropriate mitogenic signals,

cells frequently respond by undergoing apoptosis (Hueber and Evan, 1998). In this regard, it could be hypothesized that K-Ras is tolerated in an emerging tumor because it does not stimulate the RalA-PLD pathway. Ironically, the Ral pathway appears to be the most critical for H-Ras to transform human cells (Hamad et al., 2002). The data presented here indicate that K-Ras does not activate the Ral pathway in murine fibroblasts. We do not know whether this is also true for human cells. However, the observation that the transformation of human cells by H-Ras is due to activating the Ral pathway further suggests that in an emerging tumor the transformed phenotype induced through this pathway is selected against, preventing the appearance of tumors with activated H-Ras. We previously reported that expression of either PLD1 or PLD2 in 3Y1 rat fibroblasts leads to apoptosis (Zhang et al., 1999). Thus, while elevated PLD activity can contribute to a transformed phenotype, too much PLD signaling in H-Ras-transformed cells may help sensitize these cells to apoptotic cell death and ironically make H-Ras mutations less of a problem in human cancer than mutations to K-Ras because of the ability of these cells to survive.

Chapter V. PLD in Mitogenic Signaling

In this Chapter, I will discuss the significance of this dissertation work and provide a new view of the regulation and membrane localization of mitogenic PLD. Chapter III has shown that the PLD activity is mostly restricted to the caveolin-enriched light membrane microdomains (CEMMs) in response to mitogenic signals. This result has provided evidence that mitogenic signaling can be initiated in the CEMMs. Chapter IV has shown that H-Ras but not K-Ras activates PLD through the synergistic action of RalA and ARF6. This result has distinguished not only H-Ras and K-Ras signaling, but also ARF1 and ARF6 signaling. This work has underscored the mechanism of the regulation of PLD in the Ras pathway, and helped to provide a link between extracellular agonists and intracellular signaling through PLD. A new view of the dynamic regulation of PLD in mitogenic signaling cascades and a model of Ras and PLD in the membrane microdomains are provided.

5.1. Significance of the study

The work in Chapter III (published in Xu et al., 2000) has shown that the elevated PLD activity in v-H-Ras, v-Raf, and v-Src transformed cells is largely restricted to CEMMs where many signaling molecules aggregate to form signaling complexes (Okamoto et al., 1998).

These data indicate that mitogenic signals through PLD are initiated in CEMMs where many signaling molecules co-localize. PLD1 is present mostly in the heavy membrane fractions, and to a much lesser extent in the light membrane fractions, whereas PLD2 is largely restricted to the light membrane fractions. Therefore, neither the role of

PLD1 nor that of PLD2 in the mitogenic signaling can be ruled out. Some studies argued that PLD2 is the major player on plasma membrane (Liscovitch et al., 2000). But in the oncogene-transformed cells, PLD activity depends upon Ral (Frankel et al., 1999; Jiang et al., 1995b), which only interact with PLD1 but not PLD2 (Luo et al., 1998; 1997). The presence of PLD1 in CEMMs provides evidence that PLD1 may be the first activated regulator in mitogenic signaling before PLD2.

The work in Chapter III has also demonstrated that polyunsaturated fatty acid-containing PC is excluded from the CEMMs. This result supports the model that phospholipids with saturated or monounsaturated fatty acids are packed tightly with cholesterol in lipid rafts (Harder and Simons, 1997; Simons and Ikonen, 1997). As discussed in section 5.5, this result, along with the results presented in Chapter IV, also argued the localization and function of PLD function in the cell membrane.

The work in Chapter IV (published in Xu et al., 2003) has shown that H-Ras but not K-Ras elevates PLD activity by the synergistic action of RalA and ARF6.

We have shown, for the first time, that H-Ras, but not K-Ras, can activate the Ral-GDS pathway and elevate ARF6 levels. This further distinguishes the biological effects of H-Ras and K-Ras.

We also showed that the ARF that activates PLD in the CEMMs is ARF6, but not ARF1. First, although ARF1 can interact with RalA in the whole cell lysates, the percentage of the total ARF1 that binds to Ral is very low compared to that of ARF6 (see Chapter IV), suggesting it is likely that the Ral/PLD/ARF6 complex is more relevant. Second, only ARF6 co-localizes with RalA in the CEMMs, where the mitogenic PLD activity is. Third, ARF6, but not ARF1, is increased in the oncogene-transformed cells

that correlate with the elevated PLD activity. Fourth, active ARF6, but not ARF1, can activate PLD along with active RalA. Moreover, dominant-negative ARF6 but not ARF1 dramatically decreases the PLD activity by H-Ras, and dominant-negative ARF6 also can block the activation of PLD mediated by EGF (see Chapter IV for more details). All these results support the notion that ARF6 but not ARF1 is the main player to activate PLD in response to mitogenic stimuli. However, the role of ARF1 in the PLD activation in different cellular compartments cannot be excluded. As discussed above, RalA can bind to ARF1 and there is a certain amount of RalA that co-localizes with ARF1 in the Golgi membrane fractions (our unpublished data), suggesting that RalA/PLD1/ARF1 can also form a complex in certain cellular context or in certain signaling pathways.

As discussed in Chapter IV, the results in Chapters III and IV also argued that PLD1 is likely the primary target of RalA and ARF6. As discussed in sections 5.5, activation of PLD1 via RalA and ARF6 might lead to activation of PLD2 in the light membrane fractions, where both PLD1 and PLD2 are present.

The work also provides the link of the PLD in Ras signaling to tumor progression, as discussed in Chapter IV.

This work, along with others' as discussed below, makes it possible to view the PLD regulation at new levels, and provides connections between signaling cascades (see sections 5.2~5.4). Our work has also argued the membrane localization of Ras and PLD, as discussed more in Section 5.5.

5.2. Regulation of PLD at four different levels

Although PLD can be regulated in a long-term way by protein synthesis and degradation, in this dissertation, I focused on the short-term regulation of PLD activity in

signaling cascades. Under this circumstance, I viewed the regulation of mammalian PLD at four indivisible sub-levels: extracellular level (agonists), transduction level (signaling pathways), co-factor level (proteins and lipids), and dynamic level (post-translational modification and change of conformation and localization).

The transduction level of regulation and its involvement in the linkage between the extracellular level and the co-factor level in signaling cascades will be discussed in next two sections. The dynamic level of the regulation of PLD will be addressed in §5.5.

5.3. Regulation of PLD in transduction level

PLC, PKC, Ras, and PI3-Kinase pathways are believed to transduce certain extra- or intra-cellular signals to regulate PLD activity, and are considered as the transduction level of regulation. The possibility that protein kinase A (PKA) can regulate PLD activity has been recently reviewed (Choi et al., 2002) and will not be addressed here.

5.3.1. PLC/PKC Pathway

PKC pathway appeared to be involved in mediating PLD activity stimulated by different classes of agonists (see Chapter I). It was demonstrated that direct PKC stimulation of PLD1 is coupled to G protein-coupled receptors since there appeared to be loss of receptor response after the PKCα binding site on PLD1 was disrupted (Zhang et al., 1999). PLC, which leads to increase in cellular Ca^{2+} and diacylglycerol and subsequently activates PKC, provides the link between PKC-mediated PLD activation and the agonists acting through receptors PTK, PTK-associated receptors, or trimeric G protein-coupled receptors (GPCR) (for review of PLC, see Rhee, 2001). There are four different PLCs (Fig. 5.1). Some studies demonstrated that PLCγ is involved in mediating PLD activation through receptors PTK stimulated by growth factors PDGF (Hess et al.,

1998; Lee et al., 1994; Yeo et al., 1994) and insulin (Slaaby et al., 2000), through GPCRs stimulated by carbachol (Min et al., 2000), or through PTK-associated receptors by cross-linking of the B cell receptor (Hitomi et al., 1999). It is also reported that PLC-β is involved in GPCR-mediated PLD activation via PKC as an intracellular signal stimulated by α1-adrenergic agonists, such as endothelin-1 (ET-1) and angiotensin II (Eskildsen-Helmond et al., 1997).

