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REGULATION OF PHOSPHOLIPASE D ACTIVITY BY SMALL GTPases

by

JINGQING LUO

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

1997

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ABSTRACT

Regulation of Phospholipase D Activity by Small GTPases

by

JINGQING LUO

Adviser: Dr. David A. Foster

The hydrolysis of phosphatidylcholine by phospholipase D (PLD) may generate several lipid second messengers or mediators including phosphatidic acid, lysophosphatidic acid and diacylglycerol. We investigated the activation of PLD by v-Src oncogene and demonstrated a requirement for the GTPase cascade of Ras and Ral. Another small GTPase Arf, previously implicated in vesicle transport, was also found to be involved in the activation of PLD by v-Src.

The data indicate that PLD activity associates with RalA in v-Src-transformed cells *in vivo* and *in vitro*. Overexpression of RalA potentiated PLD activation by v-Src, and expression of dominant negative RalA mutants inhibited both v-Src- and v-Ras-induced PLD activity. These data indicate that RalA is involved in the tyrosine kinase activation of PLD and that PLD is a downstream target of a Ras/Ral GTPase cascade. However, the association between RalA and PLD is not sufficient for PLD activation, another factor is required. We found the presence of Arf in RalA precipitates from v-Src transformed cell membrane lysates. Arf increased the RalA-associated PLD activity and contributed to differential activity of PLD associated with RalA mutants. The data also suggested that the N-terminal

end of RalA is important for the binding of Arf and for PLD activation. Since the interaction between RalA and Arf is very weak, other molecules may be required. We also characterized the RalA associated PLD activity in v-Src transformed cells by examining the association of RalA with a purified PLD1 preparation and with a human PLD1 that was overexpressed in sf9 insect cells infected with baculovirus expressing a cloned hPLD1. Several lines of evidence demonstrate that the PLD associated with RalA in v-Src transformed cells is the Arf-responsive, PIP_2-dependent PLD1 and that this interaction is direct. Since Arf and PLD have been implicated in vesicle transport, the apparent involvement of Arf and PLD in the intracellular signals activated by v-Src suggest a role for vesicle transport in signal transduction and Arf may serve as a mediator of these two cellular functions.

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INTRODUCTION

Phospholipase D (PLD) has been implicated in a wide variety of cellular functions including responses to extracellular stimuli[1 2 3] as well as membrane trafficking and vesicular transport[4 5]. According to current research, PLD activities differ in many mammalian tissues and cell lines dependent on subcellular localization, pH optima, divalent cations, requirement of phosphatidylinositol-4, 5-bisphosphate (PIP_2), susceptibility to inhibition by fatty acids (e.g. oleate) and detergents[6], the involvement of protein kinase C (PKC), and extent of activation by small GTPases such as Arf, Rac, Rho, Cdc42 and Ras. The mechanism of PLD activation has attracted considerable attention since the reaction can generate important lipid second messengers.

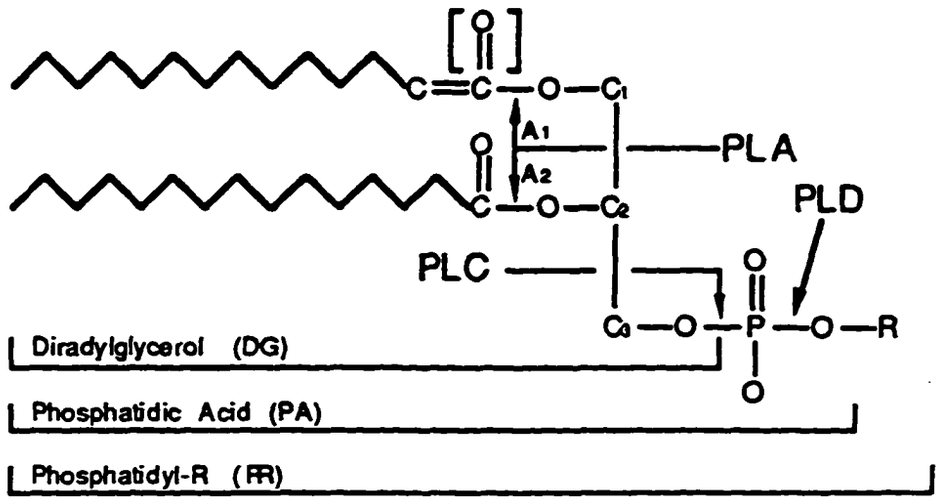
I. Functional Significance of Phospholipase D

There is little direct evidence indicating the functional significance of PLD activation . But an accumulation of indirect evidence links PLD with mitogenesis and vesicle transport. In addition, the newly reported activation of PLD by small monomeric G proteins suggested that PLD is involved in numerous cellular pathways.

1.1 The involvement of PLD signals in many cellular functions

The hydrolysis of phosphotidylcholine (PC) by PLD gives rise to phosphatidic acid (PA) and choline (Fig. 1a). PA can be then dephosphorelated by a PA phosphatase to diacylglycerol (DG), which activates protein kinase C [7 8]. PA can also be metabolized to

lysoPA, which induces a wide range of effects including mitogenesis [9]. More recently, PA itself has been implicated as a regulator of proteins involved in a wide variety of cellular responses. PA has been shown to activate the GTPase activating proteins for the small GTPase signalling molecules Ras, Rac, and ARF [10 11 12]. PA stimulates PI-kinase activity[13], which leads to the production of PIP_2 , a substrate for PLC-γ and a cofactor for PLD[14 15 16]. Additionally, a direct interaction between PA and a defined region of Raf has been reported that appears to be necessary for localizing Raf to the membrane where it is activated[17]. Another suspected role for PA is in facilitating vesicle budding and vesicular transport [12]. This hypothesis is based largely on the observation that PLD activity is stimulated by the small GTPase ARF [18 14], which has been shown to be required for vesicle budding in the Golgi[19]. Consistent with a role for PA and PLD in vesicular transport, the addition of PLD to Golgi membranes stimulated vesicle formation in the absence of Arf [20]. Another potential role for PLD and PA is the formation of stress fibers since the formation of stress fibers by lyso-PA is dependent upon PLD activity [21]. Thus, control of cellular PLD and PA levels is likely important for the control of many aspects of cell physiology. All of these findings indicate that PLD is involved in many cellular functions (Fig. 1b) through PA, DG and lysoPA as potential second messengers and possibly plays an important role in intracellular protein traffic, membrane-lipid regulation and cytoskeleton reorganization.



Phosphatidyl-R $\xrightarrow{\text{PLA}}$ Fatty acid + Lysophosphatidyl-R

$\xrightarrow{\text{PLC}}$ DG + R-PO_4

$\xrightarrow{\text{PLD}}$ PA + R

$\downarrow$ PAP

DG + PO_4^-

Figure 1a. Phospholipase and phospholipid structure. A generic glycerophospholipid, phosphatidyl-R, is shown with target sites for the various phospholipases (PLA1, PLA2, PLC and PLD). Products generated from PLDs are: a phosphatidic acid (PA) and the head group (R=choline).

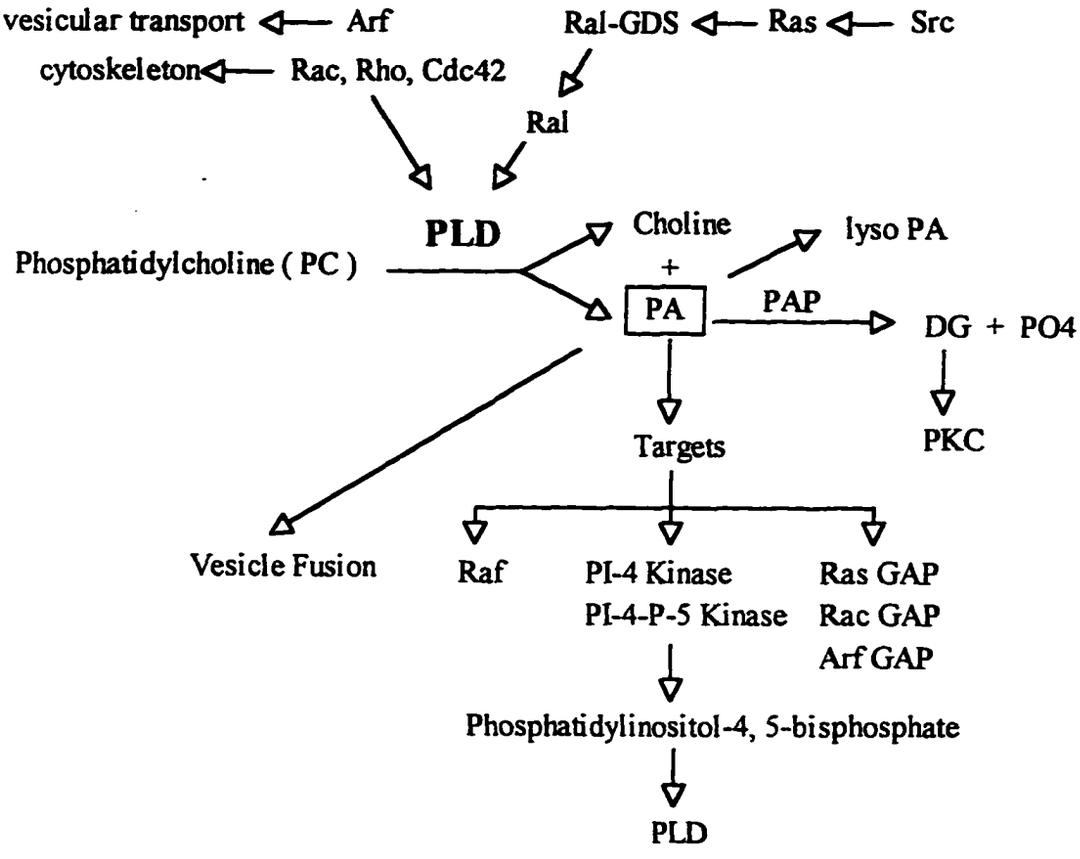


Figure 1b. PLD signals are involved in many cellular functions

1.2 Correlation between PLD activity and mitogenesis

Considerable studies indicate that DG from PLD pathway is generally produced in much larger amounts and for longer periods than that from PIP_2 hydrolysis. This may be due to constitutive activation of PLD in some cell types, suggesting that the expression of a high level of PLD activity is associated with prolonged changes in cell function[22]. Although there is little direct evidence indicating the role played by PLD in regulating cell function, it has been reported in several cases that expression of PLD activity is associated with

transformation and immortalization[23]. For example, in peripheral B lymphocytes, PLD activity can not be activated by phorbol esters. However, B-lymphocyte cell lines infected with Epstein-Barr virus, a process known to predispose the cells to immortality, have a PLD activity that is readily stimulated by phorbol esters[24]. Another example is that the infection of fibroblast cells with Rous sarcoma virus leads to their transformation as a result of the expression of the constitutively active tyrosine kinase v-Src. Studies in our laboratory have demonstrated that one of the effects of v-Src expression is activation of PLD and the prolonged production of DG[25 26]. In addition, PLD activity is elevated in response to most mitogenic stimuli [27 28 29]. Therefore, PLD is believed to be involved in mitogenesis and important for regulation of cellular functions.

II. PLD protein and its activity

PLD activities have been extensively characterized in both membrane and soluble fractions derived from mammalian tissues and cells[30 31 32 33]. But the studies have been hindered by the lack of purified PLD protein or cDNAs. Thus, for a long time, investigators could only detect PLD activity by measuring PLD specific transphosphatidylation reaction.

The hydrolysis of PC by PLD produces phosphatidic acid (PA) and choline (Fig. 2a). In the presence of alcohol, PLD catalyzes a transphosphatidylation reaction producing phosphatidylalcohol. For example, when ethanol is used, the products will be phosphatidylethanol (PEt) and choline (Fig. 2b). Phosphatidylalcohol is less subject to further metabolism than is PA, and can easily be distinguished from substrate and other products by thin layer chromatography (TLC).

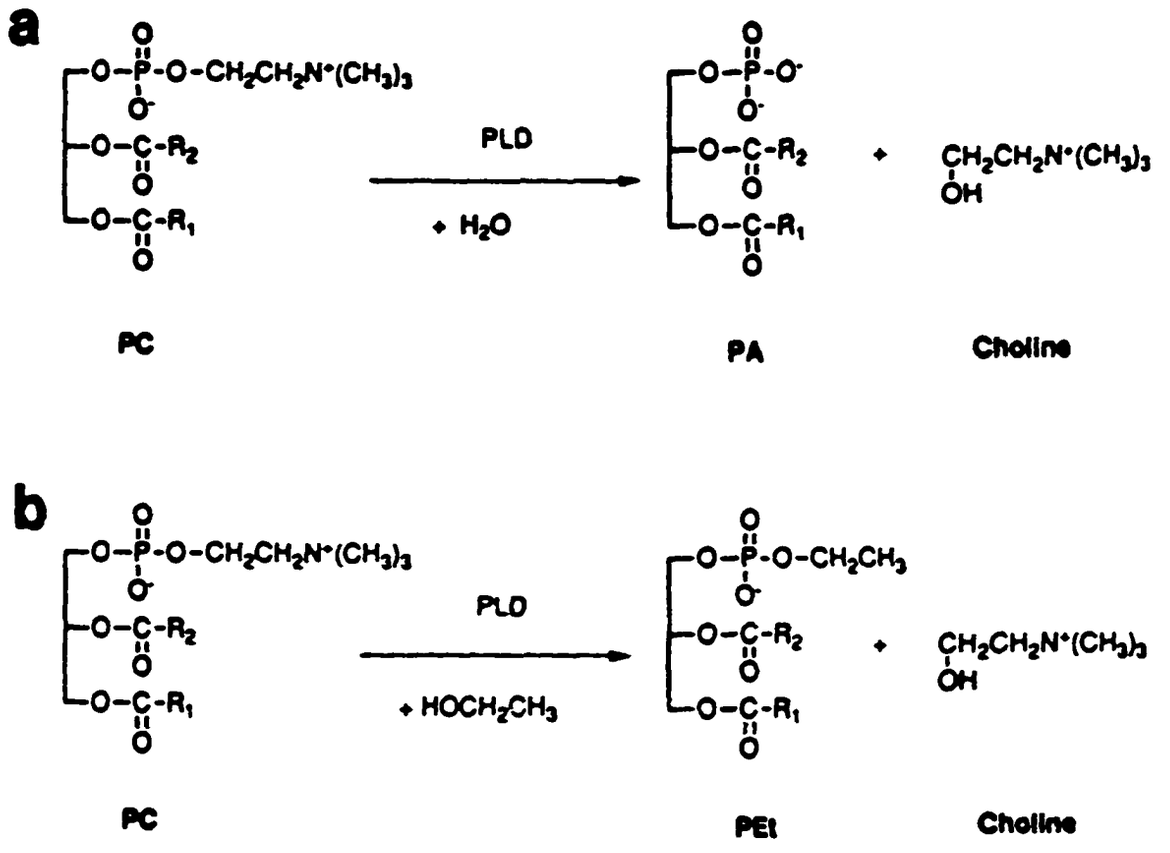


Figure 2 Reactions catalyzed by phospholipase D. a) hydrolysis, b) transphosphatidylation

PLD activity can be detected by utilizing radio-labelled phospholipids as substrate. For intact cells, phospholipids are labelled by incubation of the cells with a radioactive fatty acid (e.g. [^{3}H]-myristate). Following incubation of cells under desired conditions, the products are extracted in the presence of alcohol from the cells and separated by TLC. PLD activity is assessed by quantitation of radioactive PA and/or phosphatidylalcohol. Assays for PLD activity *in vitro* are conducted in a similar manner, with use of a radiolabelled PC as

substrate. Exogenous substrate for PLD is in the form of liposomes that contain [^{3}H]-PC, PIP_2 and phosphatidylethanolamine (PEamine). After cells are lysed with detergent, lysates are mixed with liposomes and PLD activity is determined. Recently, fluorescent probe labelled fatty acid and PC are being used in the above systems. It was found that the composition of the lipid liposomes is crucial for observing the hydrolysis of PC. For example, PIP_2 was reported as a key component of lipid liposomes. But its real role is unknown. It was suggested that the PIP_2 probably serves as a binding site for PLD or other potential cofactors that may participate in the PLD signalling systems.

