# The Involvement of Ras and Ral GTPases In v-Src-Induced Intracellular Phospholipid Signalling

by

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# ABSTRACT

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v-Src-induced activation of phospholipase D (PLD), which leads to the generation of lipid second messengers, is GTPase-dependent. Two GTPases, Ras and Ral, were found to mediate PLD activity. The involvement of Ras was demonstrated by the finding that a neutralizing antibody to Ras was able to block membrane PLD activity, and GTP bound Ras was able to interact with a cytosolic factor(s) that contributes to the activation of PLD. Ral, another Ras family GTPase, was identified as a Ras downstream effector required for the activation of PLD. First, PLD activity could be precipitated with immobilized Ral protein. Deletion of the amino terminal domain of Ral reduced the association between Ral and PLD. Second, PLD activity was inhibited by overexpression of several Ral mutants in v-Src-transformed NIH cells. Overexpression of Ral protein enhanced PLD activity in v-Src-transformed, but not in parental NIH cells. A Ras/Ral GTPase cascade model is proposed for v-Src-induced PLD activity in which Ras is activated by v-Src and interacts with RalGDS (Ral guanine nucleotide dissociation stimulator). This interaction localizes RalGDS to the membrane. Ral is then recruited and brings associated PLD to form a Ras/RalGDS/Ral-PLD signalling complex where PLD becomes activated. In addition, Ral is found to be involved in cell transformation by oncogenic Src and Ras. Therefore, the activation of PLD mediated by the Ras/Ral GTPase cascade may contribute to oncogenic Src- and Ras-induced cell transformation.

# PREFACE

This thesis is organized into eight parts. Part I reviews published work that provides an introduction to my thesis subject. Parts II and III are my published work from two articles. Part IV presents my recent work which has been submitted for publication. Part V is a summary of my thesis with a short discussion and prospect. Part VI presents tables and figures with legends. Part VII describes the materials and methods employed in my thesis study. Lastly, all references are listed in alphabetical order.

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# ABBREVIATIONS

| | |
|---|---|
| ARF | ADP ribosylating factor |
| DAG | diacylglycerol |
| EGF | epidermal growth factor |
| GAP | GTPase-activating protein |
| G protein (GTPase) | GTP binding protein |
| GDPβS | guanosine 5'-2-O-(thio)diphosphate; |
| GMP-PNP | 5'-guanyl-β, γ-imidodiphosphate |
| GTPγS | guanosine 5'-3-O-(thio)triphosphate; |
| Grab2 | growth factor receptor-bound protein-2 |
| MAPK | mitogen-activated protein kinase |
| PA | phosphatidic acid |
| PAP | phosphatidic acid phosphatase |
| PC | phosphatidylcholine |
| PEt (PBt) | phosphatidylethanol (phosphatidylbutanol) |
| PIP2(PI) | phosphatidylinostol (4,5) bisphosphate |
| $PI_3K$ | phosphatidylinositol-3-kinase |
| PLC | phospholipase C |
| PLD | phospholipase D |
| PKC | protein kinase C |
| PTK | protein tyrosine kinase |
| RalGDS | Ral guanine nucleotide dissociation stimulator |
| Sos | Son of sevenless (Ras guanine nucleotide exchange factor) |
| SRD | Schmidt-Ruppin D strain of Rous sarcoma virus |
| SH2(3) domain | *src* homology 2 (3) domain |
| TPA | 12-0-tetradecanoylphorbol 13-acetate |

# PART I. INTRODUCTION

Protein tyrosine kinases (PTKs) are generally characterized as belonging to either the receptor class or the nonreceptor class of enzymes. Those of the receptor class bind polypeptide hormones through their extracellular sequences and initiate transmission of intracellular signals by activation of their cytoplasmic kinase domains (reviewed by Ullrich & Schlessinger, 1990; Fantl & Williams, 1993). The nonreceptor class of PTKs represent a collection of cellular enzymes that lack extracellular sequences. Mutations to the nonreceptor PTKs that result in constitutive PTK activities are frequently oncogenic (reviewed by Hunter, 1991; Bolen, 1993). The activation of PTKs induces intracellular signal transduction that is associated with altering the level of intracellular second messengers, phosphorylation of cellular mediators, and regulating the activities of GTP binding proteins. These intracellular events are critically important for influencing a diverse array of cellular responses, including proliferation, differentiation, cell survival and cytoskeletal organization (reviewed by Cross & Dexter, 1991; Cantley et al., 1991).

v-Src protein is the oncogenic product of the Rous Sarcoma virus, and posseses constitutive PTK activity that causes cell transformation and induces fibrosarcomas *in vivo* (Jove & Hanafusa, 1987). The cellular counterpart of the v-Src protein, c-Src, exhibits very low kinase activity. The Src cellular proto-oncogenic protein is comprised of four major functional domains (Cantley et al., 1991): a 300 amino acid protein tyrosine kinase domain localized near the carboxyl terminal, a short amino terminal sequence required for addition of myristic acid for membrane localization, and two additional domains next to the

amino terminal named Src homology 2 and 3 (SH2 and SH3) involved in the mediation of protein-protein interactions. Phosphorylation of Src tyrosine-527 at the carboxyl terminal suppresses tyrosine kinase activity by the intramolecular interaction between phosphorylated Tyr-527 and the SH2 domain (reviewed by Cantley et al., 1991; Cooper & Howell, 1993). v-Src protein has a constitutively active tyrosine kinase and transforming ability because it lacks 527 tyrosine residue (Kmiecik & Shalloway, 1987). The activation of PTK is usually accompanying autophosphorylation (Ullrich & Schlessinger, 1990). Oncogenic Src protein phosphorylates itself at tyrosine-416 (Hunter, 1987). The phosphorylated tyrosine residue(s) of PTKs or their direct substrates often provide binding sites for SH2 domain-containing proteins (Koch et al., 1991; Mayer & Baltimore, 1993). This interaction resultes in recruitment of primary cytosolic targets to the inner plasma membrane where they become activated to serve as intracellular mediators or adaptors (McGlade et al. 1992; Sasaoka et al., 1994; Rozakis-Adcock et al, 1993; Egan et al., 1993; Li et al, 1993). These target proteins then play important roles in intracellular signal transduction pathways that transmit or amplify signals to the nucleus where gene expression is selectively regulated.

p21Ras protein belongs to a large superfamily of membrane-bound GTPases. The Ras family GTPases play critical roles in the control of normal and transformed cell growth in a wide variety of cellular processes (Bourne & McCormick, 1990; Boguski & McCormick, 1993). Some of them have demonstrable oncogenic potential. In addition to their role in regulating many aspects of growth- and differentiation-related signal transduction, members of this superfamily play an important part in regulation of cytoskeletal actin and membrane trafficking (Bourne & McCormick, 1990; Prendergast & Gibbs, 1993;

Chant & Stowers, 1995). Convincing evidence has accumulated indicating that Ras functions as a crucial mediator of many biological responses stimulated by tyrosine kinases (Egan & Weinberg, 1993; Stach et al, 1992). Ras signaling capacity is activated by GTP binding and inactivated by GTP hydrolysis. The activation of Ras by oncogenic Src protein is initiated by phosphorylation of the Shc protein (Rozakis-Adcock et al, 1992; Pelicci et al., 1992; McGlade et al., 1992; Sasaoka et al., 1994). The phosphorylated Shc provides a binding site for the SH2 domain of the Grab2 protein (Egan & Weinberg, 1993; Rozakis-Adcock et al., 1993). This interaction results in the recruitment of Sos, a guanine-nucleotide exchange factor that is complexed with Grab2 (Buday & Downward, 1993). Sos then promotes the release of GDP from the inactive Ras-GDP complex, allowing GTP to bind (Gale et al, 1993). When bound to GTP, Ras assumes an activated conformation which is necessary for the further transduction of the growth-factor induced signal (Polaskis & McCormick, 1993). This process of signal transduction through Ras is hypothesized to be mediated through the activation of Ras cellular target proteins, or effector molecules, as a consequence of direct interaction with the effector domain of GTP-bound Ras (Marshall, 1993).