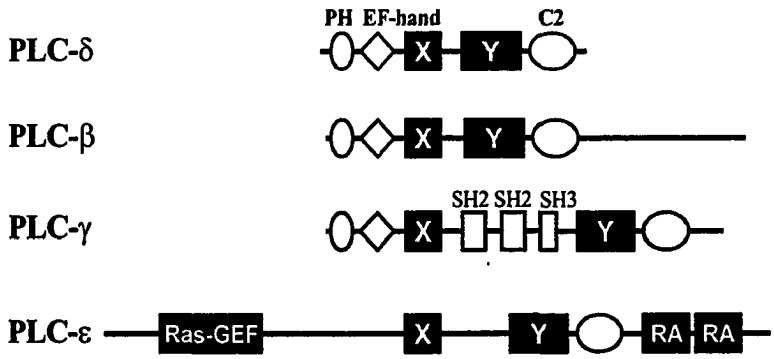


FIG. 5.1. Domain organization of the four types of PLC isozymes.
The X and Y catalytic domains as well as the PH, EF-hand, C2, SH2, SH3, Ras-GEF, and RA (Ras binding) domains are indicated. Adapted from Rhee, 2001.

PLC/PKC pathway may also attenuate PLD activation. Hydrolysis of PIP_2 will decrease the level of PIP_2, an important cofactor for PLD, which is present at a very low concentration (<0.1% of total phospholipids) (Exton, 1997b). But it is unknown if the same pool of PIP_2 is used by PLC and PLD. PKC may also act as an inhibitor for PLD activation. For example, downregulation of PKC isoforms by long-term treatment with phorbol esters or inhibition of PKCδ by rottlerin increases PLD activity (Hornia et al., 1999), and dominant-negative PKCδ can also potentiate PLD activation (Hornia et al., 1999). It is also implicated that overexpression of PKCε inhibits both phorbol ester and PDGF-induced PLD activity (Kiss et al., 1999). The mechanism of the inhibitory effect of PKC isoforms is not clear yet. Interestingly, phosphorylation of PLD through PKCα

by phorbol ester has been shown to inactivate PLD1 (Hu and Exton, 2003). It is also worth noting that PKC also decreases GEF activity of ARNO (Macia et al., 2000; Santy et al., 1999), one of the GEFs for ARF6-dependent PLD activation. PKC can also attenuate the Ras-mediated activation of Ral-GDS (Rusanescu et al., 2001), one of the GEFs for Ral-mediated PLD activation.

5.3.2. PI3-kinase pathway

PI3-kinase-dependent activation of PLD has been reported through different receptors (receptors PTK, PTK-associated receptors, and GPCRs) in response to stimulation of different agonists, such as insulin (Karnam et al., 1997; Standaert et al., 1996), agents that cross-link IgE receptors (FcεRI) (Powner et al., 2002) or IgG receptors (FcγRI) (Gillooly et al., 1999), and fMLP (Fensome et al., 1998; Nakamura et al., 1997). It is also reported that PI3-kinase is required for PLD activation by Ca^{2+}-mobilizing agents such as thapsigargin (Cissel et al., 1998). Interestingly, PI3-kinase has been implicated to mediate almost all the proteins that regulate PLD, including PKC, Ral, ARF, and Rho. Several reports indicated that PI3K is required for the membrane translocation of PKCα, -β (Bourgoin et al., 2002; Standaert et al., 1996), and GEFs for ARF proteins, such as ARNO (Blagoveshchenskaya et al., 2002; Bourgoin et al., 2002; Venkateswarlu et al., 1998b), GRP (Venkateswarlu et al., 1998a), and cytohesin-1 (Nagel et al., 1998). PI3K is also required for the activation of GEFs for Rho family proteins (Booden et al., 2002; Fleming et al., 2000; Han et al., 1998; Innocenti et al., 2003; Weiner et al., 2002). Recently, it was shown that PKC, ARF, and Rac1 are all associated with PLD1 in a PI3K-dependent manner (Powner et al., 2002). PI3K is also involved for Ral activation, since its downstream target PDK1 is important for activation of Ral-GDS

(Tian et al., 2002). However, it should not be overstated that PI3K activates GEFs for these small G proteins, since the PIP_3 generated by PI3K activation does not act as an activator, but rather a membrane recruiter. It is reminiscent of the reports that Ras-mediated activation of Rac1 could be regulated through not only PI3K-dependent mechanism (Nimnual et al., 1998; Scita et al., 1999), but also PI3K-independent mechanism (Lambert et al., 2002), in which Tiam1, a Rac1-specific GEF, is activated directly by association with GTP-bound Ras (Lambert et al., 2002).

It should be noted that changing of PIP_2 to PIP_3 decreases the level of PIP_2, a key effector for PLD activation (Brown et al., 1993). PIP_3 can also activate PLD (Hammond et al., 1997; Liscovitch et al., 1994), but it is not as potent as PIP_2. However, the significance of this change is unknown. As discussed above, PIP_3 can also recruit PKC to the membrane, which decreases the GEF activity of ARNO (Macia et al., 2000; Santy et al., 1999) and Ral-GDS (Rusanescu et al., 2001). In this regard, PI3K may somehow attenuate PLD activity. By using wortmannin, a PI3K inhibitor, one report showed that the late, but not early, phosphatidylcholine-hydrolysis induced by TNFα is not dependent on PI3K (Plo et al., 2000). Whereas other reports showed that wortmannin, which inhibits EGF-stimulated PI3K activity, can potentiate the EGF-stimulated PLD activity (Zhang and Akhtar, 1998), and wortmannin can also enhance PLD activation stimulated by the phorbol ester PMA in fibroblasts (Kiss and Tomono, 1995) and by TPA, ATP, and bradykinin in vascular endothelial cells (Natarajan et al., 1997). These results collectively support the notion that PI3K may play an inhibitory role in the regulation of PLD.

PI3K is an important downstream target of Ras, and in certain context, it might be considered within the Ras pathway.

5.3.3. Ras Pathway

In general, Ras can be activated not only through receptors PTK via GRB2/SOS, but also through GPCRs. In some cases, the GPCRs transactivate the receptors PTK, to subsequently activate Ras (Daub et al., 1996; Downward, 2003). Ras is involved in mediating PLD activation through receptors PTK induced by EGF (Voβ et al., 1999), PDGF (del Peso et al., 1997; Lucas et al., 2000; Voβ et al., 1999), and insulin (Voβ et al., 1999), and through GPCRs induced by norepinephrine (Muthalif et al., 2000), as well as by PMA (Voβ et al., 1999).