Recently, two groups have biochemically characterized what are apparently two distinct PLDs - one that is dependent upon PIP_2 and the small GTPase Arf [34 35]; and, another that is independent of Arf and PIP_2 , but stimulated by oleate [36]. The oleate-stimulated PLD has been purified from pig lung and has a MW of 190 kDa. The Arf-dependent PLD has only partially been purified from pig brain, and has proved difficult to purify for several groups. A major hurdle in isolating PLDs was recently overcome with the report of the cloning of two mammalian PLDs [37 38]. The first PLD gene identified [37], designated PLD1, is dependent upon PIP_2 and is stimulated by Arf, as was reported for the PLD partially purified by Brown et al. [14 34]. There are two splice variants of PLD1 (PLD1a and 1b) that differ by the omission of a 38 aa exon [39]. PLD1 localizes to peri-nuclear regions where PLD activity has been shown to promote Arf-mediated coated vesicle formation. A cDNA encoding a second PLD, designated PLD2, has also been cloned [38]. This PLD is also dependent upon PIP_2, but is insensitive to Arf and may be regulated *in vivo* via inhibition since, unlike PLD1, it appears to be constitutively active [38]. PLD2 localizes predominantly

to the plasma membrane and has been postulated to be involved in cytoskeletal regulation or endocytosis.

III. Activation Mechanism of PLD

Though the activation mechanism of PLD is poorly understood, existing evidence demonstrates that PLD activity can be regulated by a variety of different factors. These include the protein tyrosine kinases [26 40 41 42 43 44], the regulation by G proteins and a group of small monomeric G proteins[45 46 47 48 49 50], phorbol ester stimulation via activation of PKC [25 51 52 53 54 55 56], the activation by a number of growth factors [25 28] and Ca^{++} [57 58 59 60 61]. Such diversity of regulatory mechanisms may implicate the existence of several different PLD isoforms or predict that activation of PLD and its sequelae mediate effects of multiple cellular pathways.

3.1 The involvement of tyrosine kinase

Current investigations indicate the involvement of tyrosine kinases in the PLD activation pathway. The protein-tyrosine kinase activity of both v-Src [62] and v-Fps [63] induces DG via a PC-specific PLD and PA phosphatase. The platelet-derived growth factor and epidermal growth factor whose receptors are protein-tyrosine kinases have also been reported to induce PLD activity [25 28]. Studies from our laboratory showed that stimulating the tyrosine kinase activity of v-Src in LA90-transformed BALB/c3T3 cells by expressing a temperature-activatable derivative of v-Src that upon temperature shift, results in an increase in v-Src kinase activity and subsequent induction of PLD activity. In v-Src transformed cells, we can detect much higher PLD activity than in normal cells. Thus, PLD-mediated hydrolysis of PC

may be a common component of intracellular signals mediated by protein-tyrosine kinases. Also, it is reported that by using vanadate peroxides[40 64], which is a pharmacological agent and an enhancer for the accumulation of tyrosine phosphorylated proteins, PLD activity is stimulated in electropermeabilized HL-60 granulocytes. PLD activation by tyrosine kinase is also being investigated by using different kinase inhibitors such as erbstatin. It appears that the activation of PLD by tyrosine kinases is quite complex and may involve perhaps several mechanisms.

The connection between tyrosine phosphorylation and the stimulation of PLD activity remains unidentified. Although the first animal PC specific PLD---hPLD1 and hPLD2 have been cloned recently, endogenous PLD proteins have not been detected by protein analysis. Thus, it is not known whether PLD is itself a target for tyrosine phosphorylation or whether other proteins mediate this process.

3.2 The effects of small G proteins

Signal-transducing GTP-binding proteins can be classified largely into two groups. One is the high-molecular-weight (~90KD) or heterotrimeric GTP-binding proteins (G proteins), consisting of three subunits, α, β and γ. Another group is the low-molecular-weight (20~30KD), monomeric GTP-binding proteins (small G proteins). They can exist in two interconvertible conformational states, inactive GDP-bound state and active GTP-bound state. For almost all Ras-related GTPases, the conversion between the GDP-bound and GTP-bound conformations is modulated by regulators such as guanine nucleotide exchange factors (GEFs) which stimulate the replacement of GDP by GTP and GTPase-activating proteins (GAPs) which enhance the intrinsic GTPase activity of the GTPase (Fig. 3).

Recently, a group of small monomeric G proteins has been found to be involved in PLD activation pathway. These include Arf, Rac, Rho, Cdc 42 and Ras. They are all Ras-related GTP-binding proteins (or GTPase) [45-50] and participate in transmitting signals from cell surface to the cell nucleus.

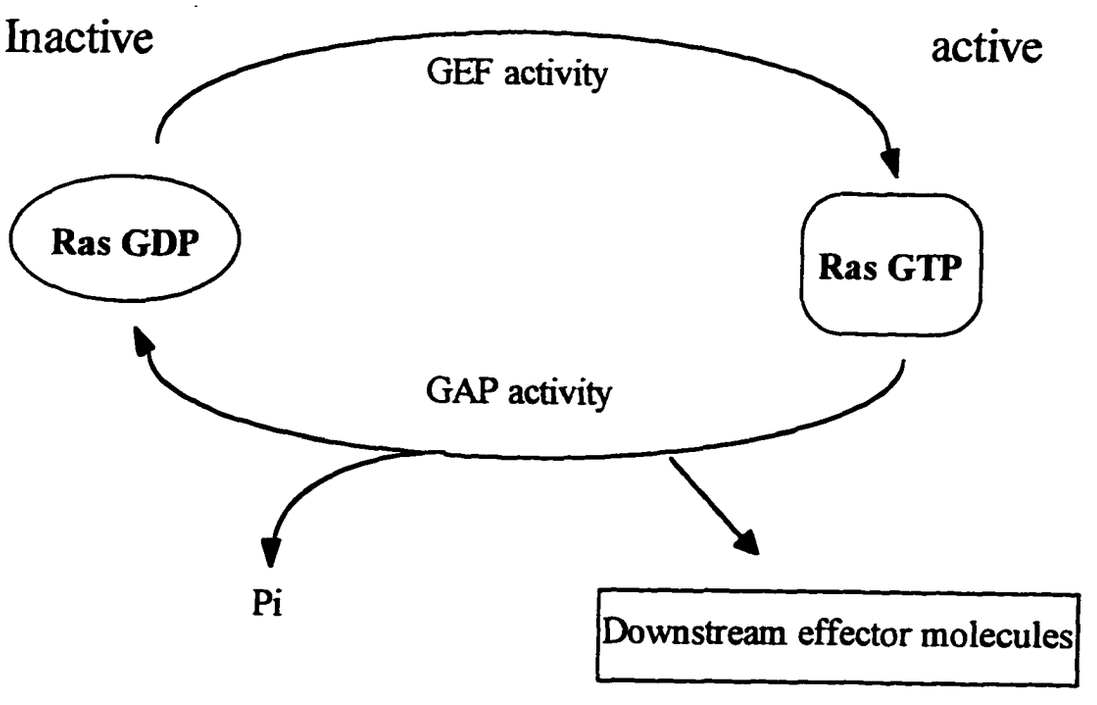


Figure 3. Cellular control mechanisms for Ras-related small GTPases. Interaction with a specific guanine nucleotide-exchange factor (GEF) results in release of GDP and binding of GTP. The GTP state of GTPase is active and interacts with its effector molecules to transmit a cellular stimulus. The signal is turned off when interacting with GTPase-activating protein (GAP) and stimulates the conversion of GTPase to its inactive GDP state.

3.2.1 Arf GTPase

Recently, several groups have demonstrated that a small monomeric G protein Arf (ADP-ribosylation factor)[46 47 50] is involved in stimulating PLD activity. Arf is a member of the Ras superfamily GTPases. It was originally identified as a protein cofactor required for the efficient modification of the α subunit of heterotrimeric G protein by cholera toxin[65]. Later, Arf protein was implicated as regulator of vesicle-mediated protein traffic [66 67]. In its GTP bound form, it facilitates coatomer binding and vesicle formation. Its GTP hydrolysis stimulates dissociation of coatomer and vesicle fusion. It is a mediator of vesicular transport in Golgi. It is clear that Arf is the cytosolic protein conferring sensitivity of the cell-free Golgi transport assay to GTPγS[68]. Immunofluorescence and biochemical data suggest that Arf is localized to the Golgi complex[69 70]. Once in the GTP-bound form, Arf will associate more stably with membrane or phospholipid micelles, while Arf-GDP is soluble. This ligand-dependent membrane association requires myristoylation of the N-terminal glycine residue. On the other hand, the nucleotide exchange of the Arf is dependent on added lipids, detergents, or both[71]. Although most of the Arf found in cells and tissues is soluble after cell lysis, the proteins likely to interact with Arf in cells, including PLD, the GTPase-activating protein for Arf (Arf-GAP) and G proteins, are all associated with membranes. All of these indicate that Arf and membrane lipids are highly regulated.

The understanding of Arf action was dramatically changed with the demonstration that Arf is an activator of membrane bound enzyme PLD. It was found that PLD activity is stimulated by GTPγS *in vitro* in a PIP_2 and cytosol-dependent manner, and a cytosolic factor required for this activity was purified and identified as Arf [46 47]. It is therefore possible that

PLD might be involved in membrane traffic. It was also be found that the Arf dependence of PLD activity was maintained through several steps of purification, suggesting an interaction between Arf and PLD. How Arf actually activates PLD and what is the real role of PLD is still not known. But the involvement of Arf in the activation of PLD possibly connects PLD signalling with vesicular transport.

In addition, several Rho family GTPases [72 73 74 75 76] are involved in stimulation of PLD activity, such as Rho and Cdc42. Recently, by using enriched preparations of porcine PLD, it was indicated that RhoA could activate this enriched PLD activity and act synergistically with Arf proteins. Also, Cdc42, which was shown to be involved in the establishment of cell polarity and the localization of budding [77 78 79], has proved to be an effective activator of PLD activity and showed similar synergistic activity with added Arf[50]. But the relationship between Arf and Rho family GTPases-mediated effects on PLD activation remains to be explored.

3.2.2 Ras GTPase

Ras proteins reside on the inner surface of the plasma membrane where they participate in transmitting signals from tyrosine kinase receptors[80 81]. Ras proteins have several downstream effector molecules (Fig. 4). By interacting with different targets, Ras proteins activate different signalling pathways. The research in our laboratory indicated that the induction of PLD activity by v-Src requires Ras. Consistent with Ras mediating v-Src-induced PLD activity, v-Ras has also been demonstrated to activate PLD [82 83]. Although the activation of PLD by v-Src is mediated by Ras, two reported Ras effector molecules, Raf-1 [84 85] and phosphatidylinositol-3-kinase [86], were apparently not involved [82]. The observation

that Ras also interacts with [87 88 89] and activates Ral-GDS [90] suggested that the activation of PLD by v-Src could be through Ral GTPase. Since the involvement of Ras is indirect, requiring a cytosolic factor for PLD activation, we investigate here the involvement of small G protein Ral in v-Src-induced PLD activation.

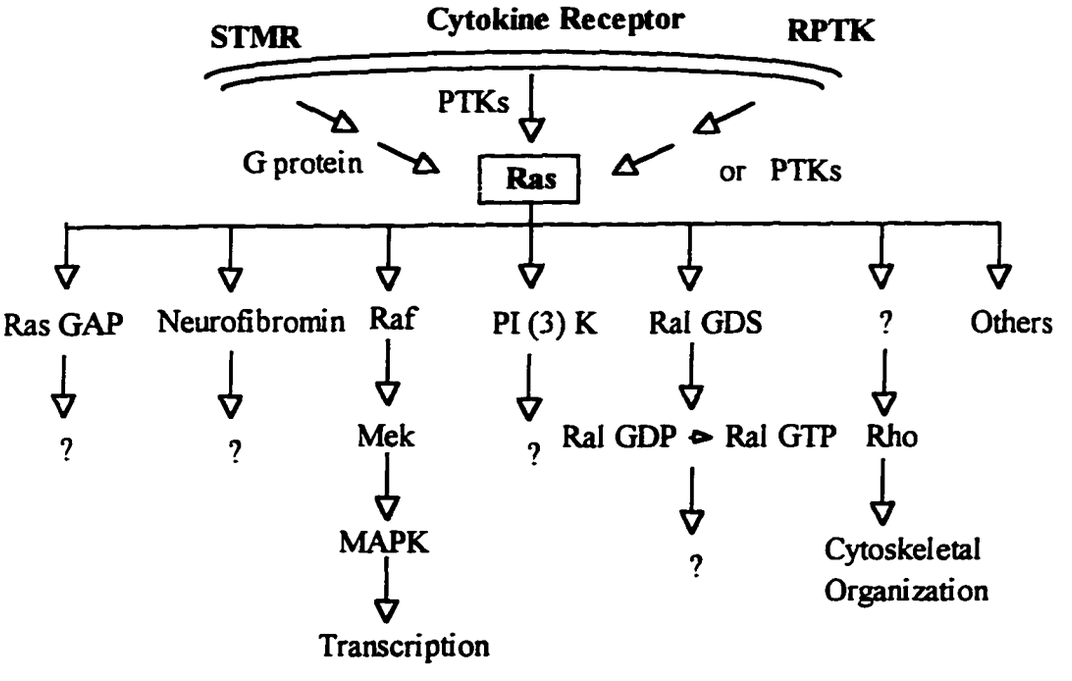


Figure 4. Downstream targets of Ras. Ras is activated in response to ligands that stimulate receptor protein tyrosine kinase (RPTK), cytokine receptors, and some seven trans-membrane receptors (STMR) that couple to heterotrimeric G proteins. It is becoming apparent that active Ras binds to a variety of proteins that may be responsible for carrying out its diverse functions.

IV. PLD activation in v-Src initiated signalling

v-Src is the first cancer-causing gene, or oncogene and was discovered over 20 years ago. It is a viral oncogene and comes from the modification of a normal cellular gene c-Src by a virus called Rous sarcoma virus, which is an avian retrovirus that causes rapid eruption of tumours in infected animals. The name of src comes from the sarcomas it causes in chicken. The Src protein is a tyrosine kinase, which plays an important role in signal transduction pathway. Figure 5 shows the domain organization of c-Src. This protein's crystal structure was described recently. It contains Src-homology (SH) domains 2 and 3, along with a catalytic kinase domain. The activity of c-Src is controlled by phosphorylation at Tyr527. Once phosphorylated on Tyr527, it will bind to SH2 domain and block the catalytic site. The crystal structure indicates that the SH3 domain also plays a crucial role in preventing the catalytic site from functioning. The main alteration in v-src is a deletion that removes Tyr527, and c-Src can be converted to an efficient oncogene by substituting Tyr527 with phenylalanine.

The results in our lab indicate that activating the kinase activity of v-Src results in stimulating phospholipase D activity. It was also found that activation of PLD by v-Src is dependent upon Ras [81] (Fig 6). Ras is involved in mediating v-Src induced PLD activation. Therefore, a signal transduction pathway from v-Src tyrosine kinase activation to elevated PLD activity exists. But the mechanism of PLD activation by v-Src is not clear. So for several years, our lab has been trying to clarify this pathway from v-Src to PLD activation.

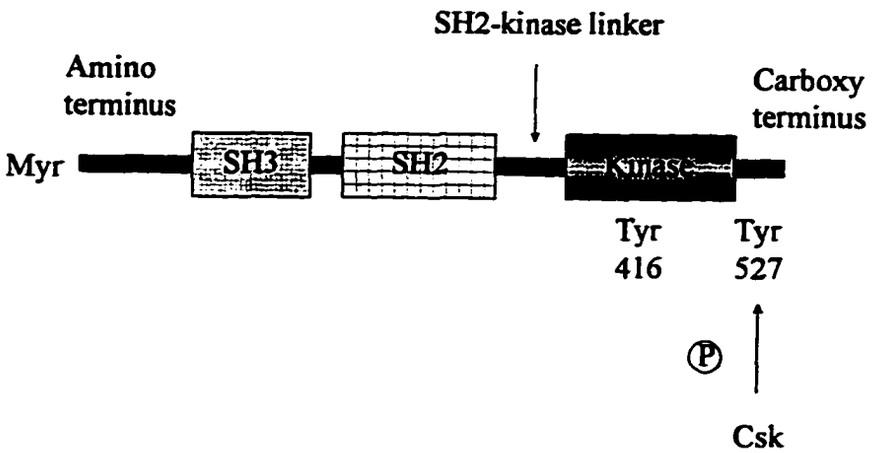


Figure 5. The domain organization of c-Src. The c-Src protein is anchored to the cell membrane through a myristylation (Myr) site. It contains Src-homology (SH) domains 2 and 3, along with a catalytic kinase domain. The activity of c-Src is controlled by phosphorylation at Tyr 527.