Ras GTPase-activating protein (RasGAP) was the first hypothesized Ras effector. Biochemical and molecular genetic evidence sugests the idea that rasGAP mediates some aspectes of Ras function by complexing with other signalling proteins although the detailed mechanism remains poorly defined (Boguski & McCormick, 1993). A better understood pathway emanating from Ras involves the activation of an evolutionarily conserved cascade of mitogen-activated protein kinases (MAPKs) including Raf, MAP kinase kinase (Mek) and MAP kinase (Erk). This kinase cascade plays an important role in regulating cell proliferation and differentiation. The first step is binding of the Raf kinase to the

active GTP-bound Ras (Moodie et al, 1993; Vojtek et al., 1993; Zhang et al, 1993). However, it seems that Ras binding itself dose not activate the intrinsic kinase activity of Raf; rather, it only localizes Raf to the plasma membrane where some unknown activation events take place (Leevers et al, 1994; Stokoe et al, 1994). Another Ras downstream target protein was found to be phosphatidylinositol-3-kinase (PI3 kinase) (Rodriguez-Viciana et al, 1994), which has been tightly correlated with changes in the growth state of cells induced by either growth factors or oncogenes. PI3 kinase was found to interact with GTP-bound Ras. Mutations in the effector domain of Ras, or an antibody thought to block access to it, prevented interaction between Ras and PI3 kinase. Finally, the level of PI3 kinase products generated *in vivo* can be increased or decreased depending on whether activated or dominant negative Ras mutants are expressed. However, it was recently reported (Hu et al., 1995) that PI3 kinase enhanced Ras activity, and that a negative mutant Ras was able to block the signaling induced by PI3 kinase in *Xenopus oocytes.* Thus, the relationship of Ras and PI3 kinase remains to be further characterized.

Recently, several groups have reported that GTP bound Ras, via its effector domain, interacts with Ral guanine nucleotide dissociation stimulator (RalGDS). Ral is a another member of the Ras family of GTP-binding proteins, and has two forms, RalA and RalB. The experiment was carried out by using a yeast two-hybrid system and an *in vitro* biochemical binding assay (Kikuchi et al.; Hofer et al.; Spaargaren & Bischoff , 1994). However, the biological function of Ral is still unknown. Mutants containing individual point mutations in the Ras effector domain were shown to be responsible for regulating distinct downstream effectors such as Raf, PI3 kinase, or Ral-GDS (White et al, 1995), which have been known to be involved in different signal transduction

pathways. The hunt for Ras downstream target proteins appears to be crucial in the elucidation of signal pathways mediated by Ras. Thus, Ras has been recognized as a central player in transmitting signals to distinct parallel downstream pathway from Ras that play their unique roles in the control of a wide variety of cellular processes.

G-proteins have been implicated in the control of phospholipid hydrolysis to generate second messengers, such as diacylglycerol (DAG) and arachidonic acid during mitogenesis (Yu et al., 1988; Diaz-Laviada et al., 1990). The hydrolysis of phosphotidylinostol (4,5) bisphosphate (PIP2 or PI) by phospholipase C (PLC) was first established as a signal transduction pathway in producing inositol trisphosphate for mobilizing $Ca^{2+}$ from the endoplasmic reticulum, and DAG for the activation of protein kinase C (PKC) (Berridge, 1993). This signaling event is rapid and transient. The activation of PI-PLC was mediated, in some cases, by a heterotrimeric G-protein, Gq (Berridge, 1993). However, the major phospholipid component, phosphotidylcholine (PC), is hydrolyzed by phospholipases C (PLC) or D (PLD) to produce DAG in a variety of cell lines (reviewed by Exton, 1994). DAG produced from PC hydrolysis is accumulated in the later phase of agonist stimulation and exists longer. Many hormones, growth factors and related agonists have now been shown to induce this response (Divecha & Irvine, 1995). There is evidence that cell transformation by v-Src or v-Ras oncogenes is associated with PC hydrolysis (Song et al., 1991; Wyke et al., 1992; Carnero et al., 1994). However, the mechanisms by which G-proteins mediate PC-PLD (or PC-PLC) hydrolysis are not clear. Recent evidence indicates that a major mechanism of DAG production is the activation of a phospholipase D (PLD), yielding phosphatidic acid (PA), which is subsquently hydrolyzed to DAG by phosphatidic acid phosphotase (PAP) (Exton, 1990; Jiang,

Y, et al., 1994). The recognition that PLD is a major enzyme involved in PC hydrolysis came from the observation that accumulation of PA sometimes preceded that of DAG, but more importantly from the demonstration that many agonists cause rapid activation of transphosphatidylation (Billah et al., 1990; Cockcroft et al., 1992). This reaction, in which the phosphatidyl group of phospholipids is transfered to a primary alcohol, such as ethanol, is only catalyzed by PLD. Transphosphatidylation has been shown to be activated by different agonists in a wide variety of cell types (reviewed by Exton, 1994).

The activation of PLD has been suggested to produce mitogenic effectors (Foster, 1993; Boarder, 1994; Exton, 1994). The primary enzymatic product of PLD is PA. Its major metabolic fates are conversion to DAG by PAP, or to lysophosphatidic acid (LPA) by phospholipase $A_2$ ($PLA_2$). DAG has been shown to activate PKC (reviewed by Nishizuka, 1992). The regulation of PKC isozymes by DAG is fairly well understood (Bell & Burns, 1991). Some studies suggested that DAG from PI hydrolysis can activate both $Ca^{2+}$-dependent and $Ca^{2+}$-independent PKC isozymes, whereas DAG from PC hydrolysis can only activate $Ca^{2+}$-independent isozymes, except for those unable to respond to DAG, e. g., PKCζ (reviewed by Hug & Sarre, 1993). LPA has been shown to be a mitogenic stimulus that induces the activation of MAP kinase via a heterotrimeric G-protein pathway (Howe, et al., 1993; Tigyi et al., 1994). Several lines of experiments suggested that PA might also be a mitogenic signaling molecule (Fukami et al., 1992; Pai et al., 1991). It was reported that PA inhibited RasGAP activity (Tsai et al, 1989; 1990), and therefore activated Ras. This effect has been suggested to be related to the mitogenic activity of PA. Also PA was shown to mediate thrombin-induced actin polymerization in fibroblasts, and the morphological change correlated with an increase in the level of PA (Ha et al, 1994).