Oncogenic H-Ras also activates PLD (Carnero et al., 1994; Jiang et al., 1995a; 1995b) and we found that both RalA (Jiang et al., 1995b) and ARF6 activation (Xu et al., 2003) are required in the PLD regulation by oncogenic Ras. Oncogenic Src is able to activate PLD (Song et al., 1991) and the activation of PLD by v-Src is through Ras activation (Jiang et al., 1995a). Src is also involved in mediating PLD activation by sphingosine 1-phosphate (Ghelli et al., 2002) and growth hormone (Zhu et al., 2002). In the growth hormone regulated-PLD activity, not only Src, but also another non-receptor tyrosine kinase JAK is involved, which activates the Ras pathway (Zhu et al., 2002). How v-Raf activate PLD is not clear, but it is implicated that both RalA and Rho are required in that pathway (Frankel et al., 1999), suggesting that there is a cross-talk between Ral-GDS, Rho-GEF, and Raf/MAP kinase pathways. It was also reported that the MAP kinase pathway is required for PLD activation induced by norepinephrine (Muthalif et al., 2000) or by H_2O_2 (Banno et al., 2001b; Ito et al., 1998). In the oncogene v-H-Ras, v-Src, and v-Raf transformed cells, ARF6 levels are elevated. In addition, dominant-negative ARF6 blocks the PLD activation by oncogene H-Ras and by EGF-

stimulation (see Chapter IV). These results suggest the involvement of Ras/ARF6 pathways in the regulation of both oncogenic signaling and growth factor signaling. As discussed above, there appears to be PI3K-dependent PLD activation and PI3K is an important downstream target of Ras, but the role of PI3K pathway in Ras-regulated PLD activation is not yet documented.

Attenuation of PLD activity by Ras has also been reported. PLD is activated in oncogenic Ras-transformed cells (Carnero et al., 1994; Jiang et al., 1995a; 1995b). The activity in Ras-transformed cells is increased in response to phorbol esters (del Peso et al., 1997; Lucas et al., 2002), but the response of PLD activation by growth factor stimulation is attenuated (del Peso et al., 1997; Lucas et al., 2002; Martin et al., 1993). In contrast, overexpression of wild-type Ras gives rise to dramatic increase of PLD activity in response to PGDF, but has no effect on phorbol ester-induced PLD activity (Lucas et al., 2000). Although recent work by Lacal's group suggested that two negative feedback mechanisms mediated by Raf1 and Ral-GDS may be involved in the dysregulation in oncogenic Ras (Lucas et al., 2002), the mechanism is still not clear.

5.3.4. Integration of PLC/PKC and Ras/PI3K pathways

5.3.4.1. In signaling of the three classes of agonists

As discussed above, both PLC/PKC and Ras/PI3K pathways are involved in signaling of all three classes of agonists.

Much evidence suggested that there is concurrent involvement or cross-talk between PLC/PKC and Ras pathways in regulation of PLD activity. First, Lacal's group reported that, although PLD activation by oncogenic Ras and Src is not dependent on PKC, activation of PLD by growth factors requires the simultaneous activation of the endogenous wild-type Ras proteins and a PI-PLC/DAG/PKC-dependent mechanism (del

Peso et al., 1997; Lucas et al., 2002). Second, PKCι, one of atypical PKCs, seem to be downstream of transforming Ras-activated PLD1, but upstream of PLD2 (Mwanjewe et al., 2001). Third, Schmidt's group also reported that the dominant-negative mutant of Ras or RalA reduces the PLD activation through PKC and both Ras/Ral and PKC are involved in PLD stimulation by PMA and growth factors (Voβ et al., 1999). Fourth, Wakelam's group showed that the antigen-stimulated activation of PLD involved the concurrent association of Rac1, ARF6, and PKCα with PLD1b at the plasma membrane in actin-rich structures (Powner et al., 2002). This activation appeared to be PI3K dependent, since up to 10 μM of PI3K inhibitor LY294002 diminished about 75% of PLD activity induced by the antigen of FcγRI (Powner et al., 2002). Moreover, ARF6 but not ARF1 was shown to regulate PLD activity in myometrial homogenates and in the cell-free system, ARF6-regulated PLD activity is controlled by βγ subunits of heterotrimeric G proteins (Le Stunff et al., 2000). This result has provided a link between heterotrimeric and monomeric G proteins.

Other reports, although they did not show the direct integration of the two pathways in PLD activation, might still provide a link between PLC/PKC and Ras/PI3K pathways. First, as discussed above, PKC translocation can be regulated by PI3K (Bourgoin et al., 2002; Standaert et al., 1996). Second, PKC was implicated in the preferential down-regulation of Ras/Ral signaling (Rusanescu et al., 2001). Third, PIP_3 is involved in the stimulation of PLCγ1 through its PH domains (Balboa et al., 1994), which links Ras/PI3K pathway to the PLC/PKC pathway. Fourth, the intracellular second messenger Ca^{2+} mediates both PKC and Ras signaling (reviewed in Cullen and Lockyer, 2002). It has also been implicated that Ca^{2+} or calmodulin regulates Ral activation

(Clough et al., 2002; Hofer et al., 1998; Park, 2001; Wang and Roufogalis, 1999); and calcium/calmodulin kinase II (CaMK II) is also involved in the phosphorylation and activation the of the Rac1-GEF Tiam1 (Fleming et al., 1999; 1998) or Tiam1 translocation induced by PDGF or lysoPA (Buchanan et al., 2000). These may provide a further link between PLC and Ras pathways. Fifth, PLCε, which has two Ras association domains and one Ras-GEF domain (reviewed in Rhee, 2001), can act as not only a Ras effector, but also a Ras stimulator (Kelley et al., 2001). Furthermore, PLD activity and/or PA were implicated in regulating the activity of PKC (reviewed in Exton, 2002a), PLC (Jones and Carpenter, 1993; Zhou et al., 1999), Ras (Tsai et al., 1989), and PI3K (Banno et al., 2001a). These results may provide further links between these pathways.

5.3.4.2. In signaling of other agonists

Other agonists, including H_2O_2, sodium fluoride (NaF), phorbol ester, and Ca^{2+}, which activate PLD, can also function through the above pathways.

The intracellular concentration of H_2O_2 is transiently increased when cells are stimulated with growth factors or cytokines (Bae et al., 1997; Lo and Cruz, 1995; Sundaresan et al., 1995). H_2O_2, when added extracellularly or generated intracellularly, may inactivate protein tyrosine phosphatase (Bae et al., 1997) and thus may enhance receptor PTK activity. As discussed in Chapter I, H_2O_2 can induce not only the tyrosine phosphorylation of EGF receptor, but also that of PLD1 and PLD2 (Min et al., 2001; Min et al., 1998a). However, the tyrosine phosphorylation of PLD2 does not directly mediate PLD activation (Mehta et al., 2003). Several reports suggested that H_2O_2-induced PLD activity was mediated by Ca^{2+} influx (Ito et al., 1997; Lee et al., 2000a), PKCα activation (Lee et al., 2000a; Min et al., 2001; Min et al., 1998a), or the MAP kinase pathway (Banno et al., 2001b; Ito et al., 1998).

Bourgoin et al. reported that the PLD activity in osteoblast-like cells induced by NaF, a specific activator of heterotrimeric G proteins, was mediated by Ca^{2+} and ARF, but not PKCα; however, activation of PKCα by TPA potentiates the NaF-induced membrane translocation of ARF and thus the activation of PLD (Bourgoin et al., 1996).

For the obvious reason, phorbol esters and/or Ca^{2+} ionophores activate PKC since they either act as pseudo substrates or increase the Ca^{2+} concentration inside the cells, respectively. However, this might not be the only reason that they activate PLD. PMA-stimulated PLD activity is also sensitive to toxins that block Rho activation (Kotter et al., 2000; Ojio et al., 1996) or partially sensitive to inhibitors of ARF activation (Kotter et al., 2000; Shome et al., 1998; 2000). Furthermore, PMA-induced PLD activity is increased by overexpressing of wild-type RhoA (Malcolm et al., 1996) or decreased by expressing dominant-negative RhoA (Meacci et al., 1999). PMA was also implicated to regulate GEFs of Rho (Kong et al., 1998) and ARF (Frank et al., 1998). Ras and Ral are also involved in phorbol ester-mediated PLD activity (Schmidt et al., 1998; Voβ et al., 1999). In a mast cell line, both Rho and PI3K were implicated to be involved in the Ca^{2+}-mobilizing agent thapsigargin-induced PLD activity (Cissel et al., 1998).