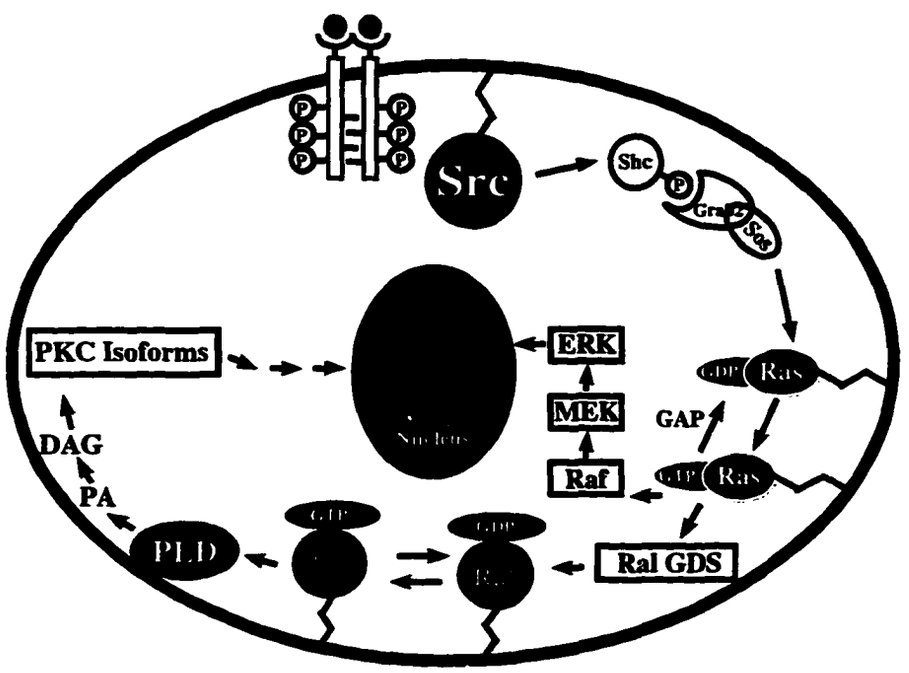


Figure 6. Ras mediates v-Src induced PLD activation

V. Summary

Taken together with the reports about PLD activation cited above, it is believed that phospholipase D is probably regulated through several pathways by different molecules. It is very possible that there are different PLD isoforms that are cell type and localization specific. In this study, the involvement of small G proteins, such as Ral and Arf, in activation of PLD activity in response to mitogenic signals v-Src and Ras are investigated. We found that the activation of PLD by v-Src involved a GTPase cascade of Ras, Ral-GDS and Ral. Arf exists in Ral/PLD complex and stimulates Ral-associated PLD activity. A model is proposed for involvement of Ral and Arf in v-Src-induced PLD activation.

EXPERIMENTAL METHODS

I. Cells and Cell Culture Conditions

Normal and v-Src-transformed murine fibroblast BALB/c 3T3 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum (HyClone). Cell cultures were made quiescent by growing to confluence and then replacing with fresh media containing 0.5% newborn calf serum for one day.

II. *in vitro* PLD Assay

An *in vitro* PLD assay that uses exogenous phospholipid substrate was developed. The substrate for PLD is presented in the form of phospholipid liposomes composed of phosphatidylethanolamine, PIP_2 and dipalmitoyl PC. 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 resuspending with sonication for 10 min at 45°C in lysis buffer. The liposomes consist of phosphatidylethanolamine/PIP_2/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 0.2 ml. The reaction mixtures were incubated for 15 min at 37°C and terminated by the addition of 2 ml of $CHCl_3$:MeOH:HCl 1.0:1.0:0.006. Phase separation was achieved by adding 1 ml of 0.1 N HCl, 1 mM EGTA. The resolution of PLD products was by thin layer chromatography (TLC) and quantification of transphosphatidylation bands.

III. Sample Preparation

Murine fibroblast cell lines BALB/c3T3, NIH3T3 and their v-Src transformed derivatives SRD and SRN will be used in all experiments.

(a) Total cell lysate preparation

Detergents octylglucoside (OG) 15 mM or Triton X-100 1% are used to lyse cells. Cells were grown to confluence at which time the media was replaced with fresh media containing 0.5% newborn calf serum for one day. Cells were washed twice with PBS buffer and treated with 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) containing 1% Triton X-100 or 15mM OG. Protease inhibitors (12 μg/ml leupeptin, 20 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride) were included just prior to lysis (30 min at 4°C). Lysates were scraped from the plates with a rubber policeman and centrifuged for 10 min at 1500 rpm; the supernatant was then centrifuged for 45 min at 30,000 rpm and the supernatant was recovered and protein concentration determined using the Bio-Rad assay. The PLD activity existing in cell lysates will be determined by using *in vitro* PLD assay.

(b) Fractionation of membrane and cytosol

Cells were grown to confluence at which time the media was replaced with fresh media containing 0.5% newborn calf serum for one day. Cells were washed twice with PBS buffer and then scraped into 3 ml hypotonic buffer containing 25 mM HEPES, pH 7.2; 0.5 mM EGTA; 0.5 mM EDTA; 1 mM DTT. The cells were disrupted with 50 strokes in a Dounce homogenizer and the homogenates were centrifuged at 1500 rpm for 10 min at 4°C

to remove nuclei and cell debris. The membrane and cytosol fractions were separated by centrifugation at 44,000 rpm for 50 minutes at 4°C. The protein concentrations of membrane and cytosol were determined by using the Bio-Rad assay. The PLD activity in membrane and cytosol fractions were determined by using *in vitro* PLD assay.

For Arf translocation assay, membrane and cytosol fractions were incubated together in the presence of 100 μM GTPγS at the protein amount ratio cytosol/membrane = 3/1. Membrane and cytosol fractions were re-isolated by centrifugation following a incubation at 30°C for 10 minutes.

IV. Assay of Ral-associated PLD Activity

(a) GST fusion protein system

The Glutathione S-transferase (GST) gene fusion system consists of a pGEX plasmid vector, a GST from Schistosoma japonicum and a target gene. They are designed for inducible, high level intracellular expression of genes or gene fragments as fusion proteins with the 26-KDa GST domain. The pGEX vectors (Fig. 7) feature a tac promoter for chemically inducible, high level expression and the lacIq gene, which allows the use of any E. coli host. The enzyme GST is used as the affinity handle for purifying fusion proteins. Expressed fusion proteins are easily purified from bacterial lysates by affinity chromatography using immobilized glutathione. This system is used here to study protein-protein interactions regarding PLD and its associated proteins.

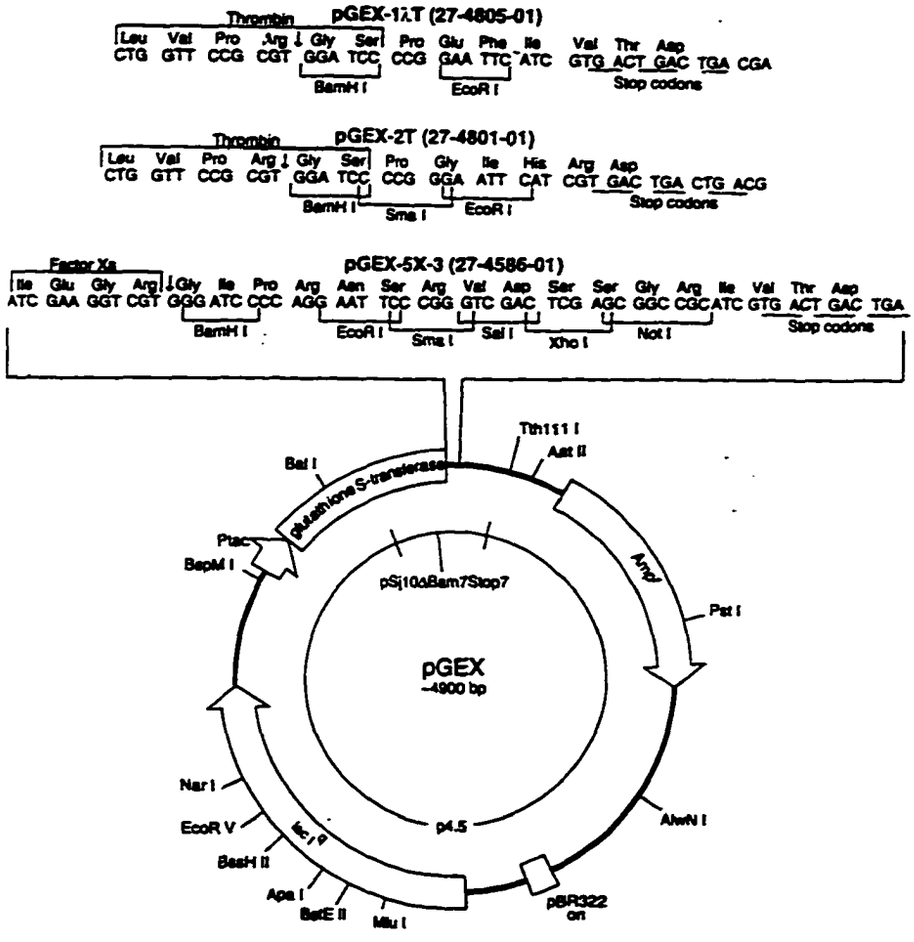


Figure 7. Map of the glutathione S-transferase fusion vectors showing the reading frames and main features

(b) Immobilized GST fusion protein binding assay

The immobilized GST fusion proteins (e.g. Ral and Ras) were loaded with GTPγS or GDPβS by incubating beads at 30°C for 30 minutes in the presence of 200 μM GTPγS or GDPβS. After washing 3 times with PBS buffer, the immobilized beads (20~40 μg fusion

proteins) were added into detergent lysate or membrane and cytosol fractions (600-800 μg protein) containing PLD activity for precipitation. After 1.5 h at 4°C, the GST fusion proteins were separated from the lysate or membrane and cytosol fractions by microcentrifugation for 30 sec and washed 3 times with cold lysis buffer. A liposome-based *in vitro* PLD assay was used to assay PLD activity associated with immobilized Ral fusion proteins. 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.

(c) Co-Immunoprecipitation Assay

Antibodies (e.g. anti-Ral) are added into cell lysates containing PLD activity. After incubation, the immuno-complex is collected by using protein A agarose beads. The PLD activity associated with the beads will be determined by using in vitro PLD assay.

V. Infection of sf9 Cells with PLD-expressing Baculovirus

PLD expressing sf9 cells were gifts of Dr. Andrew J. Morris. Recombinant baculoviruses expressing PLD1 and PLD2 were generated and propagated as described in papers [37-39]. For expression of PLD1 and PLD2, exponentially growing sf9 cells were infected with recombinant baculovirus at a multiplicity of infection of 10 for 1 h with gentle rocking. Infected cell were than grown in fresh media for 48 h prior to harvest and lysis for PLD assay.

VI. Arf Protein

The Arf protein used to stimulate PLD activity was a generous gift of Dr. Paul

Sternweis and Alex Brown. The Arf preparation was prepared as described previously by Brown et al. [34] from porcine brain. It was approximately 50% pure and contained a mixture of Arf1 and Arf3. Arf proteins (40 nM) were used together with 10 μM GTPγS for stimulating PLD activity.

VII. Western Analysis

Equal amounts of protein from both membrane and cytosolic fractions or cell lysates were subjected to SDS-polyacrylamide gel electrophoresis using an 8% acrylamide separating gel for PLD detection and 10% for Arf detection. Transfer to nitrocellulose paper. After blocking overnight at 4°C with 5% non-fat dry milk in isotonic phosphate buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4 , 4.2 mM Na_2HPO_4), the nitrocellulose filters were incubated with appropriate antibodies as described in the text. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG was used for detection using the ECL system (Amersham) and SuperSignal™ western blotting reagent (Pierce).

VIII. Purification of PLD1

PLD1 was affinity purified from sf9 cells infected with the PLD1-expressing baculovirus using PLD1 selective anti-peptide antibodies as described previously by Morris and Frohman [37-39]. Purified PLD1 proteins were a gift from Dr. Andrew J. Morris. Partially purified pig brain PLD was provided by Dr. Paul Sternweis and Alex Brown.

INVOLVEMENT OF Ral GTPase IN v-Src-INDUCED PHOSPHOLIPASE D ACTIVATION

Introduction

An early response to the tyrosine kinase activity of v-Src is an increase in phospholipase D activity[26], which leads to the generation of a variety of biologically active lipid second messengers including phosphatidic acid, lysophosphatidic acid and diacylglycerol[3]. Recently, our laboratory demonstrated that v-Src-induced PLD activity is mediated by Ras[81], although Ras involvement was indirect, requiring a cytosolic factor for PLD activation[81]. Ras was recently reported to interact with[87-89] and activate Ral-GDS[84], the exchange factor responsible for the activation of Ral GTPases. We demonstrate here that this newly identified Ras/Ral signalling pathway mediates PLD activation by v-Src.

Ral is a Ras-related small GTPase with membrane localization. It shares about 58% homology with Ras. Its N-terminal end is 11 aa longer than Ras. Ral was reported as a nontransforming Ras-related protein, having its own specific guanine-nucleotide exchange factor, Ral-GDS. What is the function of Ral GTPases in cells? Until now, two signalling molecules, whose activities may be regulated by Ral, have been identified. One is a newly identified GAP for Cdc42 and Rac, this protein is called Ral-BP1, which stands for Ral-binding-protein 1. It binds specifically to the GTP bound form of Ral, and not to other related GTPases. The other signalling molecule is the phospholipase D that is investigated here.

We found that PLD activity could be precipitated from v-Src-transformed cell lysates with immobilized RalA protein and with an anti-Ral antibody. A mutation to the region of RalA analogous to the "effector domain" of Ras did not reduce the ability of RalA to complex with PLD; however, deletion of a Ral-specific N-terminal region did. Overexpression of RalA potentiated PLD activation by v-Src, and expression of dominant negative RalA mutants inhibited both v-Src- and v-Ras-induced PLD activity. These data argue that RalA is involved in the tyrosine kinase activation of PLD through its unique N-terminus and that PLD is a downstream target of a Ras/Ral GTPase cascade.

Results

1. The association of PLD activity with RalA in v-Src transformed cells

The research from our laboratory indicates that although the activation of PLD by v-Src is mediated by Ras[81], two reported Ras effector molecules, Raf-1[84,85] and PI-3-kinase[86], were apparently not involved[81]. The recent observation that Ras also activates Ral-GDS [90] suggested that the activation of PLD by v-Src could be through Ral GTPase. We therefore examined whether GST-RalA fusion proteins (immobilized Ral) could associate with PLD activity in detergent lysates of v-Src-transformed and parental Balb/c 3T3 cells. As shown in Figs. 8a and 8b, both GTP- and GDP-loaded RalA precipitated PLD activity from both cell types. Although GTP-bound RalA was somewhat more efficient in binding PLD than GDP-bound RalA, the difference was substantially less than that reported for the binding of both Ras and Ral to other downstream effector molecules [84-86, 91]. The effect was specific for

Ral since GST-Ras was unable to precipitate PLD activity (Fig. 8b).

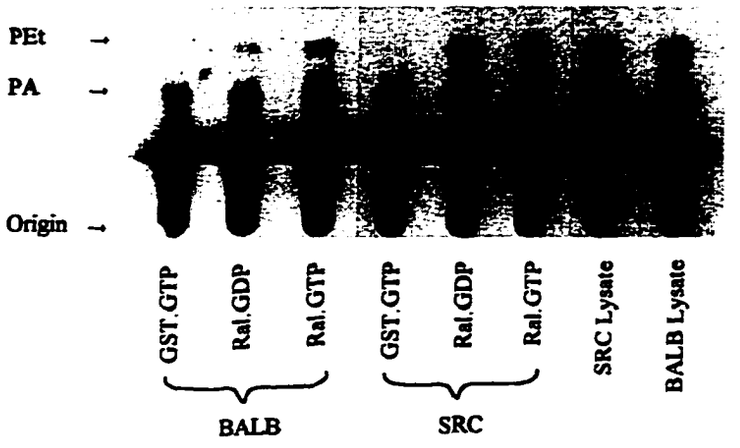


Figure 8a. PLD activity associates with RalA in v-Src-transformed cells *in vitro*. An autoradiograph of the thin layer chromatography resolution the PLD transphosphatidylation product phosphatidylethanol (PEt) and phosphatidic acid (PA) is shown. Immobilized GST and immobilized GST-RalA fusion protein preloaded with either GTPγS or GDPβS were mixed with detergent lysates from both v-Src-transformed (SRC) and parental Balb/c 3T3 (BALB) cells. The immobilized proteins were isolated by microcentrifugation and the PLD activity in the pellets was determined as described in materials and methods. The PLD activity in total cell lysates is also shown as control.

Also shown in Fig. 8a is the total PLD activity in the extracts prior to treatment with the immobilized GST and RalA fusion proteins. The amount of PLD activity observed in the total extract represents the amount of PLD activity in 40 μg protein; whereas the amount of PLD in the RalA pellets represents the amount of PLD activity extracted from 600 μg of protein. Since the amount of PEt observed in both the Ral precipitates and the total cell extracts as shown is similar, the PLD activity extracted with GTP-loaded immobilized RalA from v-Src-transformed cells represents about 6.7% of total PLD.