It has been proposed that PC-PLD stimulation is secondary to PKC activation which is initiated by DAG coming from PI-PLC hydrolysis. However, a large number of growth factors were able to promote PC hydrolysis and, in some instances, the effect appears to be unrelated to PI hydrolysis (Nanberg et al., 1990; Cook et al., 1992). It was reported that tyrosine kinases activate phospholipase D by PKC-independent mechanisms (Wyke et al., 1992; Song and Foster, 1993). Therefore, more direct mechanism(s) of activation of PC-PLD, via G-proteins, and/or tyrosine kinase-induced phosphorylation cascades, may operate. There is growing evidence to support the idea that PC hydrolysis by PLD is regulated by GTP-binding proteins (reviewed by Exton, 1994; Cockcroft, 1992). However, the mechanism by which G-proteins regulate PLD activity and what types of G-proteins are involved is not fully understood. It was recently reported (Brown et al., 1993; Cockcroft et al., 1994) that ADP-ribosylation factor (ARF), a low molecular weight GTP-binding protein involved in membrane ruffling, is a cytosolic GTP-binding protein that activates PLD activity in HL60 cells. However, the activation of PC-PLD by a nonhydrolyzable GTP analog, GTPγS, in plasma membranes isolated from some tissues can be observed in the absence of the cytosolic fraction, suggesting that a membrane-associated G-protein was also involved. In addition, some indirect evidence suggested that Rho, a monomeric GTP binding protein, was involved in the activation of PLD (Bowman et al., 1993; Malcolm et al., 1994). Finally, it was also reported (Lambeth et al., 1995) that a unknown cytosolic factor was able to enhance GTPγS-activated PLD activity after depleting ARF in human neutrophils. Thus, the involvement of G-proteins in the activation of PLD could be cell type- or agonist-dependent. The mechanism of how G-proteins are involved in the activation of PLD by tyrosine kinase v-Src is still a mystery.

Signal transduction initiated by tyrosine kinases is often associated with the activation of G-proteins (Satoh et al., 1992). G-proteins have been implicated in the activation of PLD to generate intracellular phospholipid second messengers (Boarder, 1994; Cockcroft, 1992). We previously reported that v-Src-induced PLD activation is PKC-independent (Song et al., 1993). In order to delineate the signal transduction events leading to the activation of PLD by v-Src, we have investigated the involvement of G-proteins in v-Src-induced PLD activity by utilizing biochemical and molecular genetical approaches. First, a G-protein was found to be required for v-Src-induced PLD activity, by employing a cell permeabilization system. An *in vitro* system was subsequently developed to demonstrate that Ras is a mediator, and a Ras downstream effector is required for the activation of PLD by v-Src. However, two reported Ras downstream effectors, Raf and PI3 kinase, were both ruled out as mefiators for v-Src induced PLD activation. RalGDS, a guanine nucleotide exchange factor for the Ras related Ral protein, was recently reported to be a Ras effector (Kikuchi, et al., 1994; Hofer, et al., 1994; Spaargaren, et al., 1994), and that the interaction between Ras and RalGDS is required for the activation of Ral (Urano et al., submitted for publication). Therefore, it is feasible that Ral could mediate the activation of PLD by v-Src. To test this hypothesis, we constructed a series of mutant forms of Ral and expressed them in v-Src-transformed and parental NIH 3T3 cells in which PLD activity was examined. In combination with *in vitro* binding studies, we have found that Ral is consititutively associated with PLD. Ral regulates PLD activity only in v-Src transformed cells, but not in parental NIH 3T3 cells. Ras and Ral, are both found to be required for the activation of PLD by v-Src. Ras functions upstream from Ral. The Model of a Ras/Ral GTPase cascade in mediating PLD activity in tyrosine kinase signaling is proposed. In addition, a

new line of evidence indicating the biological function of Ral in the modulation of cellular transformation by oncogenic Src and Ras will be discussed.

# PART II. Evidence That v-Src-Induced Phospholipase D Activity Is Mediated By A G-Protein

## 2.1. RATIONALE

Protein tyrosine kinase activity is frequently an early event in the transduction of intracellular signals and has been extensively implicated in transformation tumorigenesis (Fantel and Williams, 1993). As a result of constitutively active kinase activity, v-Src activates multiple intracellular signaling mechanisms that leads to the induction of gene expression (Foster, 1993; Qureshi et al., 1991; 1992). Protein kinase C (PKC) is a serine/threonine-specific protein kinase that has been implicated in v-Src-induced intracellular signaling (Nori et al., 1990; Spangler et al., 1989; Wolfman et al., 1987), although, not all intracellular signals activated by v-Src involve PKC (Qureshi et al., 1992). We have reported that v-Src-induced increases in DAG are derived from phosphatidylcholine (PC) via the action of a type D phospholipase (PLD) and a phosphatidic acid phosphatase (PAP) (Song et al., 1991). The activation of PLD by v-Src was found to be independent of PKC (Song et al., 1993). Thus, it is likely that the DAG produced by the PLD/PAP mechanism is responsible for the activation of PKC by v-Src. How v-Src might induce PLD activity and the subsequent activation of PKC is not known. G-proteins have recently been implicated in the transduction of intracellular signals by protein-tyrosine kinases. A pertussis toxin-sensitive G-protein has been implicated in the transduction of intracellular signals initiated by the insulin (Gawler et al., 1987; Krupinski et al., 1988; Luttrell et al., 1990), epidermal growth factor (Jonhson et al., 1987; Yang et al., 1991), and colony stimulating factor-1 (Imamura et al., 1988; 1990) receptors. G-proteins have also been implicated in

mediating the effects of c-Src on the β-adrenergic response (Bushman et al., 1990; Moyers et al., 1993) We previously demonstrated that the activation of PKC-dependent gene expression and phosphorylation of the PKC substrate MARCKS by the related protein-tyrosine kinase v-Fps is dependent upon a G-protein (Alexandropoulos et al., 1991). Since PKC is presumably the target of the PLD-generated DAG, these data suggest that a G-protein could be involved in the activation of PLD activity by v-Src. Additionally, non-hydrolyzable analogs of GTP, which enhance G-protein-mediated signals, have been reported to stimulate PLD activity in vitro (Bocckino et al., 1987; Hurest et al., 1990; Olson et al., 1991; Xie et al., 1991). Here, we have employed a permeabilized mammalian cell system to generate data implicating a G-protein and phosphorylation in the activation of PLD activity by v-Src.