Taken together, these results suggest that not only PKC pathway, but also ARF, Rho, or MAP kinase, which might be regulated through Ras/PI3K pathway, are involved in the activation of PLD by these agonists.

5.4. Regulation of PLD in signaling cascades

As discussed in Chapter I, three classes of agonists can activate PLD through their specific receptors: receptors PTK, receptors associated with PTK, and GPCRs. All these three classes of receptors can activate PLC/PKC pathway (reviewed by Rhee, 2001), PI3

kinase pathway (reviewed in Vivanco and Sawyers, 2002; Yart et al., 2002), and Ras pathway (reviewed in Downward, 2003; Hur and Kim, 2002; Lowes et al., 2002; Takai et al., 2001), which are all involved in PLD activation. As discussed above, other agonists, including H_2O_2, phorbol ester, and Ca^{2+}, can also be integrated into the above pathways. In addition, proteins that activate PLD, such as PKC, Ral, ARF, and Rho family proteins, are also involved in these signaling cascades. Accumulating evidence (see section 5.3 and Chapter I) supports the notion that these agonists, through their specific receptors via PLC/PKC and Ras/PI3K pathways, activate proteins such as PKC, Ral, ARF, and Rho family proteins, and subsequently activate PLD.

Thus, we suggest a model of the short-term regulation of PLD activity in the signaling cascades, and provide in Fig. 5.2. Agonists that stimulate receptors PTK or PTK-associated receptors can activate Ras, PI3K, and PLCγ pathways, and agonists that stimulate GPCRs can activate PLCβ, PLCε, and Ras/PI3K pathway or transactivate receptor PTK via Gα-GTP and subsequently activate Ras/PI3K and PLCγ pathways. Once Ras is activated, it activates downstream targets such as through Ral-GDS to activate Ral and through ARF6-GEF to activate ARF6. Activation of PI3K pathway either by the receptors or by Ras will change PIP_2 to PIP_3. PIP_3 recruits PDK1 to the membrane and ensures the activation of Ral-GDS/Ral pathway. PIP_3 also recruits ARF-GEF such as ARNO or Rac1-GEF, such as TIAM1, to the membrane and ensures the activation of ARF6 and Rac1. PIP_3 can also recruit PKC to the membrane for activation. Activation of different PLCs will change PIP_2 to IP3 and DAG. IP3 binds to the IP3-receptor on the ER membrane and increase Ca^{2+} concentration inside the cell. Ca^{2+}, together with DAG, will activate PKC. The activated Ral, ARF6, a Rho family G protein

such as Rac1, and PKC will bind to PLD, along with PIP_2, possibly as a membrane tether, to form an active multi-component complex. Although the detailed dynamic change of PLD upon activation is still unknown, it is well known that the activated PLD will utilize PC to generate PA and choline. The generated PA activates PI(4)P5 Kinase, which generates more $PI(4,5)P_2$, to further activate PLD. PA can also activate PLCγ1 (Jones and Carpenter, 1993; Zhou et al., 1999) or the GAPs of small G proteins Rho, Rac, and ARF (Ahmed et al., 1993; Randazzo and Kahn, 1994), but inhibit Ras-GAP (Tsai et al., 1989). In this way, it may further regulate PLD.

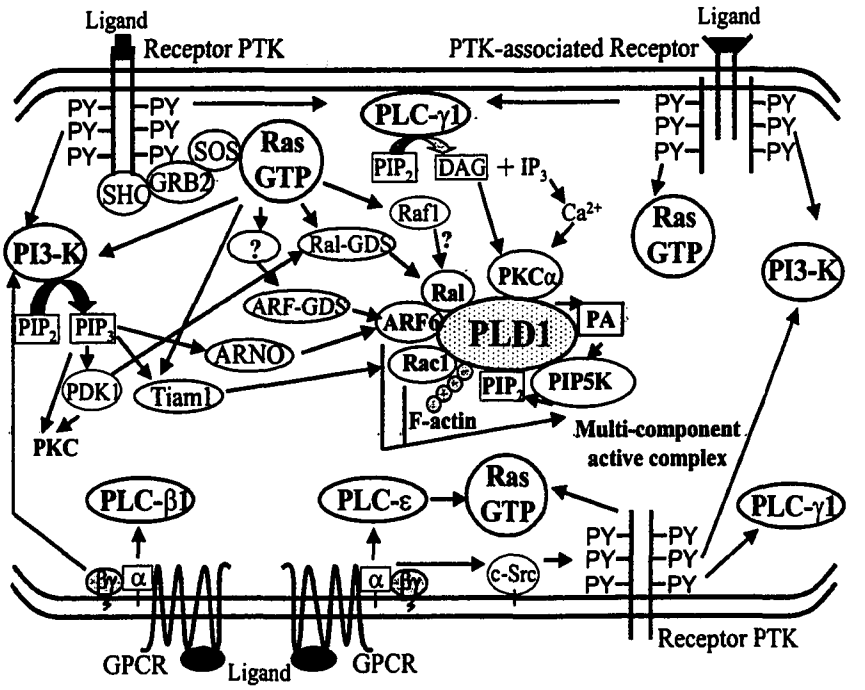


FIG. 5.2. A schematic model of the regulation of PLD1 in signaling cascades involving PLC/PKC and Ras/PI3K pathways.

Ligands binding receptors PTK can activate PLC-γ1, Ras, and PI3K pathways as indicated; ligands binding PTK-associated receptors can recruit the associated PTKs and thus activate PLC-γ1, Ras, and PI3K pathways; and ligands binding GPCR can activate PLC-γ1 and PI3K or can activate PLC-ε and thus activate Ras pathway, or alternatively, transactivate receptors PTK and thus activate PLC-γ1, Ras, and PI3K pathways. Through these pathways, RalA, ARF6, Rac1, PKCα, and F-actin can form a multi-component active complex, and the generated PA activates PIP5K to produce more PIP_2 which further activates PLD1. The attenuation of PLD activity by these pathways is not shown. Note that multiple interactions and crosstalk between different pathways may exist, even though not all might occur in the same cell. See text for details.

Interestingly, both PLD upstream regulators and downstream effectors, including Ral, ARF6, PKC, Rho, PA, and PIP_2, are all required for vesicle formation as well as actin cytoskeletal reorganization. It will be an economical way to organize the regulators and effectors altogether as an active complex to play the function. The active complex, probably with all its machinery on the new-formed vesicle, is activated further on the actin cytoskeleton in a polymerization-dependent manner and plays its dynamic role in the cell signaling.

Depending on the spatial context of cellular membrane microdomains or the temporal context of cellular signaling, not all pathways will be activated at the same extent or strength in a particular cell. Moreover, as discussed in section 5.3, all these pathways can attenuate PLD activation. How cells control the balance between these pathways and thus tune the dynamic change of PLD is poorly understood. However, we believe that the spatial-temporal regulation of PLD in these signaling cascades will draw more attention and will be extensively investigated.