To determine whether complexes between Ral and PLD exist *in vivo*, we investigated whether PLD activity could be precipitated by an anti-Ral antibody. As shown in Fig. 9, an anti-Ral, but not an anti-Ras, antibody precipitated PLD activity from v-Src-transformed cell lysates. PLD activity could also be precipitated from parental Balb/c 3T3 cell lysates by the anti-Ral antibody suggesting that an association between RalA and PLD exists prior to tyrosine kinase activation.

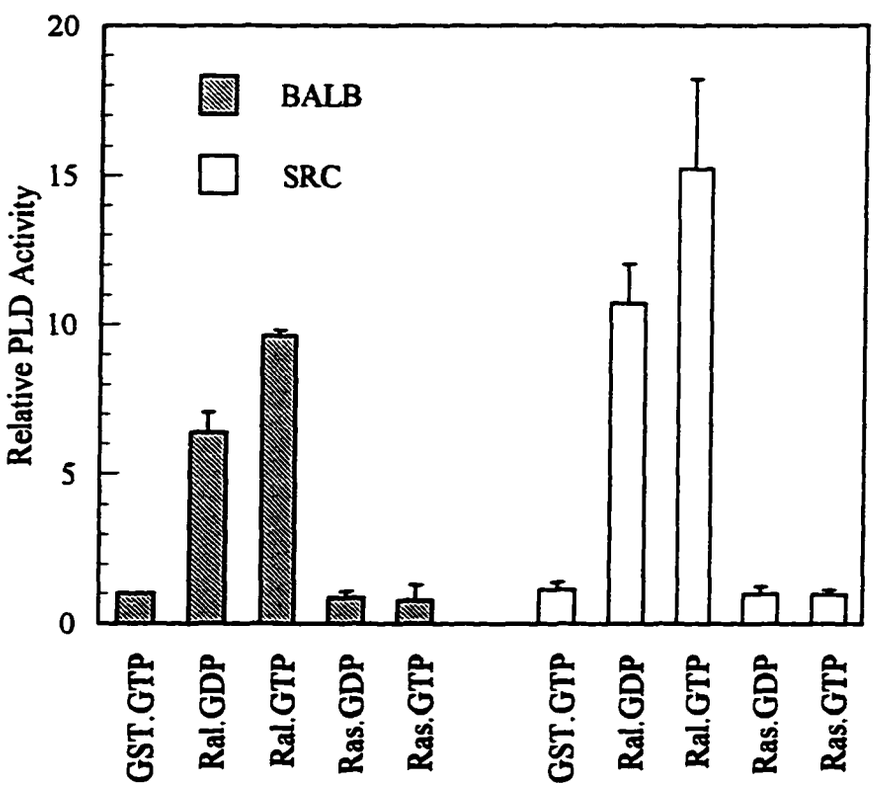


Figure 8b. Association of PLD activity is specific for RalA. Immobilized GST, GST-RalA and GST-Ras fusion proteins preloaded with either GTPγS or GDPβS as shown were mixed with detergent lysates of either v-Src-transformed or parental Balb/c 3T3 cells and the relative PLD activity in the pellets was determined as described in materials and methods. The values were normalized to the PLD activity associated with the GST control. The error bars reflect the range of the PLD activity relative to the GST controls for two independent experiments performed in duplicate exclusive to the representative experiment shown in (a).

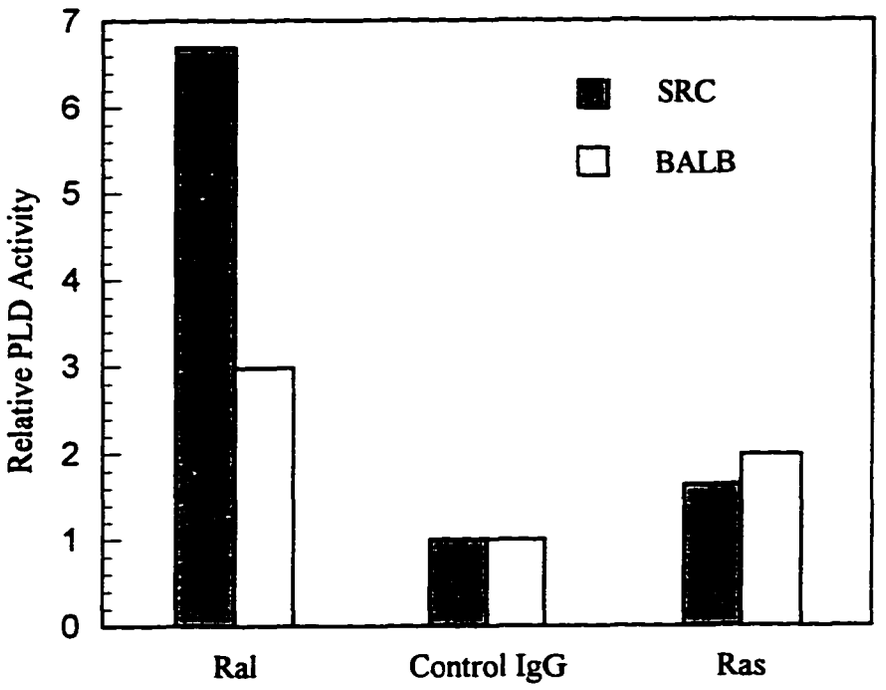


Figure 9. PLD activity associates with RalA in v-Src-transformed cells *in vivo.* Detergent lysates from v-Src-transformed and parental BALB/c 3T3 cells were treated with IgG purified from a polyclonal anti-Ral antisera, monoclonal anti-Ras antibody 259 and control rabbit IgG overnight at 4°C. The immune complexes were recovered with protein A agarose and associated PLD activity was determined as in Fig. 8a and 8b. In this experiment, an autoradiogram of the thin layer chromatography plate was scanned using a Molecular Dynamics densitometer. The relative PLD activity associated with the anti-Ral, anti-Ras and control IgG was determined by comparing the relative intensities of the phosphatidylethanol bands. The PLD activity was normalized to the PLD activity that was detected in the control IgG precipitates. The representative experiment shown is one of two performed where the results varied by less than 10%.

2. *Differential activity of PLD associated with Ral mutants*

We examined the ability of RalA mutants to associate with PLD. D49N RalA has an Asp to Asn substitution at position 49 of RalA, which is in a region of RalA analogous to the effector domain of Ras that associates with downstream effector molecules. The D49N mutation in RalA was previously shown to prevent association between RalA and a different putative downstream target molecule called Ral-BP1[91]. Surprisingly, the association between RalA and PLD was actually enhanced by the D49N mutation (Fig. 10). In contrast, deletion of 11 N-terminal residues (ΔN11) unique to Ral proteins strongly reduced the association between RalA and PLD (Fig. 10). The ΔN11 RalA deletion did not significantly effect the association of RalA with Ral-BP1 (Ref. 91 and the results from our laboratory).

3. *The effect of Ral on PLD activity in intact cells*

(a) *Wild-type RalA and activated RalA potentiated PLD activation by v-Src*

We investigated the effect of altering Ral activity in intact cells. Wild type RalA and an activated RalA mutant (Q72L) were overexpressed in v-Src-transformed and parental NIH 3T3 cells. The Q72L RalA mutant contains a mutation analogous to the activating Ras mutation at position 61. It is defective for GTPase activity as is the homologous mutation in Ras. Both wild type and activated RalA increased PLD activity in the v-Src-transformed cells, but had no effect upon the PLD activity in the parental cells (Fig. 11). In addition, purified GTP-bound RalA did not increase PLD activity in cell lysates (not shown). These

data argue that although RalA contributes to v-Src-induced PLD activation, it is not sufficient for PLD activation.

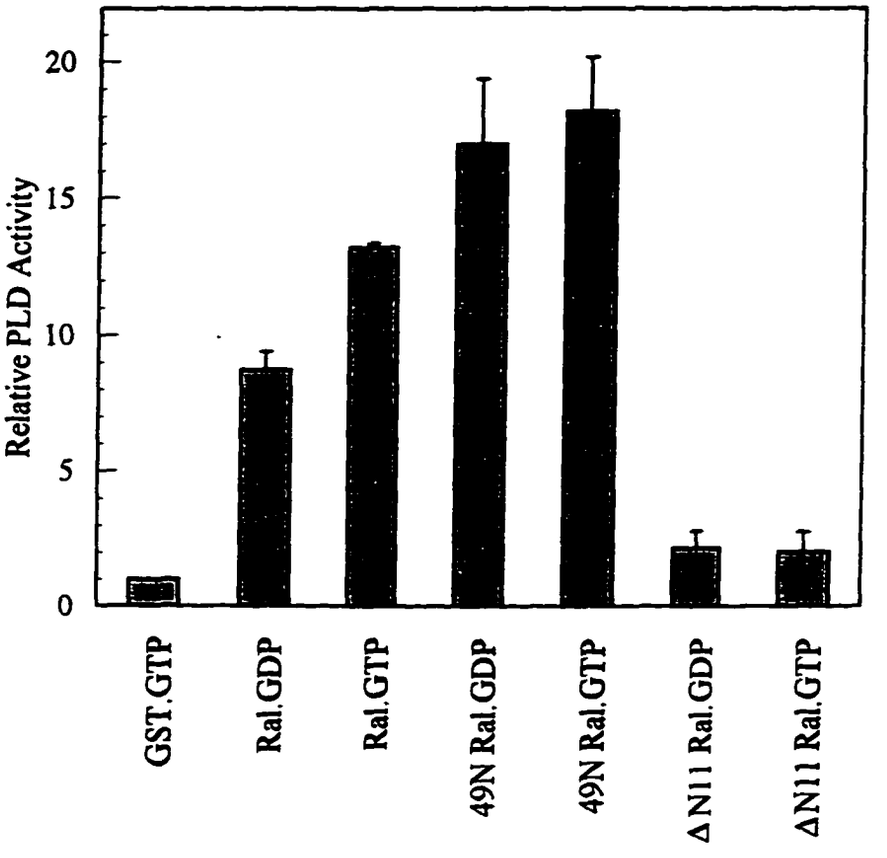


Figure 10. Differential activity of PLD associated with Ral mutants. The ability of GTPγS- and GDPβS-bound immobilized RalA mutants to associate with PLD activity in v-Src transformed cells was determined as above. PLD activity associated with wild type RalA is included for comparison. D49N contains an amino acid substitution of Asn for Asp at position 49; ΔN11 has a deletion of 11 amino acids at the amino terminus. The data was generated as in Fig. 8b and then normalized to the PLD activity precipitated with GST alone. The error bars reflect the range of the PLD activity relative to the GST control for two independent experiments.

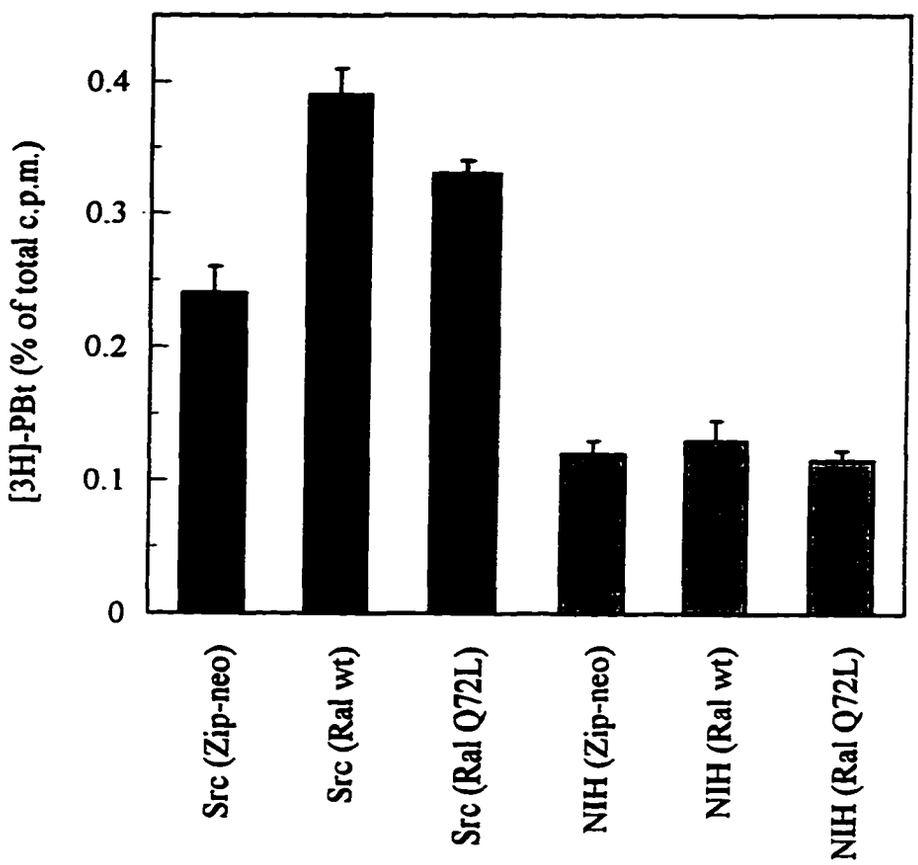


Figure 11. The effect of wild type and mutant RalA on PLD activity in NIH 3T3 cells. The effect of wild type and an activated mutant of RalA (Q72L) on PLD activity in v-Src-transformed and parental NIH 3T3 cells was determined. v-Src-transformed and parental NIH 3T3 cells were stably transfected with vectors expressing wild type and the Q72L RalA genes. RalA proteins were expressed at approximately 6 fold greater than endogenous RalA (not shown). The PLD activity in these cells was determined by measuring the level of phosphatidylbutanol (PBt) generated in the presence of exogenously supplied butanol (1.0% for 30 min). The data are the average of duplicates +/- range from representative experiments repeated at least three times. The data are presented as amount radioactivity present in the transphosphatidylation product, phosphatidylbutanol, as a percentage of the total cpm loaded onto the resolving thin layer chromatography plates. Samples are normalized for total cpm prior to loading.

(b) Dominant negative RalA mutant inhibited PLD activity induced by v-Src and Ras

Is RalA necessary for v-Src activation of PLD ? We tested the effect of a dominant negative RalA mutant (S28N) on v-Src-induced PLD activity. The S28N mutation is analogous to the dominant negative S17N mutant of Ras [92 93 94], which contains a substitution of Asn for Ser and is constitutively locked in the GDP state. Expression of S28N RalA in v-Src-transformed cells reduced PLD activity to the level observed in the parental cells or in v-Src-transformed cells expressing the dominant negative S17N Ras mutant (Fig. 12a). This result and above data support the notion that RalA plays an important role in the activation of PLD by v-Src. Further support came from studying v-Src-transformed cells expressing the N-terminal RalA deletion mutant (ΔN11) that precipitated PLD poorly *in vitro*. These cells displayed PLD activity that was reduced almost to the level observed in the parental NIH 3T3 cells. Apparently, the ΔN11 mutant that failed to bind PLD efficiently *in vitro* was not able to mediate PLD activation by v-Src *in vivo* and competed with endogenous RalA in this signalling pathway.

Since v-Src-induced PLD activity is dependent upon Ras [81] and Ras has been reported to induce an increase in PLD activity [83], we also examined the effect of the S28N RalA mutant on PLD activity in cells transformed by Ras. As shown in Fig. 12b, expression of the S28N mutant inhibited the Ras-induced increase in PLD activity. The degree of inhibition correlated well with the level of S28N Ral expression in several independent clones.