## 2.2. RESULTS

**v-Src-induced increases in PLD activity and DAG production are blocked by GDPβS.** PLDs catalyze the transphosphatidylation of substrate phospholipids to phosphatidylethanol (PEt) in the presence of exogenous ethanol (Kobayashi et al., 1987; Randall et al., 1990). This assay has been used extensively to demonstrate PLD activity (Billah et al., 1989; Bowman et al., 1993; Kusner et al., 1993). We previously demonstrated that activating the protein tyrosine kinase activity of v-Src induces the transphosphatidylation of PC to PEt in the presence of exogenously provided ethanol (Song et al., 1991; 1993). These data implicated a PC-specific PLD in the transduction of intracellular signals initiated by v-Src. Since G-proteins have been implicated in the activation of PLD (Hurest et al., 1990; Olson et al., 1991), we wished to determine whether v-Src-induced

PLD activity requires a G-protein. To accomplish this, we employed GDPβS, a non-hydrolyzable analog of GDP that inhibit signals mediated by G-proteins in permeabilized cells (Gilman, 1987). In Figure 1, it is shown that activating the protein-tyrosine kinase activity of v-Src by temperature shift, induced PLD activity in LA90 cells that had been treated with saponin to make the cells permeable to guanine nucleotides. The magnitude of v-Src-induced PLD activity in the saponin-permeabilized cells was roughly the same as that observed in the intact cells (data not shown). GDPβS inhibited the v-Src-induced increase in PLD activity in a dose-dependent manner (Figure 1). Substitution of ADPβS for GDPβS failed to block v-Src-induced PLD activity (Figure 1), demonstrating a specific effect of the guanine nucleotide.

The DAG produced in response to v-Src is derived from phosphatidic acid, the primary metabolite of PLD activity, which is hydrolyzed to DAG by phosphatidate phosphatase (Song et al, 1991). Since v-Src-induced PLD activity is blocked by GDPβS, then phosphatidic acid production and the subsequent production of DAG should also be blocked by GDPβS. As shown in Figure 2, GDPβS blocked v-Src-induced DAG production to approximately the same extent as that observed for v-Src-induced PLD activity. These data further implicate a G-protein in the activation of PLD activity by v-Src.

It has been widely reported that phorbol esters that activate PKC also induce PLD activity (reviewed by Exton, 1990 ). We previously demonstrated the PLD activity induced by v-Src could be distinguished from that induced by the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) (Song et al, 1993). This suggest ed that TPA might activate PLD activity via a mechanism that is different from the one used by v-Src. We therefore investigated the sensitivity of

TPA-induced PLD activity to GDPβS in BALB/c 3T3 cells. As shown in Figure 3, TPA-induced PLD activity was insensitive to GDPβS. Thus, GDPβS does not block all PLD and suggests some specificity for the PLD activity activated by v-Src. The data also suggest that the PLD induced by the protein kinase C isoforms activated by phorbol esters is activated by a mechanism that is different from that used by v-Src. It was recently reported that TPA-induced PLD activity does not require ATP (Conricode et al., 1992). Thus, TPA-induced PLD activity is aparently independent of both kinase and GTPase activity.

**A non-hydrolyzable GTP analog enhances v-Src-induced PLD activity.** Non-hydrolyzable analogs of GTP sustain G-protein-mediated signals because they can not be hydrolyzed by the GTPase activity of the G-protein to the inactive GDP-bound state (Gilman, 1987). If a G-protein is required for v-Src-induced PLD activity, then a non-hydrolyzable analog of GTP should enhance v-Src-induced PLD activity. In BALB/c 3T3 cells transformed by the Schmidt Rupin D strain of Rous sarcoma virus (SRD cells), the non-hydrolyzable GTP analog, GTPγS, stimulated PLD activity; whereas, ATPγS had no effect upon PLD activity in SRD cells (Figure 4). To establish whether the effect of GTPγS was on v-Src-induced PLD activity, the effect of GTPγS on PLD activity in SRD cells was compared with the effect of GTPγS on PLD activity in the parental BALB/c 3T3 cells. To distinguish v-Src-induced PLD activity from other PLD activities, the cellular phospholipids were differentially prelabeled with either [$^3$H]-myristate or [$^3$H]-arachidonate. [$^3$H]-myristate is incorporated almost exclusively into PC (Song et al., 1991), the primary substrate phospholipid for v-Src-induced PLD activity; whereas, [$^3$H]-arachidonate is incorporated into phospholipids not recognized by the PLD activated by v-Src (Song et al., 1991). Figure 5, shows that in cells prelabeled with [$^3$H]-myristate, there was a dose-dependent increase in

PLD activity in response to GTPγS that was much greater in the v-Src-transformed SRD cells relative to the parental BALB/c 3T3 cells. In contrast, if the cells were prelabeled with [$^3$H]-arachidonate, no difference between GTPγS-induced PLD activity in SRD cells and BALB/c 3T3 cells was observed (Figure 6). Thus, the ability to detect a significant GTPγS effect upon PLD activity was dependent upon prelabeling with a phospholipid precursor ([$^3$H]-myristate) that is incorporated into PC species that are substrates for the v-Src-induced PLD. The effect of GTPγS on TPA-induced PLD activity was barely additive (data not shown) and taken together with the lack of effect of GDPβS on TPA-induced PLD activity suggests that GTPγS does not stimulate TPA-induced PLD activity. These data further implicate a G-protein as a mediator of v-Src-, but not TPA-induced PLD activity.

**GDPβS preferentially inhibits v-Src-induced PLD activity in v-Src-transformed cells.** The role of G-proteins in mediating v-Src-induced PLD activity was further examined by comparing the effect of GDPβS on PLD activity in SRD and BALB/c 3T3 cells prelabeled with either [$^3$H]-myristate or [$^3$H]-arachidonate. In [$^3$H]-myristate-prelabeled cells, the observed inhibition of PEt formation by GDPβS was approximately 60% in SRD cells; whereas, in BALB/c 3T3, the observed inhibition of PEt formation by GDPβS was less than 20%. In contrast, if the cells were prelabeled with [$^3$H]-arachidonate, the observed inhibition of PEt formation by GDPβS was less than 20% in both SRD cells and the parental BALB/c 3T3 cells (Figures 7 and 8). The greater effect of GDPβS on PLD activity in SRD cells relative to BALB/c 3T3 cells observed only in [$^3$H]-myristate-prelabeled cells is consistent with the hypothesis that the elevated PLD activity in v-Src-transformed cells is dependent upon a G-protein.

**ATP is required for the GTP-dependent increase in PLD activity induced by v-Src.** The activation of PLD activity by protein kinase C was reported to be independent of phosphorylation (Conricode et al., 1992). It has also been reported that ATP potentiates GTPγS-induced PLD activity (Kushner et al., 1993). This potentiation was sensitive to the protein-tyrosine kinase inhibitor herbimycin A. Thus, PLD activity can apparently be activated by protein kinases via mechanisms that are both dependent and independent of phosphorylation. The experiments presented in Figures 1-8 contained ATP and an ATP regenerating system. We therefore determined whether the presence of ATP was essential for the effects observed in Figures 1-8 and, whether herbimycin A could block the effect. As shown in Figure 9, PLD activity is maximal in the presence of both GTP and ATP. More significantly, the stimulatory effect of ATP is drastically reduced in cells treated with herbimycin A; and herbimycin A had very little effect when GTPγS was used in the absence of ATP (Figure 9). These data suggest that the protein tyrosine kinase activity of v-Src is required for the GTP-dependent increase in PLD activity in the v-Src-transformed SRD cells.