5.5. Ras and PLD on the membrane

5.5.1. Re-visiting caveolae and lipid rafts

The traditional concept of caveolae is referred to the exclusive electron microscopic description of membrane invaginated "smooth" vesicles of 50 to 100 nm in size (see Razani et al., 2002 for a review). However, membranes with the classic morphologic features of caveolae are not found in all cells and new criteria were used to identify the caveolae or caveolae-related membranes, including (a) resistance to solubilization by the detergent Triton X-100 at 4°C; (b) a light buoyant density; (c) richness in glycosphingolipids, cholesterol, and liquid-anchored membrane proteins

(Anderson, 1998). This appeared to be a broadened concept of caveolae, partially due to the difficulties in study of the dynamic change of the caveolae structure and the limits of methodology. In a more stringent view, caveolae, lipid rafts, detergent-resistant membranes (DRMs) or detergent-insoluble glycolipid-enriched complexes (DIGs), and caveolin-enriched low-density membranes seem to be different (Brown and London, 1998; Simons and Ikonen, 1997) and these concepts are not interchangeable but overlapped with each other. For example, rafts are referred to the dynamic clustering of glycosphingolipids and cholesterol, which function as platforms for specific signaling molecules that can move within the fluid bilayer (Simons and Ikonen, 1997). Rafts can exist in the plasma membrane in the absence of caveolin or become fixed in caveolae in the presence of oligomerized caveolin (Simons and Ikonen, 1997). DRMs are presumably in a liquid-ordered state when they are isolated and contain highly enriched sphingolipids with saturated acyl chains, cholesterol, and GPI-anchored proteins. The low-density membranes are enriched in GPI-anchored protein and caveolin, suggesting that they are rafts (Brown and London, 1998).

In this dissertation, I used caveolae as a morphological concept, rafts for the functional membrane structure concept, and DRMs, low-density membranes, or CEMMs for the methodological concepts. In these regards, since the more stringent conditions were used to isolate DRMs, DRMs are presumably more related to caveolae with the assumption that the detergent does not disrupt caveolar structure, but the low-density membranes may contain both caveolae and non-caveolar rafts.

As discussed in next two sub-sections, we have to change this assumption and hypothesize that the lipid composition and the localized proteins vary in the different

regions of caveolae. The neck region of caveolae tends to be in the liquid-ordered state, but has liquid-disordered property. Whereas the non-ionic detergent can solubilize the neck region of caveolae, the non-detergent method can still keep it in the low-density fractions.

5.5.2. H-Ras on the neck: a new model

5.5.2.1. Ras localization

Biochemically, Ras proteins are excluded from DRMs (Melkonian et al., 1999; Prior et al., 2001), but both H-Ras and K-Ras are enriched in the low-density membranes (Figs. A.1 and A.2). Interestingly, K-Ras but not H-Ras is excluded from the low-density membranes when high pH method was used (see Prior et al., 2001, and Figs. A.1 and A.2). By using immuno-electron microscopy, Hancock and colleagues (Prior et al., 2001) reported that wild-type H-Ras, but not K-Ras is localized to caveolae, in spite of the detergent solubility of H-Ras proteins. Also, once H-Ras is activated, it redistributes from caveolae into the bulk plasma membrane.

There appears to be a difference between the biochemical and cytological results. To solve the discrepancies, we think the assumption that there is no artificial effect of detergent disruption on the caveolae structure is wrong. We must hypothesize that detergent not only can disrupt non-caveolar rafts, but also disrupt certain region of the caveolae, where H-Ras is. This region, as we propose here, is the neck region of caveolae, which may have certain liquid-disordered property. As discussed in the rest of this sub-section and §5.5.3, much evidence supports this idea.

Since caveolin-1 is required for caveolae formation (Liu et al., 2001a; Prior et al., 2001; Razani et al., 2001), the fact that caveolin-1 is almost gone in the H-Ras and K-Ras transformed cells (Engelman et al., 1999; Xu et al., 2003) suggests that caveolae may be

greatly reduced or do not exist in the transformed cells. As discussed in Chapter IV, H-Ras and K-Ras may present in lipid rafts and the differential functions of H-Ras and K-Ras support the idea that they localize differently. Thus, we believe that both K-Ras and H-Ras in the transformed cells are present in non-caveolae lipid rafts, but exist in different subdomains.

As addressed in the rest of this section, we suggest that inactivated and activated H-Ras may localize to the lower- and upper-neck regions of caveolae, respectively, whereas both inactivated and activated K-Ras may localize in the upper-neck region of caveolae or very possibly in non-caveolar rafts (Fig. 5.3).

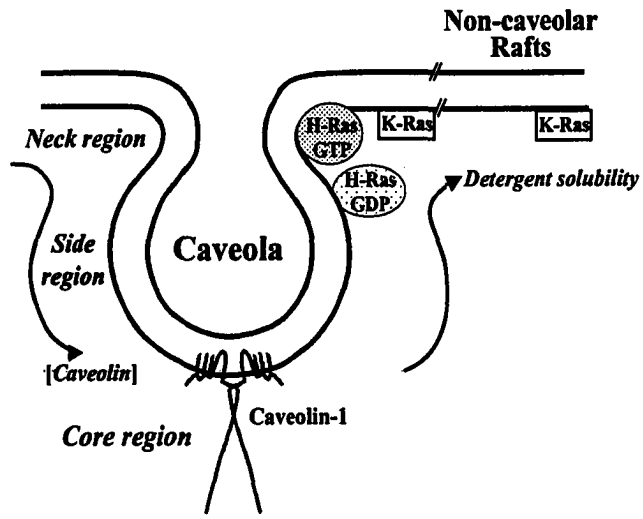


FIG. 5.3. A schematic model of the localization of Ras in caveolae and non-caveolar rafts.
Caveolin-1 exists as either a homo-oligomer of ~14 to 16 monomers (shown as a dimmer of triple palmitoylated caveolin-1 for simplicity) or as a hetero-oligomer with caveolin-2 (not shown). It is proposed that the neck region of caveolae has less caveolin and is composed of caveolae less-favored lipids, thus this region has liquid-disordered property and is detergent soluble. The dual acylation of H-Ras and the interaction of H-Ras-GDP with caveolin stabilize H-Ras with caveolae, but the prenylation of Ras is not favored by caveolae. Thus, it is proposed that H-Ras-GDP is more likely localized at the lower-neck region of caveolae. The GTP-bound form of H-Ras, however, is no longer associated with caveolin, and it localize to the upper-neck region. K-Ras is proposed to localize at the negative charged upper-neck region of caveolae or non-caveolar rafts by using the prenylation and its polybasic region. See text for details.

5.5.2.2. Factors that affect Ras localization

Consistent with the report by Prior et al. (Prior et al., 2001), Lisanti's group reported that the reconstituted caveolae-rich membranes interact with the soluble recombinant form of wild-type H-Ras, but failed to interact with the mutationally activated soluble H-Ras (Song et al., 1996a). The residues of 82-101 of caveolin-1, the so-called "scaffolding domain", is critical for the interaction.

It has been proposed that the scaffolding domains of caveolin are important for membrane localization of other proteins with caveolin-binding motifs (ΦxΦxxxxΦ, ΦxxxxΦxxΦ, and ΦxΦxxxxΦxxΦ, where Φ is an aromatic residue Trp, Phe, or Tyr, and x is any amino acid) (Okamoto et al., 1998). Proteins with caveolin-binding motifs, however, are not restricted to the caveolae, such as PKCα (Oka et al., 1997), EGFR (Waugh et al., 1999), and PLD1 (Kim et al., 1999a; Xu et al., 2000), suggesting that either the conformational change of the signaling molecules or other signals are involved in the decision of the localization process.

In the case of Ras, whereas one signal is the dynamic change of Ras proteins by activation, another signal appears to be the post-translational modification: prenylation and palmitoylation for H-Ras and prenylation as well as the presence of polybasic region for K-Ras.

By using different approaches, however, several reports suggested the notion that acylation, but not prenylation is used for targeting proteins to lipid rafts (Melkonian et al., 1999; Zacharias et al., 2002). Since the above studies either used a stretch of the specific modified sequences with a caveolae-resident protein as a reference, or used detergents, we interpret the results to mean that prenylation is not favored for caveolae. In the case of Ras proteins, the C-terminal prenylation seems to be a disadvantage for them to target

to caveolae. On the other hand, some reports (McCabe and Berthiaume, 2001) suggested that both the acylation and the protein-protein interaction facilitate the acylated proteins to transfer to the lipid rafts.