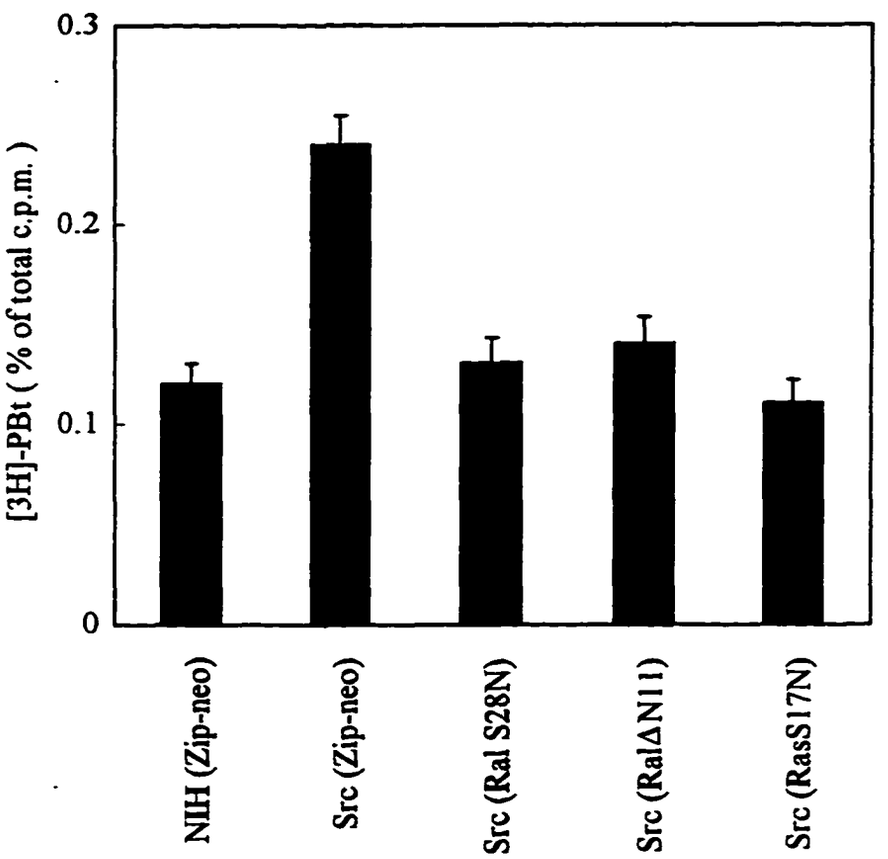


Figure 12a. v-Src induced PLD activity is inhibited by a dominant negative RalA mutant. The effect of potential dominant negative RalA mutants was compared with the effect of a dominant negative Ras mutant on PLD activity in v-Src-transformed NIH 3T3 cells. The v-Src-transformed NIH 3T3 cells were stably transfected with plasmids expressing the S28N and ΔN11 mutants of RalA and the dominant negative S17N Ras mutant. The PLD activity was determined as described in Fig. 11.

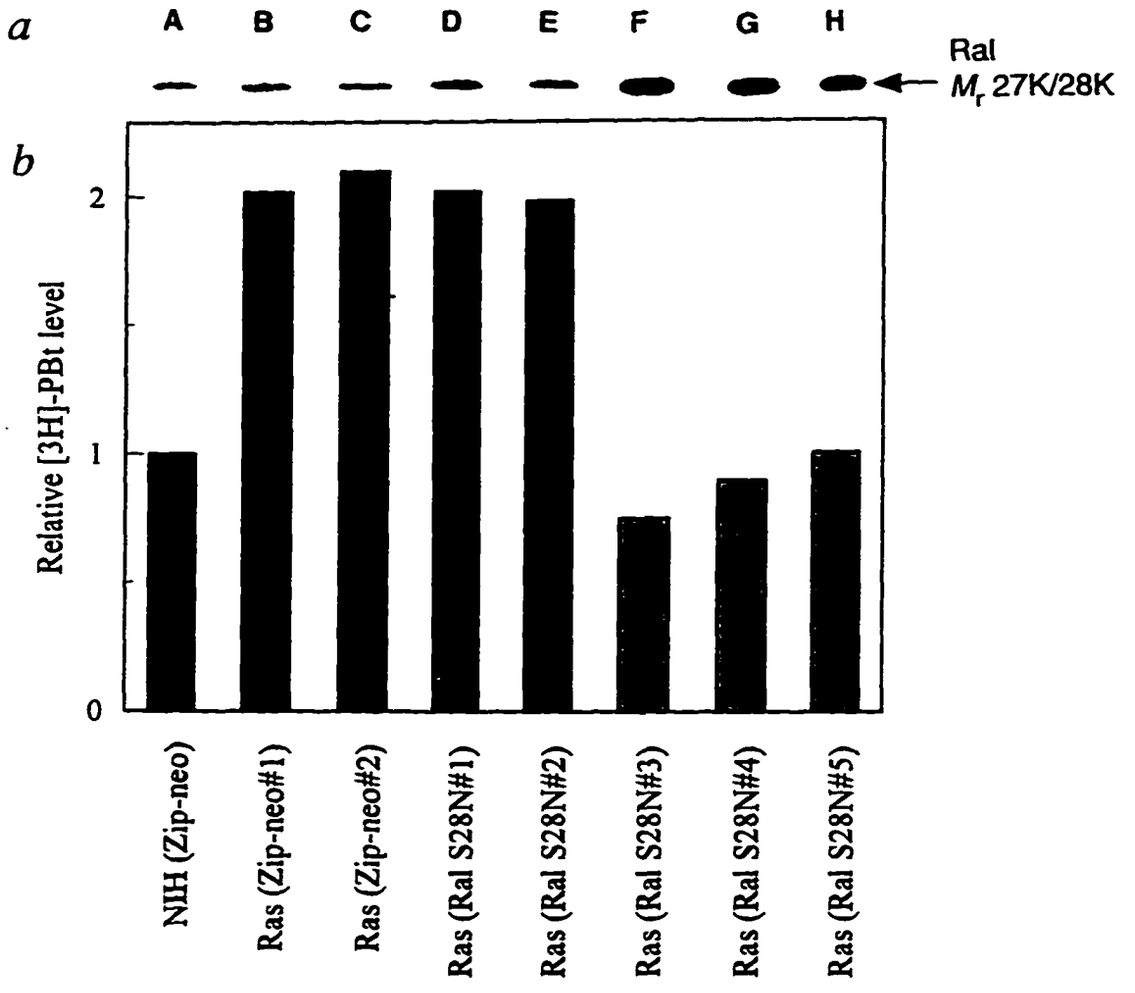


Figure 12b. Ras-induced PLD activity is inhibited by a dominant negative RalA mutant. NIH 3T3 cells were transformed by a vector expressing v-Ras (pv-HaRas) or the parental vector (pZip-neoSV(X)[26]. The Ras-transformed cells were then co-transfected with the vector expressing the S28N RalA mutant and pCEP4 (Invitrogen), which expresses the resistance gene for hygromycin. The ratio of the S28N vector to pCEP4 was 10:1. Hygromycin selection was used because the Ras-transformed cells already expressed the G418 resistance gene present in the S28N vector. Five independent hygromycin-resistant clones were picked and expression of Ral protein (endogenous and S28N) was determined by Western analysis and the relative levels of Ral protein were determined using densitometer tracing of the autoradiogram (a). The relative levels of PLD activity (normalized to the level of PLD activity in NIH 3T3 cells) were then determined as in Fig. 11(b).

Discussion

The experiments described here argue strongly that RalA mediates the tyrosine kinase activation of PLD through a novel interaction with N-terminal sequences unique to Ral. A model of how RalA might function in this capacity is outlined in Fig. 13. v-Src activates Ras, which would then bring Ral-GDS to the plasma membrane. Ral-GDS would then bring RalA and constitutively associated PLD into a tyrosine kinase/Ras signalling complex. Since activated RalA (Q72L) is unable to activate PLD activity in non-transformed NIH 3T3 cells, an additional signal induced by v-Src must be required for elevating PLD activity. The observation that Ras-transformed cells also display elevated PLD activity suggests that this additional signal is downstream of Ras. Possible mediators of this signal are the Ras related Rho and ARF proteins, which have both been shown to stimulate PLD activity in vitro [18 46 48 49 95 96]. Genetic evidence linking RhoA and Ras has been reported [97]; a connection between ARF and Ras remains to be demonstrated.

The significance of PLD activation by tyrosine kinases is unknown; however, PLD activity has been found to be elevated in response to all of the tyrosine kinases where it has been examined [3]. The ability of PLD to generate multiple lipid second messengers via its primary metabolite phosphatidic acid suggests that PLD likely contributes significantly to cellular responses activated by Src and mediated by Ras. In this regard, it is possible that PLD activation via RalA contributes to the recently reported ability of RalA to enhance both Ras- and Raf-induced cellular transformation [90].

This investigation was published in *Nature*, **23**, 409-412, 1995.

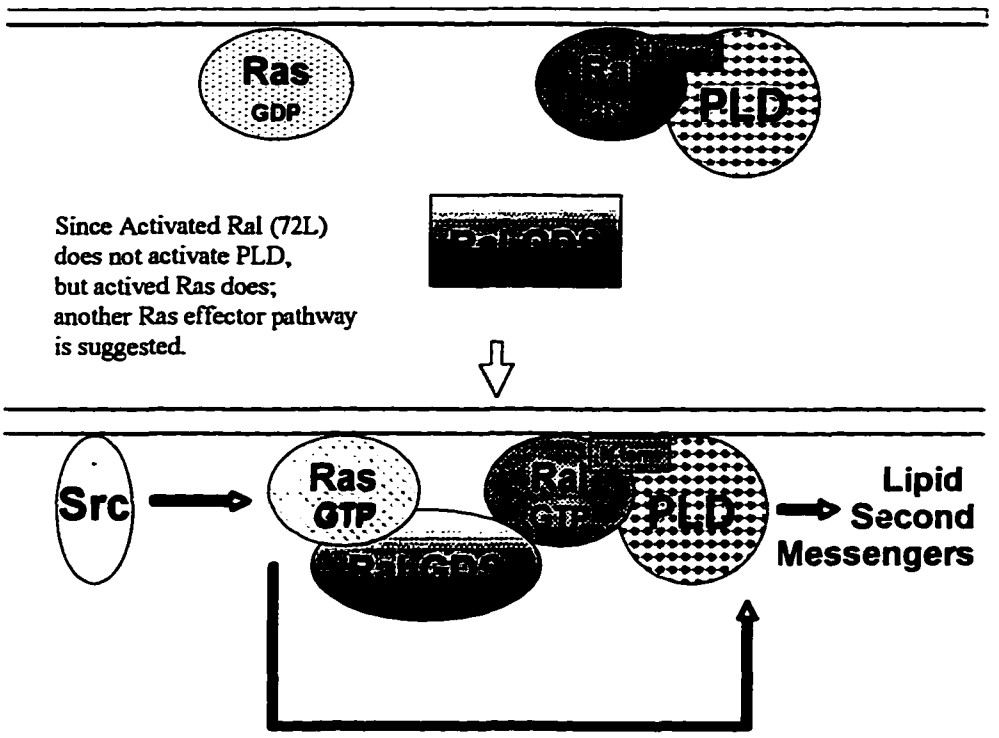


Figure 13. Model for RalA involvement in v-Src-induced PLD activation. It is proposed that RalA, which is a membrane-bound protein, serves as an anchor for PLD through its amino terminus. Ral-GDS could then serve as a conduit for bringing PLD into a complex with activated Ras. An additional Ras downstream effector molecule(s) is postulated to account for the ability of activated Ras, but not activated Ral to increase PLD activity in non-transformed NIH 3T3 cells.

RalA INTERACTS DIRECTLY WITH THE Arf-RESPONSIVE, PIP_2-DEPENDENT PHOSPHOLIPASE D1

Introduction

Since we demonstrated that a type D phospholipase (PLD) activated in v-Src- and v-Ras-transformed cells associates with the Ral-family GTPase RalA, a characterization of RalA-associated PLD was investigated. Two mammalian PLDs, designated PLD1 and PLD2, have been cloned. PLD1 is activated by Arf family GTPases, and is dependent upon phosphatidylinositol-4,5-bisphosphate (PIP_2). PLD2 is dependent upon PIP_2 but active in the absence of Arf. Another mammalian PLD that is stimulated by oleate has been characterized, but a gene for this PLD has not been cloned. In this research, we present evidence that the PLD associated with RalA is the recently cloned PLD isoform PLD1. First, the PLD activity precipitated by RalA from v-Src-transformed murine fibroblasts was stimulated by Arf, dependent upon PIP_2, and inhibited by oleate. Second, immobilized RalA could precipitate both PLD1 activity and PLD1 protein from sf9 insect cells infected with baculovirus expressing PLD1. Third, a series of RalA mutants precipitated PLD activity from PLD1-expressing insect cells with the same pattern as that observed in v-Src-transformed murine fibroblasts. And finally, immobilized RalA could precipitate PLD1 from a purified PLD1 preparation. These data argue that the PLD associated with RalA is the Arf-responsive, PIP_2-dependent PLD1 and that this interaction is direct.

Results

1. RalA-associated PLD from murine fibroblasts is stimulated by Arf, dependent upon PIP_2 and inhibited by oleate

Several reports have implicated Arf in the activation of PLD [14 37 47 98], and a PLD that is responsive to Arf (PLD1) has now been cloned [37 39]. The previous research indicates that PLD activity associated with RalA in murine fibroblasts [82] and that this PLD activity is elevated by the oncogenic stimuli of v-Src [26] and v-Ras [81 82]. We therefore investigated whether the PLD associated with RalA from v-Src-transformed fibroblasts is affected by Arf. Lysates were prepared from v-Src-transformed cells and treated with either GST or GST-RalA fusion protein attached to glutathione beads (immobilized RalA) and PLD activity in the presence and absence of Arf was determined. In Fig. 14a, it is shown that the PLD activity precipitated from v-Src-transformed fibroblasts using the immobilized GST-RalA fusion protein is substantially elevated by an Arf preparation consisting of Arf1 and Arf3 [34]. No PLD activity was detected in the GST precipitates either in the absence or presence of Arf (Fig. 14a). Thus, like PLD1, the PLD associated with RalA from v-Src-transformed fibroblasts is stimulated by Arf. The Arf-dependent PLD1 is dependent upon PIP_2 [14 37]. We therefore examined the PIP_2-dependence of the RalA-precipitable PLD activity, and as shown in Fig. 14b, the RalA-precipitable PLD from v-Src-transformed fibroblasts was completely dependent upon PIP_2. PLD1 was also shown to be inhibited by oleate [37], and oleate also inhibited the RalA-precipitable PLD (Fig. 14c). These data indicate that the PLD associated with RalA from v-Src-transformed murine fibroblasts has properties that are similar to those reported for PLD1.

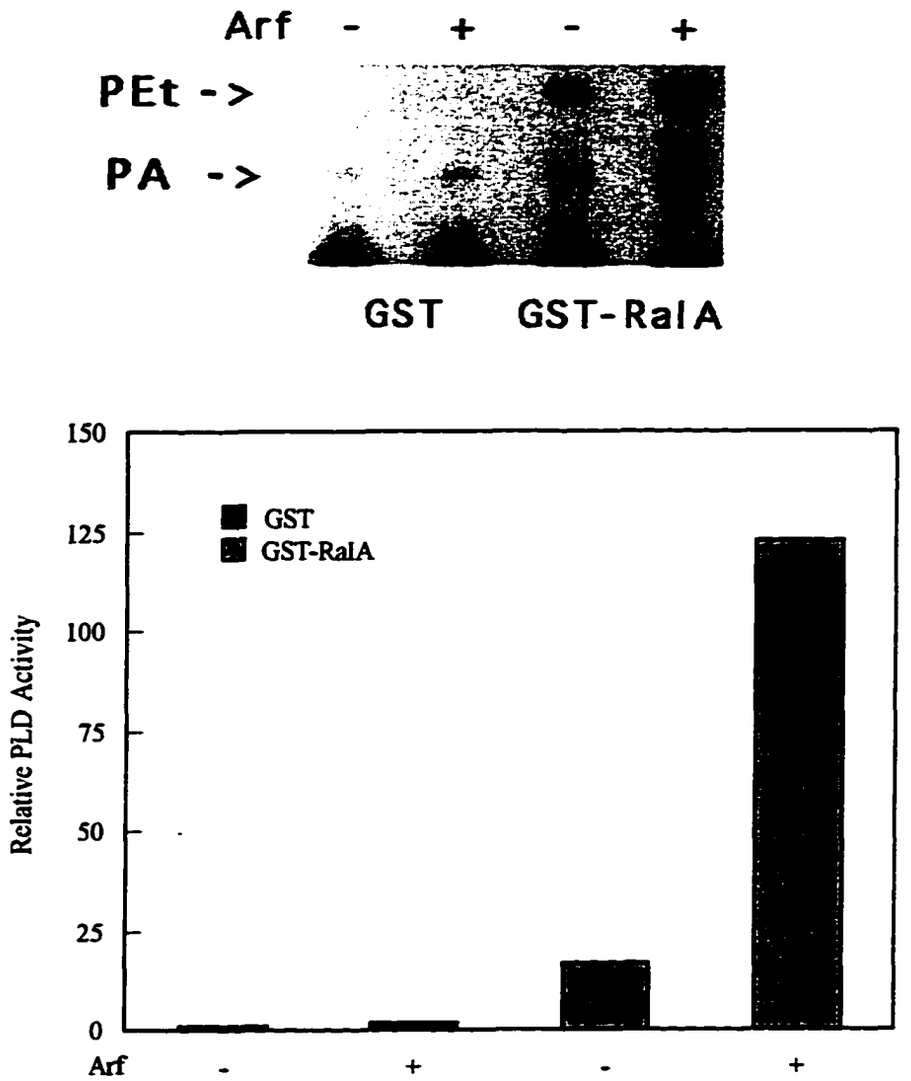


Figure 14a. RalA-associated PLD from murine fibroblasts is stimulated by Arf. Lysates from v-Src-transformed Balb/c 3T3 cells were treated with GTPγS-loaded GST and GST-RalA fusion protein at 4°C for 1.5 h. The GST and GST-RalA proteins were recovered by centrifugation and the PLD activity in the pellets was determined as described previously in the presence and absence of partially purified Arf proteins (~40 nM) as shown. 600 μg of protein from the v-Src-transformed cell lysates was used for the precipitations. Thin layer chromatography of the transphosphatidylation products are shown in the top panel and the relative PLD activity (PEt levels) normalized to background PLD activity recovered in the GST controls is shown in the lower panel. The data are from a representative experiment (repeated 3 times) where the PLD activity was normalized to background PLD activity in the GST control in the absence of Arf.