**PLD activity in SRD cells is insensitive to both cholera and pertussis toxins and aluminum fluoride.** Several heterotrimeric class G-proteins are substrates for ADP-ribosylation by bacterial toxins (Gilman, 1987). ADP-ribosylation by cholera toxin blocks GTP hydrolysis and therefore stimulates signals mediated by G-proteins that are substrates for cholera toxin. We previously demonstrated that cholera toxin does not induce phosphorylation of the PKC substrate MARCKS in either avian or murine fibroblasts (Spangler et al., 1989). Since the activation of PLD activity presumably results in the activation of PKC, these data suggest that the G-protein implicated here is not a cholera toxin substrate. Consistent with our previous observations, cholera toxin had no effect upon PLD

activity in either SRD or BALB/c 3T3 cells (data not shown). Pertussis toxin, which blocks some G-protein-mediated signals (Gilman, 1987), also had no effect on PLD activity in SRD cells (data not shown). Therefore, the G-protein implicated here is not likely a substrate for either cholera or pertussis toxin. Aluminum fluoride (10 mM NaF; 20 μM $AlCl_3$), which has been reported to specifically enhance heterotrimeric G-protein-mediated intracellular signals (Kahn et al., 1991) did not enhance PLD activity in SRD cells (data not shown). Although these data do not identify the putative G-protein implicated here, they eliminate a large number of candidate heterotrimeric G-proteins.

## 2.3. DISCUSSION

In recent years, it has become apparent that a substantial number of biological functions are regulated by GTPase activity (Bourne et at ., 1990; 1991). Data presented here suggest that the induction of PLD activity induced by the protein tyrosine kinase activity of v-Src is regulated by a G-protein. We recently demonstrated that HaRas is required for the transduction of at least two distinguishable intracellular signals activated by v-Src (Qureshi et al., 1992). These v-Src-induced intracellular signals could be distinguished on the basis of a differential sensitivity to PKC and a dominant negative mutant of Raf-1 in murine fibroblasts (Qureshi et al., 1991; 1992). A PKC-dependent intracellular signal activated by v-Src was sensitive to a dominant negative HaRas mutant (Alexandropoulos et al., 1993). A Raf-1-dependent intracellular signal activated by v-Src was also sensitive to the dominant negative HaRas mutant - with HaRas functioning upstream from Raf-1 (Alexandropoulos et al, 1992). Since the G-protein-dependent DAG produced in response to v-Src is likely responsible for

the activation of PKC (Song et al., 1993), the G-protein implicated here likely functions upstream from PKC. Thus, the data presented here implicate a third GTPase requiring step in the transduction of intracellular signals activated by v-Src. G-proteins have also been implicated in the ability of c-Src to modify the β-adrenergic response (Bushman et al., 1990; Moyer et al., 1993). Thus, GTPase activity apparently regulates at least four distinguishable effects of Src.

There are two major classes of G-proteins: heterotrimeric and monomeric (Bourne et al., 1990; Gilman, 1987). The involvement of G-proteins of the heterotrimeric class in the transduction and amplification of intracellular signals has been well established for intracellular signals initiated by membrane receptors of the seven transmembrane domain class (Bourne et al., 1991; Gilman, 1987); however, there have been several reports suggesting the involvement of heterotrimeric class G-proteins in some of the signals activated by protein tyrosine kinases including the receptors for insulin (Foster, 1993; Krupinki et al., 1988; Luttrell et al., 1990), epidermal growth factor (Johnson et al., 1986; Yang et al., 1991), colony stimulating factor-1 (Imamura et al., 1988) and the *fps* and *src* gene products (Moyers et al., 1993). In addition, it has been reported that α subunits of heterotrimeric class G-proteins can be phosphorylated on tyrosine residues by the insulin receptor (Krupinski et al., 1988) and c-Src (Hausdorff et al., 1992). Thus, it is becoming apparent that the role of heterotrimeric G-proteins may extend beyond intracellular signals initiated by the seven transmembrane domain receptors. While no heterotrimeric class G-protein that mediates intracellular signals initiated by protein-tyrosine kinases has been unambiguously identified, there are a large number of heterotrimeric class G-proteins that have been identified on the basis of sequence homology to known heterotrimeric class G-proteins without a defined function (Simon et al., 1991).

Thus, there are many candidate G-proteins for protein-tyrosine kinase-initiated signaling systems where heterotrimeric G-proteins have been implicated. The recent report of tyrosine phosphorylation of G-protein $\alpha$ subunits by c-Src (Hausdorff et al., 1992) suggest the possibility that the G-protein required for the v-Src-induced activation of PLD activity could be a direct substrate of v-Src. The lack of stimulatory effect by either cholera toxin or aluminum fluoride toxin or an inhibitory effect by pertussis toxin would tend to rule out a substantial number of heterotrimeric class G-proteins including $G_s$, the G-protein phosphorylated by c-Src in vitro (Hausdorff et al., 1992); however, there is substantial homology between the heterotrimeric class G-proteins (Simon et al., 1991) and if c-Src can phosphorylate $G_s$, v-Src might phosphorylate another heterotrimeric G-protein.

The monomeric G-protein HaRas has been implicated in the transduction of intracellular signals initiated by v-Src (DeClue et al., 1991; Nori et al., 1991; Smith et al., 1986; Stacy et al., 1991). It was recently reported that PLD activity could be activated in neutrophils by the Ras-family monomeric G-protein Rho (Bowman et al., 1993). The monomeric G-protein family of ARFs (ADP ribosylation factors) has also been shown to activate PLD in vitro (Brown et al., 1993; Cockroft et al., 1994). Thus a growing body of evidence implicates monomeric G-proteins in the activation of PLD. The lack of a stimulatory effect by aluminum fluoride on v-Src-induced PLD activity supports the involvement of a monomeric G-protein since aluminum fluoride has been reported to have no effect upon monomeric G-proteins (Kahn et al., 1991). Although, the putative G-protein that mediates v-Src-induced PLD activity remains to be identified, the data presented here define an additional GTP-dependent event in v-Src-induced intracellular signals and further demonstrate the importance of GTPase activity

as a master switching mechanism in the transduction of intracellular signals initiated by protein-tyrosine kinases.

# PART III. Ras Mediates The Activation Of Phospholipase D By v-Src

## 3.1. RATIONALE

There is a strong correlation between the activation of phospholipase D (PLD) and mitogenesis (Boarder, 1994; Foster, 1993). Protein tyrosine kinase activity is also widely implicated in mitogenic signaling (Fantl et al., 1993) and commonly leads to an elevation of PLD activity (Plevin et al., 1991; Song et al., 1991; Kaszkin et al., 1992). Ras proteins have been shown to mediate intracellular signaling pathways activated by tyrosine kinases (Egan and Weinberg, 1993), and have been implicated in v-Src-induced transformation (Nori et al., 1991). Ras has been also reported to be involved in the activation of phospholipase C (Cockcraft and Gomperts, 1985) and phospholipase $A_2$ (Bar-Sagi and Feramisco, 1986). We demonstrated previously that v-Src activates a PLD activity which can be distinguished from the PLD activity induced by phorbol esters that activate protein kinase C (Song and Foster, 1993). As discussed in part II, v-Src-induced PLD activity is dependent upon a G protein(s). Here, data is presented implicating the monomeric G protein Ras in the activation of PLD by v-Src.