5.5.2.3. Model of Ras localization

Therefore, at least two forces, dual acylation and Ras-caveolin interaction, drive wild-type H-Ras to localize to the caveolae, but another force, the prenylation, disturbs the H-Ras membrane localization. It is reasonable to propose that these forces together may place the wild-type H-Ras not in the core region but in the lower-neck region of caveolae, where less caveolin molecules are present (Fig. 5.3). This region should have certain liquid-disordered property. Once Ras is activated, more likely it migrates to the more liquid-disordered upper-neck region of the caveolae (Fig. 5.3). In this hypothesis, the perturbation of caveolae structure by detergent has to be considered. No matter if Ras is activated or not, in either case, detergent will extract the perturbed H-Ras from the lipid rafts, with the activated H-Ras having higher detergent solubility than the wild-type H-Ras (Prior et al., 2001). As addressed in the next sub-section, the information about the regulation and localization of PLD isoforms supports this notion.

5.5.3. Dynamic change of PLD on the membranes: a new model

5.5.3.1. PLD localization

Methodologically, PLD2 is present in DRMs (about 15%) (Czarny et al., 2000; Sciorra et al., 2002) and enriched in the low-density membrane (Czarny et al., 2000; Xu et al., 2000), whereas PLD1 is excluded from DRMs, but there is certain amount of PLD1 present in the low-density membrane (Kim et al., 1999a; Xu et al., 2000). As discussed

below, we hypothesized that PLD2 is mainly localized to the upper-side region of caveolae, but certain PLD1 is in the upper-neck region or in the non-caveolar rafts.

5.5.3.2. Factors that may affect PLD membrane localization

There are several factors that may affect PLD membrane localization. These include (1) PLD-caveolin interaction, (2) acylation, (3) PC with saturated acyl chains, (4) PIP_2 with a polyunsaturated acyl chain, (5) change of the ionic charge by generation of PA, (6) participation of other proteins, such as RalA and ARF6, (7) actin polymerization, and (8) other factors.

Caveolae favored factors:

Both PLD1 and PLD2 have the consensus caveolin binding sequence in CRIII, which is Y/FxYxxxxF/Y. This aromatic residue-containing sequence was assumed to interact with the choline headgroup of PC to either increase the rate of catalysis or limit the specificity of PLD for PC, and alternatively interact with caveolin (Frohman et al., 1999). Both PLD1 and PLD2 were reported to interact with caveolae-1 and caveolin scaffolding peptide (residues 82-101). And interestingly, for PLD activity in DRM, presumably PLD2, low concentrations of caveolin peptide [1-10 μM] increases PLD activity about 40%, but high concentrations decreases PLD activity with nearly complete inhibition at a concentration of 50 μM (Czarny et al., 1999); whereas for PLD1, caveolin-1 peptide inhibits the basal and PKC-dependent PLD activity with the IC50 of about 0.5 μM (Kim et al., 1999a). However, there is no stimulatory effect for purified PLD1 or PLD2 by caveolin-1 peptide (Czarny et al., 2000).

Just like Ras, the acylation of PLD also plays an important role for membrane interaction. As discussed in chapter I, both PLD1 and PLD2 are palmitoylated at two

cysteine residues (Sugars et al., 1999; Xie et al., 2002). PLD1 is predominantly in the perinuclear regions, but blocking palmitoylation shifts PLD1 to the plasma membrane (Sugars et al., 1999). Palmitoylation-deficient PLD1 can still associate with the crude membranes, but the association is weakened (Sugars et al., 2002; Xie et al., 2001). Interestingly, palmitoylation-deficient PLD2 seems to bind membranes stronger than the deficient PLD1 (Xie et al., 2001; 2002). How the palmitoylation of PLD1 prevents it from moving to the plasma membrane but still allows PLD2 to be targeted to the plasma membrane is not known, but only when palmitoylated, certain amount of PLD1 is targeted to the CEMMs (Han et al., 2002b).

Caveolae less-favored factors:

Although it is unknown how and whether the PLD-caveolin interaction and the dual acylation are sufficient to drive PLD1 and PLD2 to localize differently, the following factors which do not appear to be favored by caveolae or are susceptible to detergent extraction, may place PLD1 in a less liquid-ordered region.

First, the PLD key factor, $PI(4,5)P_2$, has interesting properties on membrane. PLD has two PIP_2 binding regions: high selective PH domain and high affinitive polybasic motif (Sciorra et al., 2002) (see Chapter I). Mutation study showed that the PH domain, but not the polybasic motif, is required for PLD2 localization to the DRMs (Sciorra et al., 2002). PIP_2, with highly unsaturated acyl chains, is enriched in DRMs (Pike and Casey, 1996). However, whereas the total recovery of PIP_2 by either detergent or detergent-free method is about 90%, the total recovery of phosphatidylinositol, the precursor of PIP_2, is only 20% by using the Triton X-100 extraction procedure, but is near 100% when using the detergent-free method (Liu et al., 1998). In the terms of

distribution, both PIP_2 and phosphatidylinositol are present higher in the low-density fractions by detergent-free method compared to that by detergent method (Liu et al., 1998). It was also argued that $PI(4,5)P_2$ can exist in the low-density membranes in the absence of caveolin (Liu et al., 1998) or the synthesis of PIP_2 takes place in the low buoyant density non-caveolar membrane (Waugh et al., 1998; 2001). Interestingly, EGF receptor was also reported to localize in the non-caveolar rafts (Waugh et al., 1999).

Second, for the substrate of PLD, we have shown that PC with a polyunsaturated fatty acyl chain is excluded from the light membrane microdomains (see Chapter III and Xu et al., 2000). This is consistent with the notion that the polyunsaturated acyl chain is not favored in the cholesterol and glycosphingolipid-containing rafts (Harder and Simons, 1997; Simons and Ikonen, 1997). It was reported that lateral interaction between cholesterol and phosphatidylcholine favors the saturated phospholipids rather than unsaturated ones by using absorption spectroscopy and fluorescence polarization measurements (Lagane et al., 2002). However, some studies argued that phospholipids are relatively depleted from DRMs compared to sphingolipids (Brown and London, 1998; Brown and Rose, 1992).

Third, for the PLD product, although the idea is argued below, polyunsaturated PA, in the presence of Ca^{2+}, was proposed to play important roles in the formation of membrane curvature for vesicle budding (reviewed in Rizzo and Romero, 2002 and citations therein). Since polyunsaturated PC is excluded in the light membrane domains and can not be used by PLD (see Chapter III), the generated PA is the PA with saturated acyl chains, but not as the polyunsaturated ones as proposed in Rizzo and Remero's model (Rizzo and Romero, 2002). Thus, we proposed that the polyunsaturated PIP_2 and

the saturated PA function together to play the role just as the PA with a polyunsaturated acyl chain.

Fourth, for the proteins that regulate PLD1, RalA and ARF6 are both detergent soluble (our unpublished results) just like PLD1 (Czarny et al., 1999); however, both proteins are co-localized in the light membrane fractions (see Chapter IV) and both are required for PLD activity. Interestingly, it was reported that binding of myristoylated ARF6 to PIP_2-conatining vesicles could create defects in the membrane bilayers by electron spin resonance study (Ge et al., 2001).