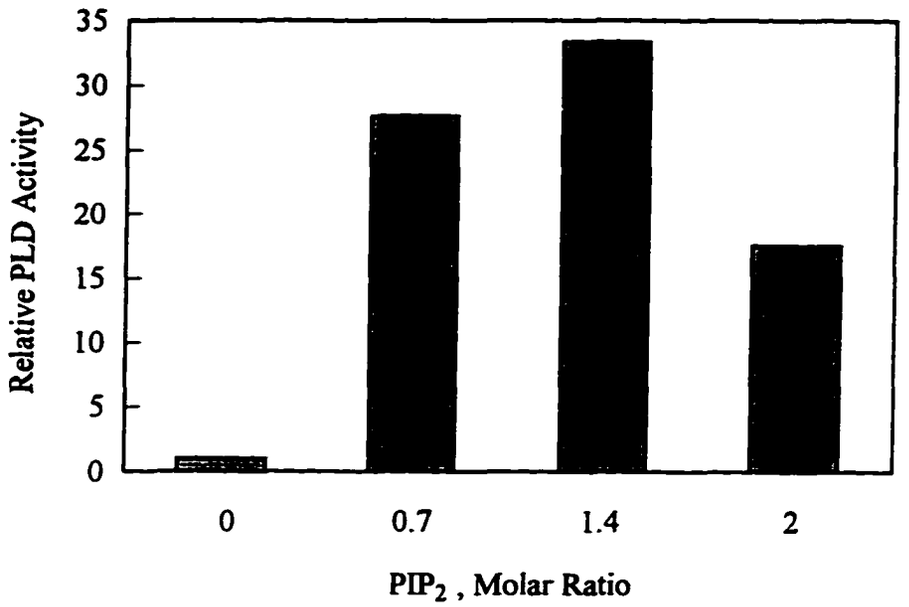


Figure 14b. RalA-associated PLD from murine fibroblasts is dependent upon PIP_2. RalA associated PLD activity from v-Src-transformed cells (in the absence of Arf) was determined using liposomes with varying concentrations of PIP_2 as shown.

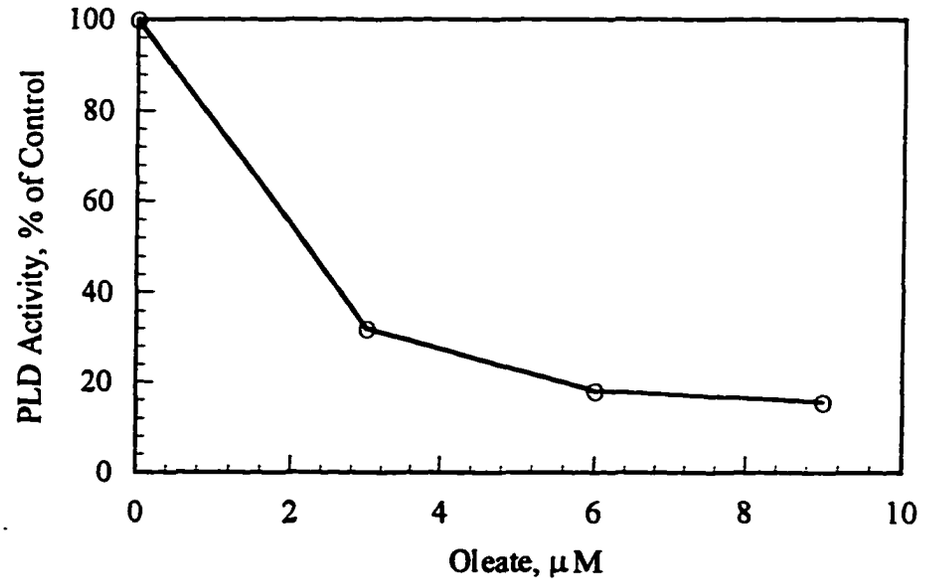


Figure 14c. RalA-associated PLD from murine fibroblasts is inhibited by oleate. RalA-associated PLD activity from v-Src-transformed cells was determined in the presence of increasing concentrations of oleate as shown.

2. RalA precipitates PLD1 from baculovirus-infected sf9 cells overexpressing PLD1

We next examined the ability of RalA to precipitate PLD activity from insect cells that were infected with baculoviruses containing PLD1 cDNA [37 39]. Lysates were prepared from uninfected and infected sf9 cells and treated with either GST or GST-RalA and PLD activity in the precipitates was determined. As shown in Fig. 15a, immobilized RalA precipitated PLD activity from lysates of sf9 cells expressing PLD1 by greater than 50 fold over background. No PLD activity was detected in RalA precipitates from lysates of uninfected sf9 cells. We next determined whether PLD1 protein could be detected in the RalA precipitates. Western analysis of lysates from the PLD1-expressing cells with an antibody raised against PLD1 revealed the expected 120 kDa protein (Fig. 15b).

3. RalA mutants precipitate PLD activity from PLD1-expressing insect cells with the same pattern as that observed in v-Src-transformed murine fibroblasts

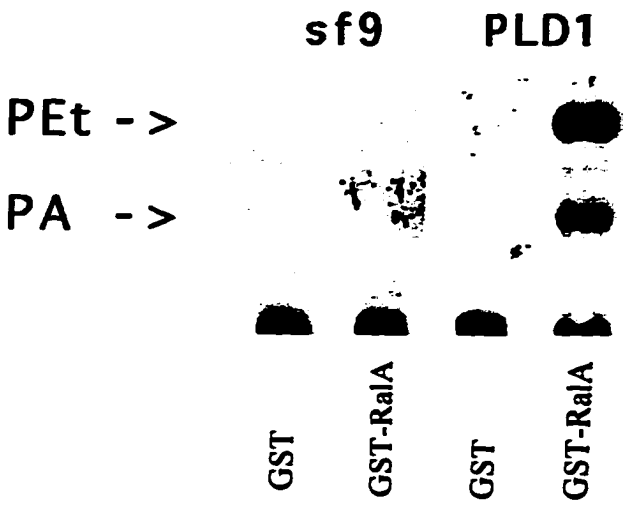
We next investigated the ability of RalA mutants to precipitate PLD activity from the PLD1-expressing sf9 cells. We previously demonstrated that the ability of RalA to precipitate PLD activity from v-Src-transformed Balb/c 3T3 cell lysates was enhanced by an effector domain mutation in RalA (D49N) and reduced by an amino terminal truncation to RalA (ΔN11) [82]. We therefore compared the ability of these RalA mutants to precipitate PLD activity from v-Src-transformed Balb/c 3T3 cells and PLD1-expressing sf9 cells. In Fig. 16, it is shown that the D49N mutant precipitated more PLD activity and the ΔN11 mutant precipitated very little PLD activity from both the PLD1-expressing sf9 cells and the v-Src-transformed cells. The similar RalA-association pattern of PLD1 and the PLD activity in v-Src-transformed murine fibroblasts suggests that the PLD associating with RalA in the

v-Src-transformed cells is the murine equivalent of PLD1. Attempts to identify PLD1 protein in the RalA precipitates from the murine fibroblasts were unsuccessful. This suggested that, as described previously [37], PLD1 is expressed at very low levels. We also used human A431 cells where PLD activity could also be precipitated by RalA and were unable to see the p120 PLD1. Thus, these results are not likely due to the inability of the antibody raised against the human protein to recognize the murine protein.

4. Immobilized RalA precipitates PLD1 from a purified preparations of PLD1

The data presented above strongly suggest that RalA interacts with PLD1. However, the data do not indicate whether the interaction is direct or indirect. To determine whether there is a direct interaction between RalA and PLD1, we examined the ability of purified bacterially synthesized RalA to precipitate PLD1 from an affinity-purified preparation of PLD1 [37,39]. The highly purified PLD preparation was treated with immobilized GST or GST-RalA fusion proteins. After incubation, they were recovered by centrifugation and were subjected to Western blot analysis using the anti-PLD1 antibody. As shown in Fig. 17, GST-RalA precipitated PLD1 from the purified PLD preparations. Densitometric analysis of the Western blot indicated that the RalA proteins precipitated approximately 25% of the PLD1. Since both the PLD1 and RalA preparations used were highly pure, the data suggest that the interaction between RalA and PLD1 is direct. We were unable to precipitate detectable PLD2 protein [38] under the same conditions using purified PLD2 protein (not shown).

(a).



(b).

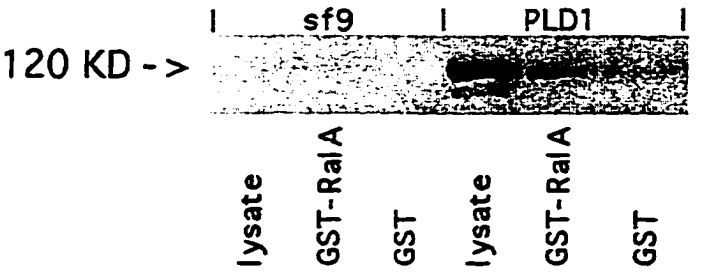


Figure 15. Immobilized RalA precipitates PLD1 activity and protein from baculovirus-infected sf9 cells overexpressing PLD1. (a). Uninfected sf9 cells or sf9 cells infected with PLD1-expressing baculovirus were lysed and 20 μg protein from the lysate was treated with either immobilized GST or GST-RalA fusion protein. The GST and GST-RalA precipitates were recovered by centrifugation and the pellets were analyzed for PLD activity as in Fig. 14. (b). Uninfected and PLD1-expressing sf9 cells were lysed and the lysate was then treated with either immobilized GST or GST-RalA. The lysates and the recovered GST and GST-RalA precipitates were then subjected to Western blot analysis using an antibody raised against PLD1. 10 μg of lysate protein was loaded onto the gel for direct Western analysis, and for the GST and GST-RalA precipitations, 30 μg of lysate protein was used.

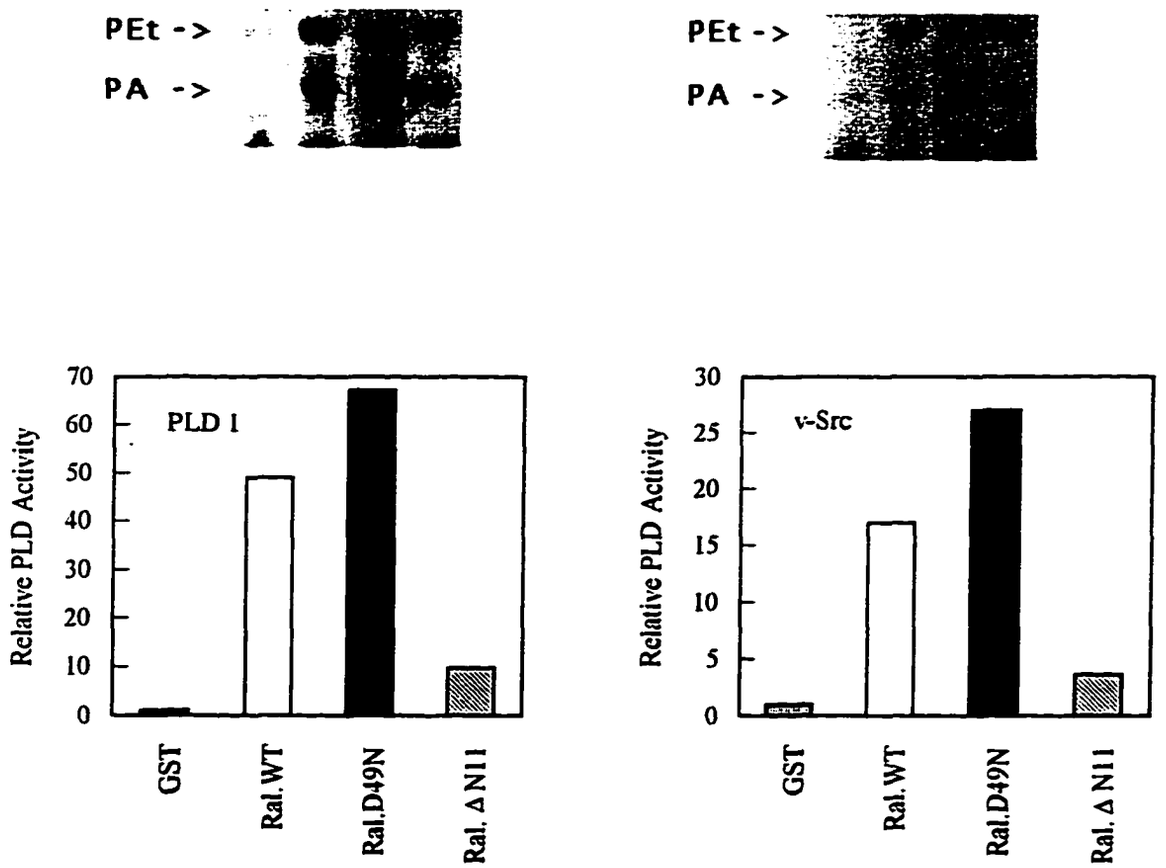


Figure 16. The ability of RalA mutants to precipitate PLD activity from murine fibroblasts and baculovirus-infected sf9 cells overexpressing PLD1. Lysates of PLD1-expressing sf9 cells (PLD1) and v-Src-transformed Balb/c 3T3 (v-Src) were treated with GTPγS-loaded immobilized GST-RalA or the D49N and ΔN11 GST-RalA mutants as shown. GST-RalA fusion proteins were recovered and the pellets analyzed for PLD activity as in Fig. 14. 20 μg of protein from the sf9 cell lysate and 600 μg of protein from the v-Src-transformed cell lysate were used in the precipitations. Thin layer chromatography of the transphosphatidylation products are shown in the top panels and the relative PLD activity (PEt levels) normalized to background PLD activity recovered in the GST controls is shown in the lower panels.

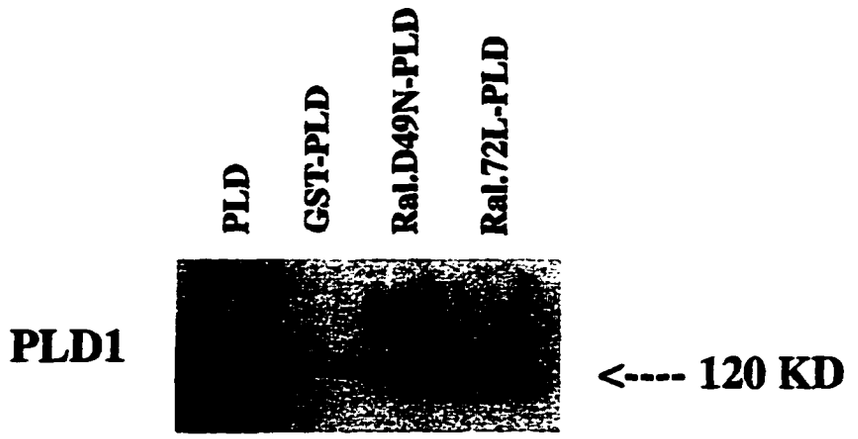


Figure 17. Immobilized RalA precipitates PLD1 from a purified PLD1 preparation. PLD1 was affinity-purified from baculovirus-infected cells as described in materials and methods. The purified PLD preparation (0.12 μg protein; 0.10 nmoles) was treated with immobilized GST, GST-RalAD49N and GST-RalAQ72L (50 nmoles). The immobilized GST and GST-RalA proteins were recovered by centrifugation, washed 3 times with PBS. The precipitates were subjected to Western blot analysis using anti-PLD1 antibody. In lane 1 (left lane labeled PLD) is the PLD1 preparations prior to treatment with either GST or GST-RalA proteins.

Discussion

Our previous studies demonstrated an interaction between RalA and PLD activity in v-Src-transformed cells [82]. The present work establishes that the PLD associated with RalA is the recently cloned PLD1 [37]. The PLD associated with RalA from v-Src-transformed cells, like PLD1, was stimulated by Arf and dependent upon PIP_2. RalA precipitated PLD1 activity from insect cells overexpressing PLD1 and an antibody raised against PLD1 recognized a protein in RalA precipitates from the PLD1-expressing sf9 cells. Purified RalA was able to precipitate PLD1 from a purified PLD1 preparation, suggesting that the

interaction between RalA and PLD1 is direct.