## 3.2. RESULTS

**GTPγS and cytosol dependent PLD activity in v-Src transformed cells.** To examine the mechanism of PLD activation by v-Src, we developed an in vitro PLD assay to examine PLD activity in isolated membranes where greater than

90% of the increased PLD activity in v-Src-transformed cells fractionated. In vitro PLD activity was optimized in membranes isolated from v-Src-transformed cells that had been prelabeled with [$^3$H]-myristate. [$^3$H]-myristate is incorporated almost exclusively into phosphatidylcholine, the substrate for the PLD activated by v-Src (Song et al., 1991; 1993). Maximal PLD activity, as determined by the transphosphatidylation of phosphatidylcholine to PEt in the presence of exogenously provided ethanol, was dependent upon cytosol and the non-hydrolyzable GTP analog GTPγS. Cytosol and GTPγS had a much smaller effect on PLD activity in membranes from BALB/c 3T3 cells. The effect of cytosol and GTPγS on PLD activity in the v-Src-transformed cells appeared to be synergistic in that the increase in PLD activity in the presence of both GTPγS and cytosol was greater than the sum of individual effects either GTPγS or cytosol alone (Table 1). The synergistic effect of cytosol and GTPγS and the increased PLD activity in membranes from v-Src-transformed cells was not observed when the cells were prelabeled with [$^3$H]-arachidonate, which is incorporated into phopspholipids (including phosphatidylcholine) not utilized by the PLD activated by v-Src (Song and Foster, 1993). If cells were pretreated with the protein-tyrosine kinase inhibitor genistein, the effect of cytosol and GTPγS was reduced to that observed in the parental BALB/c 3T3 cells (Table 1). Genistein treatment reduced cellular phosphotyrosine content by greater than 50% at 4 hr and by greater than 90% overnight (data not shown). Thus, the pattern of PLD activity in membranes from v-Src-transformed cells can be clearly distinguished from that in the parental BALB/c 3T3 cells, and from that observed in membranes from v-Src-transformed cells treated with genistein or prelabeled with [$^3$H]-arachidonate. The differences observed in the in vitro PLD activity in the membranes isolated from the v-Src-transformed and parental BALB/c 3T3 cells were identical to the differences observed previously in intact cells (Song et

al., 1991; Song and Foster, 1993; H. Jiang et al., 1994) and strongly suggest that the increased PLD activity in the membranes from the v-Src-transformed cells is due to v-Src.

**Inhibitory effect on PLD activity by a neutralizing antibody to Ras.** The monomeric G protein Ras has been implicated in v-Src-initiated intracellular signals and transformation (Smith et al., 1986; Nori et al., 1991; DeClue et al., 1991; Qureshi et al., 1992). To determine whether Ras contributes to the G protein requirement for v-Src-induced PLD activity, we examined the effect of the neutralizing Ras monoclonal antibody Y13-259 (Furth et al., 1982; Smith et al., 1986) on PLD activity in membranes from v-Src-transformed cells. Y13-259 antibody was incubated with the membrane fraction (where Ras localizes) for 45 min prior to addition of cytosol and GTPγS. As shown in Figure 10, pretreatment with Y13-259 reduced PLD activity to about half that observed in the untreated cells. This effect could be competed away with an excess exogenous Ras protein. A non-neutralizing Ras antibody (Y13-238) had little or no effect on the PLD activity in membranes isolated from the v-Src-transformed cells (Figure 10). Interestingly, the effect of Y13-259 on PLD activity was dependent upon the presence of the cytosolic fraction as shown by the lack of effect of Y13-259 on the PLD activity membranes treated with only with GTPγS. Y13-259 had little or no effect upon the PLD activity in membranes from v-Src-transformed cells in the absence of cytosol (Figure 10). Y13-259 also had no effect upon the PLD activity observed in membranes isolated from BALB/c 3T3 cells or in membranes isolated from v-Src-transformed cells that had been prelabeled with [$^3$H]-arachidonate (Figure 11). Additionally, if the v-Src-transformed cells were treated with the protein tyrosine kinase inhibitor genistein, the effect of Y13-259 was lost (Figure 11). Thus, the inhibitory effect of the neutralizing Ras antibody

is likely specific for v-Src-induced PLD activity. These data demonstrate a functional requirement for Ras for the elevated PLD activity in membranes from v-Src-transformed cells.

**Depletion of cytosol effect on PLD activity by an immobilized GTPγS bound Ras.** As shown in Figure 10, the effect of Y13-259 was dependent upon the inclusion of the cytosolic fraction, suggesting a cytosolic downstream Ras effector molecule. We therefore examined whether immobilized Ras could deplete the cytosolic fraction of a factor(s) required for v-Src-induced PLD activity in a GTP-dependent manner. Upon separation of cytosolic and membrane fractions, the cytosolic fraction was incubated with immobilized Ras proteins loaded with either GTP (non-hydrolyzable GMP-PNP) or GDP. The immobilized Ras proteins were spun out and the cytosolic fraction was added back to the membranes and PLD activity was determined. As shown in Figure 12, there was a GTP-dependent depletion of the stimulatory effect of the cytosolic fraction on PLD activity in membranes isolated from v-Src-transformed cells. These data implicate a cytosolic factor as a GTP-dependent target of Ras function in the v-Src-induced activation of PLD. Although the identity of putative cytosolic factor(s) binding to Ras remain to be determined, the data demonstrate a GTP-dependence for Ras function in v-Src-induced PLD activity.

**Down regulation of v-Src-induced PLD activity by overexpression of a dominant negative mutant Ras.**The data presented above strongly implicate Ras in v-Src-induced PLD activity in a cell free system. To test for Ras involvement in the activation of PLD activity by v-Src in intact cells, we transfected a dominant negative Ras mutant (Feig and Cooper, 1988) into NIH 3T3 cells transformed by v-Src. NIH 3T3 cells were used instead of BALB/c 3T3

cells because of a higher transfection efficiency. As a control for the effects of the Ras mutant, we used a dominant negative Raf-1 mutant (Kolch et al., 1991) that was shown previously to block v-Src-induced transformation without inhibiting v-Src-induced PLD activity in BALB/c 3T3 cells (Qureshi et al., 1993). Plasmids expressing the dominant negative Ras and Raf-1 mutants also expressed the selectable G418 resistance gene. G418-resistant colonies were selected and pooled to avoid clonal variation. Overexpression of Ras and Raf-1 proteins was confirmed by Western blot analysis (data not shown). The G418-selected v-Src-transformed cells expressing the Ras and Raf-1 mutants had a reduced ability to form colonies in soft agar. There was a greater than 80% reduction in colony number for the Ras mutant and greater than 70% reduction for the Raf-1 mutant (data not shown). Additionally, colonies that did form in soft agar were smaller than those observed for the v-Src-transformed cells. These data are consistent with previous results showing that both Raf-1 and Ras are required for v-Src-induced transformation (Smith et al., 1986; Qureshi et al., 1993). The inhibitory effect of the Ras and Raf mutants on v-Src-induced transformation suggested that in addition to being expressed, the dominant negative mutants were also functional. In v-Src-transformed NIH 3T3 cells prelabeled with [$^3$H]-myristate, expression of the dominant negative Ras mutant reduced PLD activity to the level of PLD activity observed in the parental NIH 3T3 cells (Figure 13). If the cells were prelabeled with [$^3$H]-arachidonate instead of [$^3$H]-myristate, there was no observable difference in the PLD activity between the v-Src-transformed cells and the v-Src-transformed cells expressing the dominant-negative Ras mutant (Figure 13). As demonstrated previously in BALB/c 3T3 cells (Qureshi et al., 1993), expression of the dominant negative Raf-1 mutant did not inhibit PLD activity in v-Src-transformed NIH 3T3 cells (Figure 13). The inability of the Raf-1 mutant to inhibit PLD activity suggests that the elevated levels of PLD activity in

cells expressing v-Src is not due to secondary effects of transformation since the transformed phenotype is inhibited in these cells. The data presented in Figure 13 provide evidence in intact cells that v-Src-induced PLD activity is mediated by Ras.