5.5.3.3. A model for PLD localization and activation

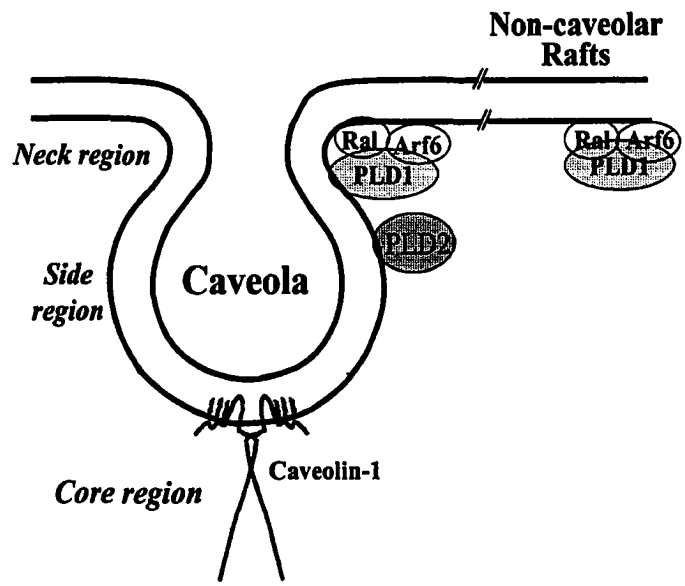


FIG. 5.4. A schematic model of the localization of PLD in caveolae and non-caveolar rafts.
It is proposed that PLD1 is localized to the upper-neck region of caveolae, which is liquid-ordered phase region but has liquid-disordered property, and PLD2 is localized to the upper-side region, which has less liquid-disordered property. The dual palmitoylation of PLD and the PLD-caveolin interaction are favored by caveolae, but the substrate—PC with saturated acyl chains, the regulator—PIP_2 with a polyunsaturated acyl chain, the product—the negative charged PA, and the stimulator—the membrane defect-generator ARF6 are all less-favored by caveolae. It is still unknown what factor(s) generates the differential localization for PLD1 and PLD2, but the two different PIP_2 binding sites, the phosphorylation of PLD isozymes, the interaction between Ral and PLD1 may play a role in this process. Activation of PLD may promote the generation of vesicle. See text for details.

Each of these factors, which either targets PLD to the membrane, or functions as a substrate, a product, or a regulator of PLD, seems to localize to a liquid-ordered phase domain with certain liquid-disordered property. Due to the presence of these factors, the dual acylation of PLD, and PLD-caveolin interaction, we would like to propose a model for the localization and action of the PLD1 and PLD2 on the membrane microdomains: PLD2 is localized to the upper-side region of caveolae where there is still certain amount of PIP_2, but PLD1 is localized to the less liquid-ordered neck region. PIP_2 with a polyunsaturated acyl chain and PC with saturated acyl chains are more enriched in the neck region of caveolae. ARF6, Ral, H-Ras, and EGFR also seem to localize to this region, which represents the intermediate phase domains between liquid-ordered and liquid-disordered states, but is closed to liquid-ordered state. That might be why the detergent could somehow displace those proteins and lipids or alternatively, some other methods could separate them from caveolae.

Once PLD1 is activated on the neck region of caveolae, PLD1 catalyzes the hydrolysis of PC to form PA and choline. Temporary binding of choline in CRIII of PLD1 further prevents it from interacting with caveolin-1. Activation of PI(4)P5K by PA will generate more $PI(4,5)P_2$ in the microdomains and the generated PIP_2 will further activate PLD1. As this is happening, the polymerization of actin will further activate PLD1. The formation of PA along with PIP_2 changes the membrane curvature dynamically. This change is migrated to the upper-side region of caveolae and PLD2 may move from the more caveolin region to the less caveolin region, where more PIP_2 is present. In this way, PLD2 is also activated, since binding of PIP_2 is favored and a lower level of caveolin-1 somehow has a stimulatory effect on PLD2 (Czarny et al., 2000;

1999). Further activation of PLD changes the membrane microdomains dramatically. The dramatic change of membrane composition in the subdomains of the caveolae may have two fates: (1) the dynamic migration of membrane causes it to incorporate into the bulk plasma membrane and to be internalized (Mineo et al., 1999); (2) this change, probably along with the help of dynamin (Henley et al., 1998; Hinshaw, 2000; Oh et al., 1998), causes the fission of the vesicle. Interestingly, the PIP_2-containing vesicles can promote the vesicle transportation along microtubules mediated by the Unc104 (KIF1A) kinesin motor (Klopfenstein et al., 2002), but in the presence of cholesterol and sphingomyelin, only low concentration of $PI(4,5)P_2$ is needed to provide a trigger for Unc104-mediated membrane transport. It was also found that ARF proteins interact with mitotic kinesin-like protein 1 (MKLP1) in a GTP-dependent fashion (Boman et al., 1999). Whether and how the formed vesicles move on the microtubules will be an interesting area to study.

5.5.3.4. Other factors that might affect PLD membrane localization

It is still not known if phosphorylation of PLD by PKC is involved in this process. Once signals are transduced, PKCα can be recruited to the membrane and bind to PLD1 to activate it (reviewed in Exton, 2002b). Binding of PLD1 with PKC may prevent PLD1 from binding to caveolin-1 (Kim et al., 1999a). Intriguingly, the phosphorylation of PLD1 by PKC takes place exclusively in the light membranes (Han et al., 2002b; Kim et al., 2000) and the phosphorylation is palmitoylation-dependent (Han et al., 2002b). Depletion of cholesterol dramatically reduces the phosphorylation of PLD in the light membranes, but does not affect the PLD1 activity (Kim et al., 2000). As discussed in Chapter I, phosphorylation of PLD1 may act as a negative feedback for the regulation of

PLD1 (Hu and Exton, 2003), but it is not clear whether the phosphorylation will reinforce the binding between PLD1 and caveolin-1 with the help of other unknown factors.

5.5.3.5. The homeostasis of caveolae and rafts

In addition to the explanation of the caveolae-containing vesicle formation and membrane internalization, our model can also explain the homeostasis of caveolae and rafts. On the plasma membrane, the border regions between the non-caveolar rafts and the heavier membranes are not only enriched with saturated phosphatidylcholine and polyunsaturated PIP_2, but also have more PA than the core-region of the rafts. Since palmitoylation-deficient PLD2 binds to membrane stronger than the palmitoylation-deficient PLD1 (Xie et al., 2002), it is more likely that normal PLD isoforms have different properties for membrane binding. We proposed that PLD2 tends to bind to the near-border region, which is a little far from the less liquid-ordered border region of the rafts, and PLD1/RalA complex associates with the PIP_2-enriched border region. In this way, PLD2 can use its highly selective PH domain (Sciorra et al., 2002) with the help by the polybasic motif to bind to PIP_2 in the near-border region, whereas PLD1, with the help of Ral, may use its highly affinitive but less selective polybasic PIP_2-binding motif (Sciorra et al., 2002) to bind to PIP_2 on the more negative-charged border region.

Since the generation of caveolae seems to be a negative module, we proposed that PLD activity is very low under the non-stimulatory condition. As cholesterol and caveolin associate with the liquid-ordered core region of the rafts, some caveolin molecules are accessible to PLD2 and somehow activate it. The generated PA will be quickly pushed to the intermediate region since it is not favored in the near-border region, but PLD2 will stay there associated with caveolin. PA will activate PI(4)P5K, which

generates more PIP_2 in the intermediate region, where PLD1 is localized. Activation of PLD1 by PIP_2 will generate more PA and subsequently change the membrane curvature. When more caveolin is bound to the rafts, PLD2 is inhibited. Gradually, the caveola is formed with PLD2 on the upper-side region and PLD1 on the neck region.

The *de novo* generation of caveolin-containing vesicles from ER can also be achieved by this way. They are subsequently fused to lipid rafts of the plasma membrane.