Previous work implicated PLD in the intracellular signals activated by the oncogenic tyrosine kinase v-Src [26 94]. The PLD activated by v-Src was dependent upon both Ras [81 91] and RalA [82]. In addition to mediating the activation of PLD by Src and Ras, RalA also mediates the transformed phenotype induced by Ras [90], suggesting a role for PLD in the transformed phenotype induced by v-Ras. Since Ral-GDS, the guanine nucleotide exchange factor for RalA, is a downstream effector molecule of Ras [90], the activation of PLD by v-Src is apparently regulated by this GTPase cascade of Ras/Ral-GDS/RalA. However, the association between RalA and PLD is actually enhanced by effector domain mutations to RalA and this association is not significantly sensitive to GTP [82]. Additionally, whereas an activated RalA mutant enhanced PLD activity in v-Src-transformed cells, activated RalA was unable to elevate PLD activity in parental NIH 3T3 cells [82]. Therefore, it is likely that another factor(s) is/are involved in the activation of Ral-associated PLD. The sensitivity of the RalA-associated PLD to Arf suggests that Arf could be such a factor. Whether the transduction of signals through Src and Ras involves Arf remains to be determined. It has been speculated that Arf facilitates vesicle budding and fusion by generating the acidic phospholipid PA through PLD activation[12] and PLD has recently been shown to stimulate vesicle budding in Golgi membranes [20]. Thus, if Arf does play a role in regulating PLD1 activation by Src and Ras, the signaling mechanism may involve vesicle formation or the alteration of some other property of membranes.

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INVOLVEMENT OF ADP RIBOSYLATION FACTOR (Arf) IN RalA/PLD COMPLEX FOR PHOSPHOLIPASE D ACTIVATION

Introduction

Previous research demonstrated that activation of PLD by Src involves a GTPase cascade of Ras and RalA [82] with a direct interaction between RalA and the recently cloned PLD1. Since RalA by itself was not sufficient for PLD activation and Arf was reported as an important activator of PLD activity, we investigated the effect of Arf on RalA-associated PLD activity.

Recently, the involvement of Arf in activation of membrane bound PLD has generated much interest in a role for PLD in vesicle transport. There is evidence indicating that PLD mediates the Arf-dependent formation of Golgi coated vesicles [99,100]. Therefore, Arf may mediate both vesicular traffic and PLD signalling pathways. The role of Arf in vesicular transport is well established. But the mechanism of PLD activation by Arf is not known. In this research, it was found that Arf can stimulate RalA-associated PLD activity. Purified PLD1 is inactive in the absence of the Ras superfamily GTPase Arf, however, the PLD activity precipitated by RalA from v-Src-transformed cell lysates is active without exogenously provided Arf. This observation suggests the presence of Arf or another activator of PLD1 in the RalA precipitates from these cells. In addition, the research from our laboratory indicated that brefeldin A, which inhibits Arf GDP/GTP exchange, inhibited the PLD activity in v-Src-transformed cells suggesting a requirement for Arf in the activation

of PLD by v-Src. Since Arf and PLD are reported stimulators of vesicle formation in the Golgi, it is possible that the vesicle formation and trafficking are involved in the intracellular signals mediated by the Ras/RalA GTPase cascade.

Results

1. Involvement of an activator for PLD activation

Since RalA by itself is not sufficient for PLD activation and PLD associated with RalA in v-Src transformed cells is PLD1, we compared PLD activity between partially purified PLD and PLD from v-Src transformed cells and examined the effect of exogenous Arf on PLD activities. Partially purified pig brain PLD was provided by Dr. Paul Sternweis's Lab. and this PLD was believed to be PLD1.

The results in Figure 18a show that partially purified PLD is inactive in the absence of Arf, indicating that its activity is Arf dependent. However, PLD activity from v-Src transformed cell lysates is active. We also examined the RalA associated PLD activity from both preparations. We found that (Fig. 18b) partially purified PLD can associate with Ral, but no activity can be detected in the absence of Arf. Therefore, PLD is inactive when it binds to the Ral and needs a factor (Arf) for activation. This observation further supports the hypothesis that the association between RalA and PLD is not sufficient for PLD activation. In Src cell lysates, we can detect high RalA associated PLD activity without exogeneously added Arf. Thus, PLD in Src cell lysates is active. Since purification procedure removes some factors, the above data suggested that a factor is involved in activation of PLD activity.

Because Arf was reported as an activator of PLD activity and plenty of Arf proteins can be detected in Src cell lysates (data not shown), Arf probably is an activator presented in RalA/PLD complex.

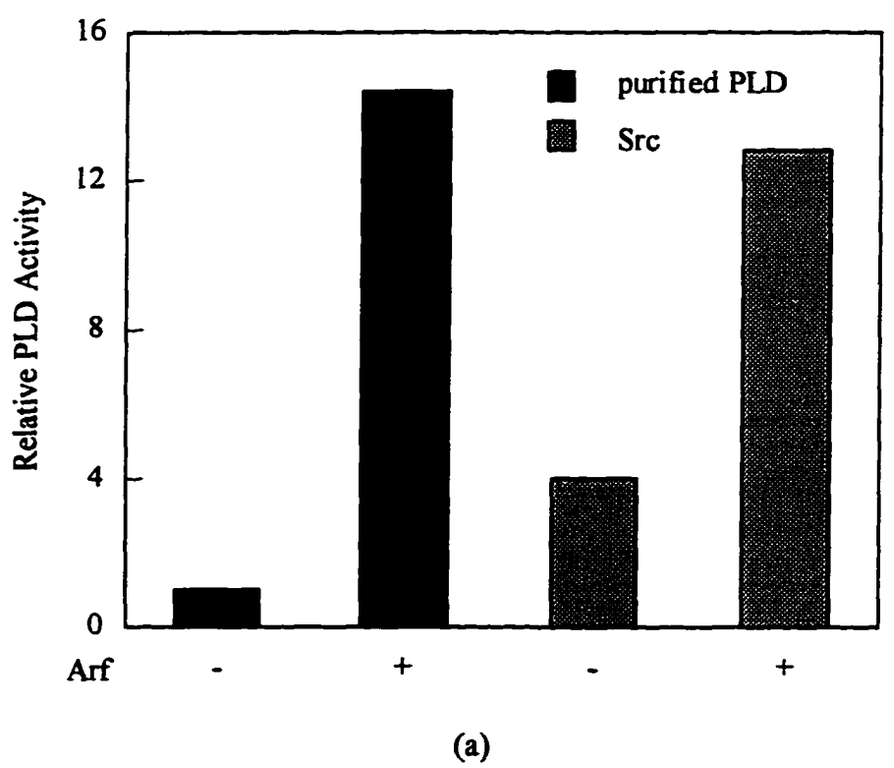
2. *Ral associates with hPLD1 and facilitates activation*

Recently, the first animal PC-specific PLD - hPLD1 has been cloned. We further investigated the effect of exogenous Arf on RalA associated hPLD1 activity. The sf9 cell lysates which were infected with bacculovirus expressing a cloned hPLD1 were used. The data shown here (Fig. 19) indicated that hPLD1 activity could not be detected in the sf9 cell lysates (extract) unless when Arf is added, but we can detect very high Ral-associated hPLD1 activity in the absence of Arf. It suggests that when hPLD1 binds to Ral, it is more active, therefore, it can not be further stimulated significantly by Arf. Probably, Ral facilitates the association between PLD and endogenous Arf or another factor for PLD activation. We checked Arf protein level in sf9 cell lysates and found that very small amount of Arf proteins exist. It is possible that when RalA precipitates PLD, it recruits Arf proteins into the complex and greatly increases PLD activity there.

3. *Differential activity of PLD associated with Ral and an N-terminal Ral mutant*

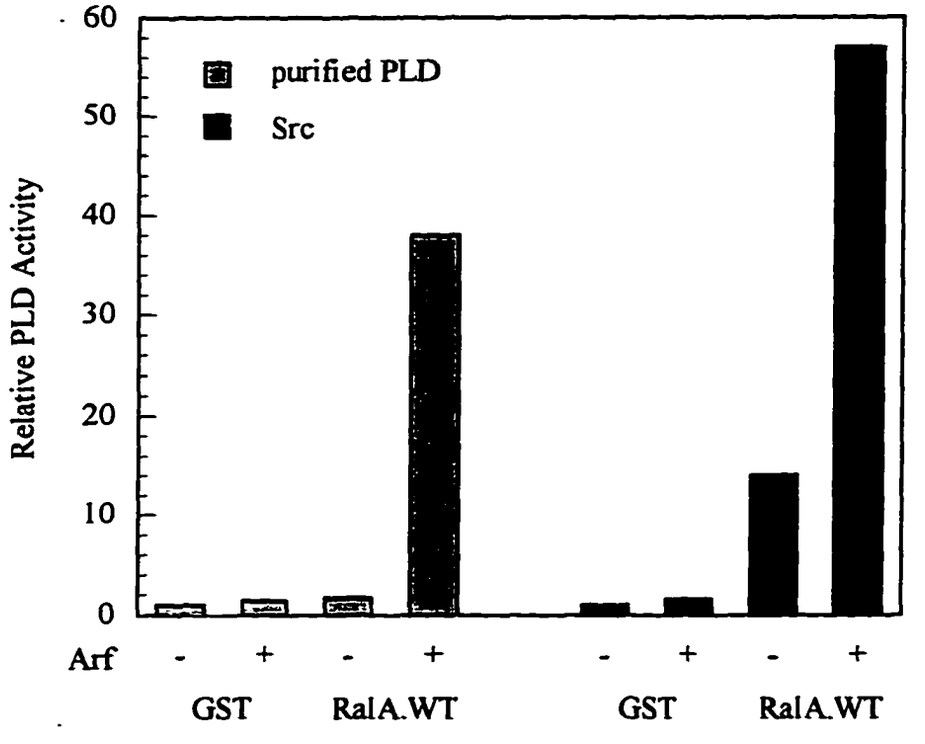
Immobilized GST-RalA fusion proteins can precipitate PLD activity from v-Src transformed cell lysates. But an N-terminal deleted RalA mutant (ΔN11) reduced the ability of RalA to complex with PLD. Since the 11 amino acids on N-terminal end of Ral is Ral specific, it does not exist in other Ras family members, we investigated the effect of Arf on ΔN11 associated PLD activity in both v-Src transformed cell lysates and PLD1 overexpressing sf9 cell lysates. Fig. 20 shows the differential activity of PLD associated with

wild type RalA and an N-terminal RalA deletion mutant. Although much lower PLD activity was detected on N-terminal deleted RalA mutant than wild type RalA in the absence of Arf, when Arf was added, this activity was greatly increased. Therefore, PLD activity bound to N-terminal deleted RalA mutant is less active than in wild type RalA and can be activated by Arf. It is possible that N-terminal deletion prevents the binding of an activator of PLD to the RalA or causes conformational change. This effect can be compensated by Arf. The N-terminal end of RalA seems to be important in precipitating PLD in an active form and very likely, it is important for binding Arf or another factor for PLD activation.



(a)

Figure 18. An activator is required for detecting partially purified PLD activity



(b)

Figure 18. An activator is required for detecting partially purified PLD activity. (a). PLD activity in partially purified pig brain PLD (4.5 μg) or Src cell lysate proteins (40 μg) were measured as described in materials and methods. (b). Immobilized GST or GST-RalA fusion proteins were mixed with either partially purified PLD 35 μg or Src cell lysates 600 μg. After incubation at 4 °C for 1.5 hours, the beads were collected and the PLD activity associated with beads were detected by using standard *in vitro* PLD assay. In (a) and (b), 40nM Arf proteins together with 10μM GTPγS were added to stimulate PLD activity.

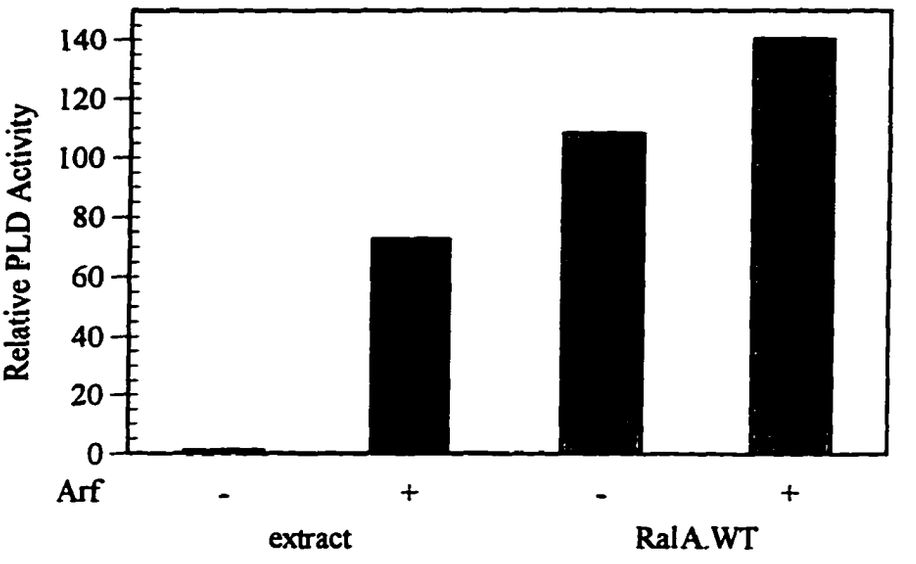


Figure 19. RalA facilitates association of an activator for PLD activation. PLD1 expressing sf9 cells were lysed by 15mM OG lysis buffer. 20 μg lysate proteins were used to detect PLD activity as shown in "extract". "RalA.WT" indicates the PLD activity associated with immobilized RalA fusion proteins after incubation of 40 μg sf9 cell lysates with GST-RalA fusion proteins.

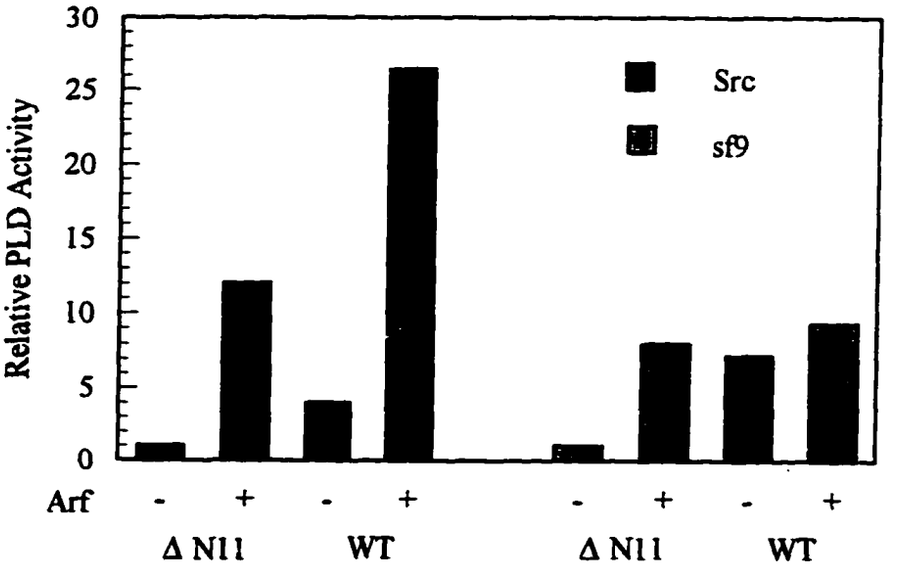


Figure 20 N-terminal end of RalA is involved in binding of an activator for PLD activation. Src cell lysates (~600 μg) and PLD1 overexpressing sf9 cell lysates (~40 μg) were treated with immobilized RalA wild type fusion proteins and N-terminal deleted RalA mutants (20 μg fusion proteins were used for each sample). After incubation, PLD activity in RalA precipitates were determined by using *in vitro* PLD assay as described in materials and methods.

4. Arf is present in Ral/PLD complex in v-Src transformed BALB/c3T3 cells

Is Arf the specific factor for PLD activation? Next, we examined if Arf is present in RalA/PLD complexes by using western blot analysis. The membrane fractions of v-Src and v-Ras transformed cells were used. Since Arf is a cytosolic protein, the cytosol and membrane fractions were incubated together in the presence of GTPγS and membrane fraction were re-isolated by centrifugation. In this way, Arf are forced to translocate from cytosol fractions to membrane fractions. Arf proteins associated with immobilized RalA beads were examined before and after Arf translocations in membrane fraction. From the results, we can see that Arf is present in GST-Ral precipitates (Fig. 21b). More Arf proteins can be detected in membrane fractions (Fig. 21a) and in RalA precipitates after Arf translocation. Comparing the binding of Arf and the binding of PLD activity by RalA, we found that the amount of Arf in different RalA precipitates correlates with the amount of PLD activity precipitated by different RalA mutants. That is, we can detecte more Arf proteins in effector domain mutant D49N precipitates which bound more PLD activity from Src cell lysates than wild type RalA. When N-terminal deleted RalA mutant ΔN11 was used, a lower amount of Arf protein could be detected in ΔN11 RalA precipitates which bound very little PLD activity from cell lysates. Therefore, Arf plays an important role in activating PLD activity by existing in RalA complex.