### 3.3. DISCUSSION

The monomeric G proteins Rho (Bowman et al., 1993; Malcolm et al., 1994) and ADP ribosylation factor (ARF) (Brown et al., 1993; Cockroft et al., 1994) have recently been reported to be regulators of PLD activity. ARF and Rho have been implicated in the regulation of membrane traffic and cytoskeletal assembly (Ridley and Hall, 1992; Kahn, 1993). Thus, the PLD activated by mitogenic stimuli like v-Src may be distinct from the PLD activated by non-mitogenic stimuli. Consistent with this, C3 exoenzyme of *C. botulinum*, which inhibits Rho family G proteins (Ridley and Hall, 1992), had no effect upon v-Src-induced PLD activity (our unpublished results). What role Ras may play in the activation of PLD by v-Src is not yet clear. Attempts to activate PLD activity directly with purified Ras in cell membranes and to isolate PLD activity with immobilized Ras proteins in detergent lysates of v-Src-transformed cells were not successful (our unpublished results), suggesting that PLD is not a direct target of Ras. However, it was recently reported that PLD activity is elevated in v-Ras-transformed cells (Carnero et al., 1994), suggesting that an activated Ras may increase PLD activity in intact cells. Several recent reports have demonstrated a physical interaction with potential Ras effector molecules including Raf-1 (Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1992; Zhang et al., 1993), phosphatidylinositol-3-kinase (Rodriguez-Viciana et al., 1994), and Ral guanine nucleotide releasing factor

(Kikuchi et al., 1994; Hofer et al., 1994). As demonstrated in Figure 12, Ras-GTP binds to a soluble factor that is required for the cytosol to activate PLD in membranes from v-Src-transformed cells. Since the dominant negative Raf-1 mutant does not prevent PLD activation, this factor is not likely to be Raf-1. We have determined that phosphatidylinositol-3-kinase localizes with the membrane fraction in v-Src-transformed cells (our unpublished data); thus, the cytosolic factor is not likely to be phosphatidylinositol-3-kinase either. A possible role for Ral guanine nucleotide releasing factor or other yet to be identified downstream target of Ras in v-Src-induced PLD activity remains to be determined; however, data presented here establish that Ras is a component in the signaling machinery activated by v-Src that results in PLD activation.

# PART IV. Involvement Of Ral GTPase In v-Src-Induced Phospholipase D Activation And Cell Transformation

## 4.1. RATIONALE

In Part III we have presented evidence that the v-Src-induced PLD activity is mediated by Ras through a cytosolic factor(s) (Jiang, 1994 and 1995). Two reported effectors of Ras, Raf-1 (Moodie, et al., 1993; Vojtek, et al., 1993) and phosphatidylinositol-3-kinase are both unlikely to be involved (Rodriguez-Viciana, et al., 1994). Recently, Ral-GDS, the exchange factor for the Ras related Ral proteins, was reported to interact with Ras, R-Ras and Rap1A in yeast and in vitro (Kikuchi, et al., 1994; Hofer, et al., 1994; Spaargaren, et al., 1994). It has been recently reported that among three GTPases, Ras, R-Ras and Rap1A, only Ras activates Ral-GDS and RalA in vivo (Urano, et al., 1995), raising the possibility that this novel Ras signalling pathway mediates the activation of PLD by v-Src.

## 4.2. RESULTS AND DISCUSSION

**RalA physically associates with PLD and mediates PLD activity in vivo**

To investigate the possibility that RalA is involved in the activation of PLD, we examined the ability of GST-RalA fusion proteins, bound to glutathione sepharose (immobilized Ral), to associate with PLD activity in detergent lysates of v-Src-transformed Balb/c 3T3 cells. As shown in Figure 14a, immobilized Ral was able to precipitate PLD activity from v-Src-transformed cell lysates. PLD was precipitated more efficiently if RalA was preloaded with GTPγS, however

GDPβS-bound Ral also precipitated significant levels of PLD activity. The effect was specific for Ral in that neither GTP- nor GDP-bound immobilized Ras was able to precipitate PLD activity (Figure. 14b). Immobilized Ral precipitated less PLD activity from non-transformed Balb/c 3T3 cell lysates, consistent with our previous observation that PLD activity is elevated in response to v-Src (Song, et al., 1991). PLD activity was precipitated with an anti-Ral but not an anti-Ras antibody from both v-Src-transformed and parental Balb cell lysates (Figure 14c), implying that complexes between Ral and PLD exist in vivo.

We next mapped the region of RalA responsible for the interaction with PLD by examining the ability of RalA mutants to associate with the enzyme. We first investigated an Asp to Asn substitution at position 49 of RalA, because this is in a region of the protein that is similar to the region of Ras that is known to bind to downstream targets and because this mutation has already been shown to block the binding of Ral to a different putative downstream target (Cantor, et al., 1995). Surprisingly, this mutant (D49N) associated with PLD more strongly than wild type RalA and there was no significant difference between the GTP and GDP loaded forms (Figure 14d). In contrast, deletion of 11 amino terminal residues (ΔN11) that are unique to Ral proteins reduced the association between GTP-bound RalA and PLD by more than 60 % (Figure 14d). Thus, this region of Ral may constitute an additional effector domain not present in Ras.

To determine the significance of the Ral/PLD interactions in vivo, we examined the effect of altering RalA activity in intact cells. We first examined the effect of overexpressing wild type RalA (approximately 8-fold) (Figure 15) in v-Src-transformed NIH 3T3 cells. v-Src-transformed cells already have 2-fold greater PLD activity than that observed in the parental NIH 3T3 cells.

Nevertheless, the v-Src-transformed cells overexpressing wild type RalA displayed PLD activity that was enhanced by an additional 65% (Figure 16). Overexpression of RalA containing a mutation (Glu72Leu) analogous to the activating Ras mutation at position 61 also led to an increase in PLD activity in v-Src-transformed cells; although interestingly, the enhancement in PLD activity was less than that observed for wild type RalA (Figure 16) even though both forms of RalA were expressed at similar levels (Figure 15). In contrast, overexpression of wild type or activated mutant of RalA did not elevate PLD activity in the parental NIH 3T3 cells. In addition, purified RalA bound to GTP did not increase PLD activity in cell lysates (our unpublished results). Taken together, these data argue that although RalA contributes to v-Src-induced PLD activation, it is not sufficient for PLD activation.