Whereas the level of caveolae can be regulated by the transduction level of caveolin generation, the reduction of caveolae can also be explained by internalization of caveolae—although certain level of caveolin can be recycled, the subsequent degradation of caveolin results in the reduction of caveolae.

5.6. Future directions

PLD is a very important molecule in cell signaling. Reports regarding the mechanism of its regulation and function in the cell are still limited. In this dissertation we have investigated the localization and regulation of PLD in mitogenic signaling and we also tried to provide a model to view the whole image of the regulation of PLD in the cell signaling. Our work, along with others', supports this model. Most studies will focus on the molecular basis regarding the spatial-temporal regulation and the function of PLD in the mitogenic signaling. The future study on the crystal structure and the knockout of mammalian PLD isoforms will help us understand the mechanism. The study of the dynamic change of PLD on the membrane and the role of PLD in oncogenesis are challenging areas. Further study of PLD will continue to provide the link between mitogenic signaling and vesicular transport.

Appendix

A.1. Supplemental data

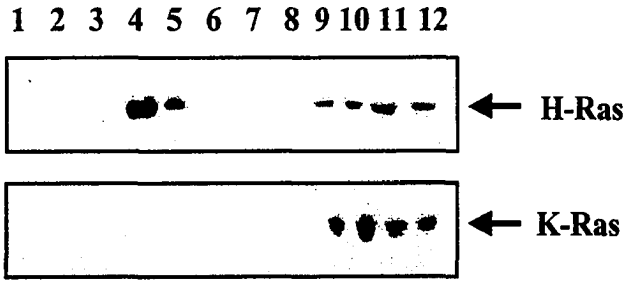


FIG. A.1. There appears to be differential localization of H-Ras and K-Ras in the presence of high pH.
Quiescent NIH 3T3 cells transformed by v-H-Ras (H-Ras) and v-K-Ras (K-Ras) were scraped into 2 ml of 500 mM Na_2CO_3, pH 11 as described (Song et al., 1996a; 1996b) instead of the neutral pH buffer as described in Chapter II. The cells were disrupted by Dounce and Polytron homogenization and then by sonication. The lysates were mixed with 2 ml of 90% sucrose (W/V) prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl). This solution was then overlaid with 4 ml of 35% and 4 ml of 5% sucrose (W/V) in MBS containing 250 mM Na_2CO_3 to form a discontinuous gradient in an ultracentrifuge tube. After the ultracentifugation, 12 fractions were recovered and subjected to Western blot analysis using antibodies to H-Ras and K-Ras, respectively.

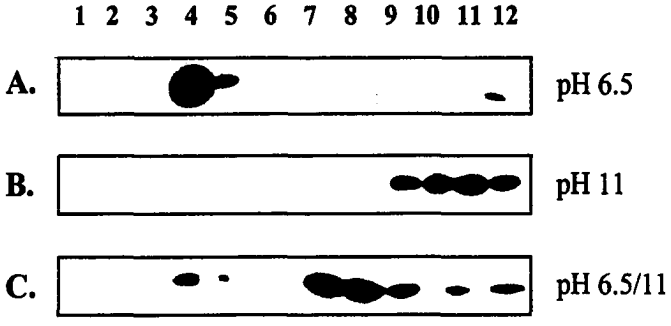


FIG. A.2. Sodium carbonate with high pH removes K-Ras from CEMMs.
Quiescent NIH 3T3 cells were scraped into 2 ml of MBS (A) or 500 mM Na_2CO_3, pH 11 (B and C) as in Fig. A.1. After homogenization and sonication, 2 ml of the lysates were mixed with 2 ml of 90% sucrose (W/V) prepared in MBS. This solution was then overlaid with 35% and 5% sucrose (W/V) in MBS (A and C) or in MBS containing 250 mM Na_2CO_3 (B). After the ultracentifugation, 12 fractions were recovered and subjected to Western blot analysis using the antibody to K-Ras (A, B, and C).

A.2. Density, refractive index, and concentration data for sucrose solution.

Table A.1. Density, refractive index, and concentration data for sucrose solution at 20^0C.

% by Weight	Density (g/cm3)	Refractive Index, ηD	% W/V	Molarity (M)	% by Weight	Density (g/cm3)	Refractive Index, ηD	% W/V	Molarity (M)
0	0.9982	1.3330			34	1.1463	1.3883	38.97	1.138
1	1.0021	1.3344	1.00	0.029	35	1.1513	1.3902	40.30	1.177
2	1.0060	1.3359	2.01	0.059	36	1.1562	1.3920	41.62	1.216
3	1.0099	1.3374	3.03	0.089	37	1.1612	1.3939	42.96	1.255
4	1.0139	1.3388	4.06	0.119	38	1.1663	1.3958	44.32	1.295
5	1.0179	1.3403	5.09	0.149	39	1.1713	1.3978	45.68	1.334
6	1.0219	1.3418	6.13	0.179	40	1.1764	1.3997	47.06	1.375
7	1.0259	1.3433	7.18	0.210	41	1.1816	1.4016	48.45	1.415
8	1.0299	1.3448	8.24	0.211	42	1.1868	1.4036	49.85	1.456
9	1.0340	1.3464	9.31	0.272	43	1.1920	1.4056	51.26	1.498
10	1.0381	1.3479	10.38	0.303	44	1.1972	1.4076	52.68	1.539
11	1.0423	1.3494	11.47	0.335	45	1.2025	1.4096	54.11	1.581
12	1.0465	1.3510	12.56	0.367	46	1.2079	1.4117	55.56	1.623
13	1.0507	1.3526	13.66	0.399	47	1.2132	1.4137	57.02	1.666
14	1.0549	1.3541	14.77	0.431	48	1.2186	1.4158	58.49	1.709
15	1.0592	1.3557	15.89	0.464	49	1.2241	1.4179	59.98	1.752
16	1.0635	1.3573	17.02	0.497	50	1.2296	1.4200	61.48	1.796
17	1.0678	1.3590	18.15	0.530	51	1.2351	1.4221	62.99	1.840
18	1.0721	1.3606	19.30	0.564	52	1.2406	1.4242	64.51	1.885
19	1.0765	1.3622	20.45	0.597	53	1.2462	1.4264	66.05	1.930
20	1.0810	1.3639	21.62	0.632	54	1.2519	1.4285	67.60	1.975
21	1.0854	1.3655	22.79	0.666	55	1.2575	1.5307	69.16	2.020
22	1.0899	1.3672	23.98	0.701	56	1.2632	1.4329	70.74	2.067
23	1.0944	1.3689	25.17	0.735	57	1.2690	1.4351	72.33	2.113
24	1.0990	1.3706	26.38	0.771	58	1.2748	1.4373	73.94	2.160
25	1.1036	1.3723	27.59	0.806	59	1.2806	1.4396	75.56	2.207
26	1.1082	1.3740	28.81	0.842	60	1.2865	1.4418	77.19	2.255
27	1.1128	1.3758	30.05	0.878	61	1.2924	1.4441	78.83	2.303
28	1.1175	1.3775	31.29	0.914	62	1.2983	1.4464	80.49	2.351
29	1.1222	1.3793	32.54	0.951	63	1.3043	1.4486	82.17	2.401
30	1.1270	1.3811	33.81	0.988	64	1.3103	1.4509	83.86	2.450
31	1.1318	1.3829	35.09	1.025	65	1.3163	1.4532	85.56	2.500
32	1.1366	1.3847	36.37	1.063	66	1.3224	1.4558	87.28	2.550
33	1.1415	1.3865	37.67	1.100	67	1.3286	1.4581	89.02	2.864

The molecular weight of sucrose is 342.3. Adapted from Beckman Coulter Ultracentrifuge.

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