We also examined RalA associated PLD activity in membrane fractions before and after Arf translocation. Figure 22 shows that much higher RalA associated PLD activity can be detected in Arf translocated membrane fractions. Therefore, when more Arf proteins in their GTP bound state is present in membrane fractions, they enhance PLD activity there.

This further supported the role of Arf for activating PLD in RalA complex.

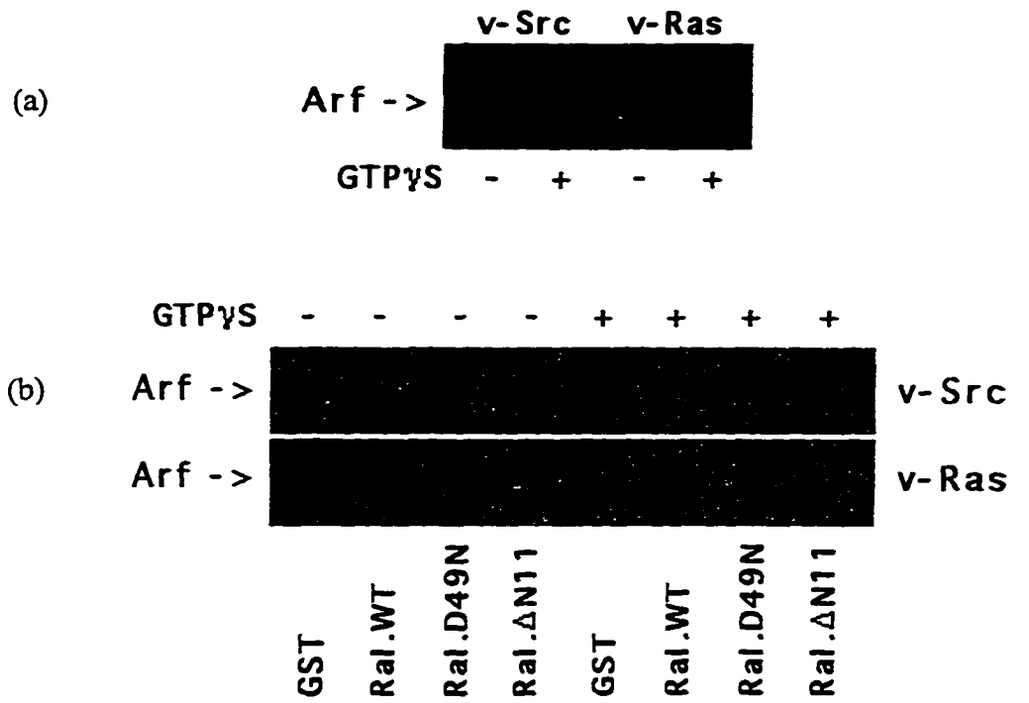


Figure 21. Western blot analysis of Arf protein in RalA precipitates. (a). Membrane proteins (20 μg for each lane) from v-Src and v-Ras transformed BALB/c3T3 cells were subjected to western analysis using antibody (1D9) specific to protein Arf. (b). Membrane fractions before and after Arf translocation from v-Src and v-Ras transformed BALB/c3T3 cells were treated with immobilized GST control, GST-RalA wild type, GST-RalA effector domain mutant D49N and N-terminal deleted RalA mutant ΔN11. The proteins on the beads were subjected to western analysis and blot with anti-Arf antibody 1D9. In (a) and (b), GTPγS "-" stands for the membrane fractions without Arf translocation. GTPγS "+" stands for Arf translocated membrane fraction.

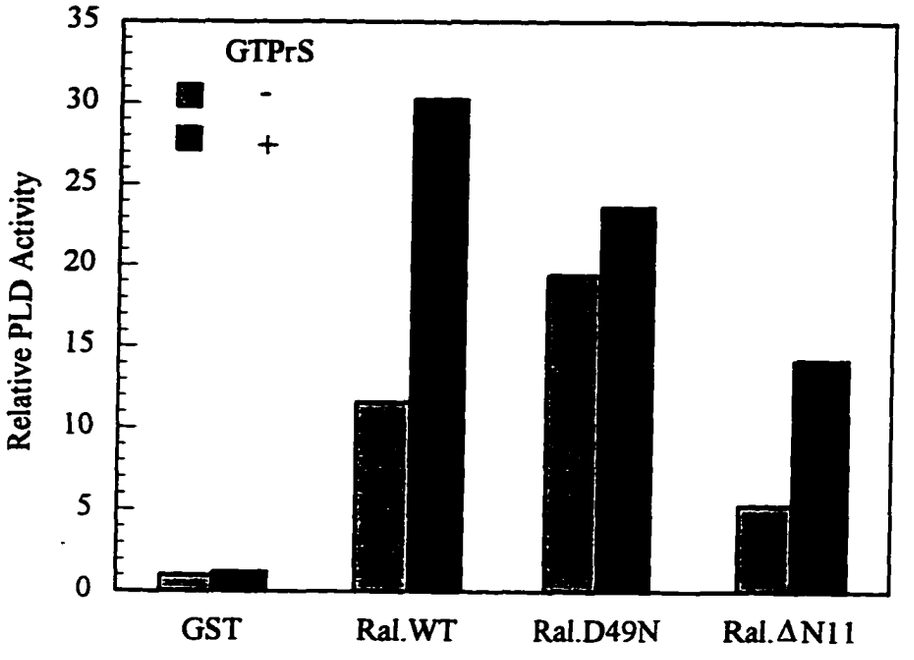


Figure 22 The effect of membrane translocation of endogenous Arf on PLD activity. The membrane fractions before and after Arf translocation from v-Src transformed BALB/c3T3 cells were treated with immobilized GST control, GST-RalA wild type, GST-RalA effector domain mutant D49N and N-terminal deleted RalA mutant ΔN11. PLD activity in different RalA precipitates were determined as described in materials and methods. GTPγS "-" and "+" are the same as in Fig. 21.

5. The weak interaction between RalA and Arf GTPase

To determine whether Arf interacts directly with RalA, we examined the interactions between RalA and Arf by using purified Arf proteins from porcine brain, which contains a mixture of Arf1 and Arf3 proteins. After incubation of immobilized GST-RalA fusion

proteins with Arf, western blot analysis was used to detect Arf proteins in RalA precipitates. A monoclonal antibody (1D9) against Arf protein was used. The result is shown in Figure 23. Comparing with Arf protein control, very weak Arf was detected in RalA precipitates. It suggested that the association between RalA and Arf is not direct or at least very weak. Other molecules probably are involved in RalA/PLD/Arf complex. A western blot analysis of purified PLD1 in RalA precipitates is shown as control, indicating direct interaction between RalA and PLD1.

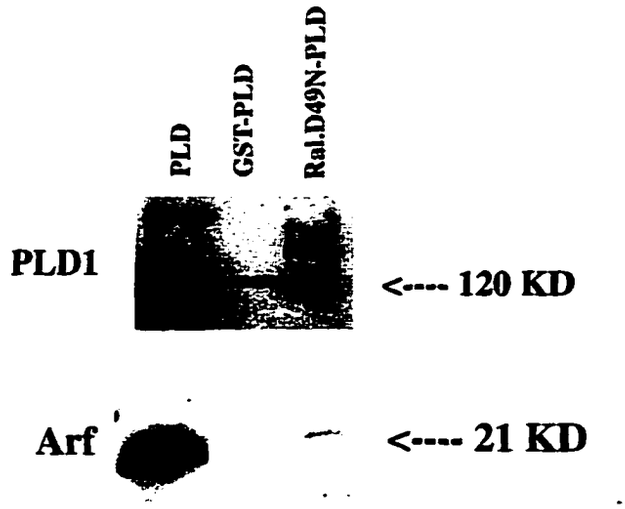


Figure 23. Western blot analysis of Arf and PLD1 in RalA precipitates. Immobilized GST control and RalA effector domain mutant D49N were incubated with either purified Arf proteins (1.6μg) from porcine brain or PLD1 (0.12μg) which was affinity purified from baculovirus-infected-cells. After collecting the beads, proteins on the beads were subjected to western blot analysis by using antibodies against Arf or PLD1.

Discussion

The present work demonstrates that Arf is a very important activator involved in PLD activation by existing in RalA/PLD complex although the interaction between RalA and Arf is very weak. We also indicate that RalA facilitates the association of PLD with an activator (probably Arf) and N-terminal end of RalA plays a critical role in precipitating PLD in an active form probably by interacting with the activator. But, how does Arf function in RalA/PLD complex and how different regions of Ral affect the binding of PLD or Arf are still not clear. It is very possible that other molecules besides Arf are involved since the association between Arf and RalA is not strong. Therefore, the functional significance of PLD activation by Arf remains to be explored.

Arf and PLD1 have been reported to mediate vesicle formation in the Golgi [18 19]. Therefore, the activation of PLD mediated by Ras and Ral may involve vesicle formation and transport. According to current reports, PLD1 localizes solely to peri-nuclear regions (endoplasmic reticulum, Golgi, and late endosomes) where PLD activity has been shown to promote Arf-mediated coated vesicle formation [99]. PLD activity stimulated by Arf is inhibited by Brefeldin A (BFA) [100], a fungal metabolite that prevents binding of Arf to Golgi membrane and inhibits secretion. BFA also inhibited Src and Ras induced PLD activation (data from our laboratory). Moreover, Golgi from BFA resistant cell lines that contain a tightly bound Arf species had very high basal PLD activity; this activity was not further stimulated by exogenous Arf and was resistant to BFA [100]. It suggests that the population of Arf responsible for stimulating vesicle formation from Golgi also activates PLD. In addition,

the products PA from hydrolysis of PC by PLD was proposed to facilitates formation of stable binding sites for coatomer, leading to budding of coated vesicles [99]. PA was also reported to play a role in receptor-stimulated secretion. All these investigations together with our research demonstrated that PLD signalling is involved in membrane traffic and vesicle formation and Arfs may serve as mediators between these two signalling system. Based on our results, we proposed a model for Ral-associated PLD activation by Arf or another activator, and N-terminal end of Ral might facilitate the association of Arf or other activator for PLD activation (Figure 24).

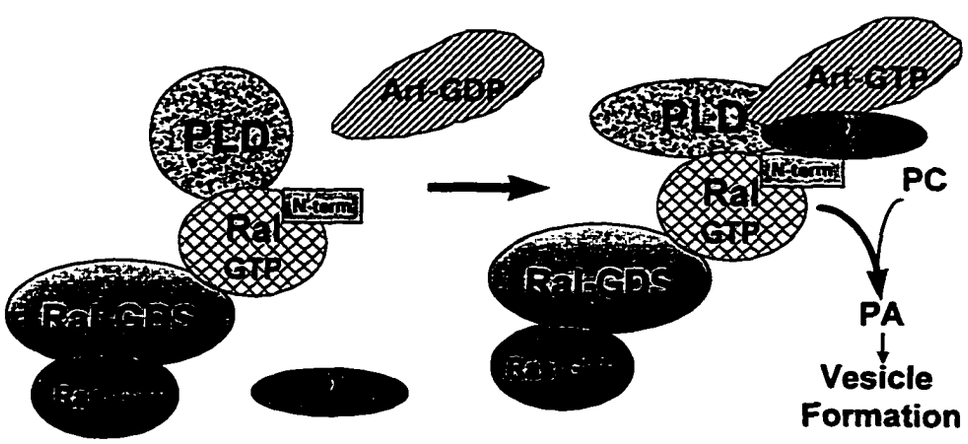


Figure 24. A model for RalA-associated PLD activation by Arf

CONCLUSION

Phospholipase D is activated by several factors. In this research, the activation mechanism for v-Src-induced PLD by a group of monomeric small G proteins, such as Ral and Arf, has been studied. An *in vitro* assay to detect PLD activity was developed, and using an immobilized GST-fusion protein system, the association between RalA and phospholipase D was investigated. We found that:

1. Elevated levels of PLD activity could be precipitated from v-Src-transformed cell lysates with immobilized GST-RalA fusion protein and with anti-Ral antibody, indicating that the Src- and Ras-induced PLD activity is associated with RalA.
2. RalA interacts directly with Arf-responsive, PIP_2 dependent PLD1. Several lines of evidence demonstrated that RalA-associated PLD in v-Src-transformed cells corresponds to the recently cloned PLD1.
3. Overexpression of wild type RalA and constitutively activated RalA mutant increased v-Src-induced PLD activity. Expression of dominant negative RalA mutants inhibited v-Src and Ras induced PLD activation. Therefore, RalA is apparently required for v-Src- and Ras-induced PLD signals.
4. PLD activity in non-transformed cells could not be stimulated by overexpression of either wild type RalA or an activated RalA mutant, indicating that the association between RalA and PLD is not sufficient for PLD activation.
5. Arf GTPase is an activator of RalA-associated PLD activity. Arf was found to be present in RalA/PLD complexes from v-Src-transformed cells and to enhance RalA-

associated PLD activity. A weak interaction existed between RalA and Arf GTPase, suggesting an indirect interaction.

6. hPLD1 overexpressed in sf9 cells was inactive in the absence of Arf. RalA-associated hPLD1 was active under the same conditions, suggesting that RalA facilitates the binding of Arf or another activator for PLD activation.
7. Arf increased PLD activity associated with a RalA mutant missing N-terminal 11 amino acids, which failed to bind PLD activity efficiently *in vitro*, indicating that N-terminal end of RalA is important for precipitating PLD in an active form.

In summary, the studies indicate that the activation of PLD by v-Src involves a GTPase cascade of Ras and RalA with a direct interaction between PLD1 and RalA. RalA activity alone is not sufficient for PLD activation and another activator is required. Arf is an essential stimulator for PLD activity induced by v-Src. Arf is present in RalA/PLD complexes and enhances RalA-associated PLD activity. The N-terminus of RalA seems to be important for the binding of Arf and for PLD activation. A weak interaction between RalA and Arf is detected, implicating that other molecules may be involved.

Arf and PLD1 are reported to stimulate vesicle formation in the Golgi. The involvement of Arf for the formation of coatomer-coated vesicles is likely to stimulate PLD activity since in Golgi-enriched membranes, the formation of coatomer-coated vesicles does not require exogenous Arf and GTPγS when the membrane has high basal PLD activity. The investigation we performed here further supports the role of Arf for PLD activation by existing in RalA/PLD complexes. RalA seems responsible for association of Arf for PLD

activation.

The role played by PLD in vesicular transport probably is through PA, the hydrolysis product of PC by PLD. Several mechanisms may explain the putative role of PA production on membrane traffic. PA may alter the biophysical properties of the lipid bilayer and promote budding and fusion. PA also acts as second messenger and regulates Arf-GAP function. The generation of PA by bacterial PLD allows coatomer to bind without addition or stimulation by Arf. Therefore, the requirement of Arf for PLD activation and subsequent production of PA are very important events in the regulation of vesicle formation and transport. In addition, the evidence that PIP_2 is required for activation of Arf-GAP by PA and may serve as an exchange factor for Arf further implicate PLD in the role of vesicular transport, since PIP_2 is also a very essential co-factor for PLD activation.

Recently, more and more evidence links PLD activation with different stimuli, such as insulin and m3 muscarinic acetylcholine receptor [101 102]. Arf is a mediator of these signalling pathways. Our investigation suggests the involvement of Arf in v-Src induced PLD activation and RalA as a mediator of Arf activation of PLD. All of these studies implicate that PLD is a target of Arf regulation in response to various external stimuli. Arf plays a role in transducing intracellular signals and regulating production of signalling molecules. There is still insufficient data to demonstrate the mechanism of PLD activation by Arf. Further studies on subcellular localization of different proteins and on the roles they play may help us to better understand the significance of the involvement of Arf and PLD in membrane traffic and vesicular transport. The results from our investigation probably established a good starting point for searching relevent factors involved in activation of PLD by Arf in response to

mitogenic signal v-Src.

Our research suggests that RalA may act as a bridge between PLD and Arf. Although Arf and PLD have been implicated in membrane trafficking and vesicular transport, the role of RalA is yet to be explored. The focus of our future work will be on establishing a possible role for RalA in vesicle formation and fusion; especially how its association with PLD and Arf affects downstream signals. An *in vitro* method will be developed to investigate whether RalA is a critical factor for vesicle budding and fusion and whether RalA affects this procedure through association with PLD and Arf. Different RalA mutants may be a good tool for this study. In addition, studies on subcellular localization of RalA, PLD, Arf and their association may further clarify the functional significance.

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