We next tested whether RalA activity is necessary for v-Src activation of PLD by examining the effect of a dominant negative RalA protein (Ser28Asn). This mutant RalA protein contains a mutation homologous to the dominant negative Ser17Asn mutant of Ras (Feig et al., 1988) that is locked in an inactive conformation and suppresses exchange factor activation of endogenous Ras (Farnsworth, 1991; Schweigehoffer, et al., 1993). Expression of the S28N RalA reduced PLD activity in the v-Src-transformed cells to the level of PLD activity observed in the parental NIH 3T3 cells or in v-Src-transformed cells expressing the dominant negative S17N Ras mutant (Figure 17) (Jiang, et al., 1995). This effect was observed only in cells prelabeled with [$^3$H]-myristate, which is incorporated almost exclusively into phosphatidylcholine (PC) - the substrate for the v-Src-induced PLD (Song, et al., 1991 and 1993). If the cells were prelabeled with [$^3$H]-arachidonate, which is incorporated into phospholipids not utilized by the v-Src-induced PLD, there was no observable difference in the PLD activity

between the v-Src-transformed cells and the v-Src-transformed cells expressing the S28N RalA mutant (Figure 17). This also indicated that inhibitory effect of RalA mutants on PLD activity was specific. If a dominant inhibitory mutant RalA down regulated the activation of PLD by v-Src, this might indicate that a RalA effector is involved. Indeed, overexpression of an effector domain mutant RalA (D49N) resulted in inhibition of PLD activity in v-Src-transformed cells (Figure 17). These data and those in Figure 16 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 also not able to mediate PLD activation by v-Src in vivo and competed with endogenous RalA in this signalling pathway.

The experiments described here argue strongly that RalA proteins mediate the tyrosine kinase activation of PLD. A model of how RalA might function in this capacity is outlined in Figure 18. v-Src and presumably other membrane tyrosine kinases activate Ras; and, as demonstrated by others (Urano, et al., 1995), Ras activates Ral-GDS by targeting it to RalA at the plasma membrane. In the process of activating RalA, Ral-GDS brings Ral and associated PLD into a tyrosine kinase/Ras signalling complex. Since activated RalA (Q72L) is unable to activate PLD activity in non-transformed cells, it is postulated that the catalytic activity of PLD is elevated by some, as yet undefined additional event induced by v-Src. Cells transformed by Ras also display elevated PLD activity (Carnero, et al., 1994; our unpublished data), this additional factor is likely a component of a signalling pathway downstream of Ras. Possible mediators are the Ras related

Rho proteins, which have been shown to activate PLD activity in vitro (Bowman, et al., 1993; Malcolm, et al., 1994; Siddiqi, et al., 1995). There is genetic evidence indicating that RhoA is activated by Ras (Ridley, et al., 1992); however, the biochemical mechanism has yet to be elucidated. The ARF GTPase has also been shown to activate PLD in vitro (Brown, et al., 1993; Cockroft, et al., 1994; Lambeth, et al., 1995); however, a connection between ARF and Ras signals has not been reported. The inhibition of PLD activity in v-Src-transformed cells by the inhibitory mutant RalA (S28N), which prevents GDP/GTP exchange, implicates a protein interacting with the GTP-sensitive RalA effector domain. This is consistant with our observation that a effector domain mutant RalA (D49N) was also able to block PLD activity in v-Src-transformed cells. A gene for a protein that binds to the RalA effector domain has been cloned and encodes a GTPase activating protein (GAP) for Rho family GTPases (Cantor et al., 1995). This protein, designated Ral-BP1, could contribute to the activation of PLD, however, since activated RalA, which binds to this Rho family GAP, does not activate PLD in non-transformed cells, there must still be an additional Ras-activated factor required for PLD activation. The proposed model, in which the Ral/PLD complex is recruited by binding to Ral-GDS, is consistent with our finding that a constitutively active RalA mutant (Q72L) enhanced PLD activity to a lesser extent than wild type RalA. GTP-bound GTPases have a lower affinity for exchange factors than their inactive GDP-bound counterparts (Boguski, et al., 1993); thus, the Q72L RalA mutant, which is bound to GTP constitutively, would not be brought into a complex by Ral-GDS as efficiently as wild type RalA, which would exist in the GDP-bound form. Finally, since the GDP-bound form of RalA was able to associate with PLD, and an anti-Ral antibody precipitates PLD activity from both v-Src-transformed and parental Balb/c 3T3 cells, it is

cosiderable that Ral is constitutively associated with PLD and mediates PLD activity upon its recruitment to Ras/RalGDS complex.

We previously demonstrated that the PLD activated by v-Src could be distinguished from the PLD activated by phorbol esters that activate protein kinase C (Song, 1993), suggesting multiple mechanisms for activating PLD. Consistent with there being at least two mechanisms for PLD activation, phorbol ester-induced PLD activity was not affected by the dominant negative S28N RalA mutant (our unpublished data). Thus, PLD activity can apparently be induced by both Ral-dependent and Ral-independent mechanisms. In addition, if PLD activated by Src and mediated by Ras and Ral is distinct from that mediated by ARF, there may be some functional similarities between ARF and Ral in the activation of PLD. It was recently reported that amino terminal mutations to ARF prevent the activation of PLD (Zhang et al., 1995) and that a peptide from amino acids 2 to 17 of ARF prevented PLD stimulation by ARF (Fensome et al., 1994). Thus, it appears that ARF and Ral may mediates PLD activity through amino terminal sequences. The significance of PLD activation by tyrosine kinases is not well understood, although PLD activity has been found to be elevated in response to all of the tyrosine kinases where it has been examined. 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 mediated by Ras.

**A Role for RalA in Src transformation.** Cell transformation by Src depends on Ras (Nori et al.,; Declue et al., 1991). It has been suggested that Ras mediates multiple signal pathways leading to cell transformation (White, et al., 1995). A key event for Ras transformation involves the direct physical

association between Ras and its effector molecules (Marshall, 1993). RalGDS, which is responsible for the activation of Ral (Urano et al., 1995), was found to be another Ras effector (Kikuchi et al., 1994; Hofer et al., 1994; Spaargaren et al., 1994). Whether the Ras-RalGDS pathway is involved in Ras transformation has not been determined. We have established v-Src transformed NIH cell lines expressing various mutant of RalA genes, wild type, activated mutant (Q72L) and dominant inhibitory mutant (S28N) and deleted amino terminal domain (ΔN11). These cell lines displayed different levels of PLD activity as discussed above. Interestingly, we have noticed that cell morphology also varied among these transformed cells. To examine whether RalA indeed play a role in mediating Src tranformation, a soft agar colony-formation assay was employed to evaluate transformation properties.

We first examined the effect of wild type and an activated RalA (Q72L), which is analogous to the Q61L oncogenic substitution in Ras, on v-Src-induced transformation. Both forms of RalA genes overexpressed in Src transformed cells enhanced the transformed phenotype. The wild type RalA increased the formation of colonies with >0.5 cm in diameter by 25-35%, while RalA (Q72L) increased by 15-25% (Table 2). We also examined the oncogenic potential of the RalA gene. Both wild type and activated RalA genes (Q72L) were not sufficient to induce cell transformation as colonies failed to form in soft agar. Thus, it appears that RalA lacks potent transforming activity on its own, but potentiates cell transformation by Src. The reason that activated RalA had a lesser stimulatory effect than wild type RalA in Src transformation may be explained by the following. Activated Ras resulting from induction by tyrosine kinase v-Src, interacts with RalGDS (Urano et al., 1995), which consequently activates Ral. Due to the fact that activated GTPases have a lower affinity for their exchange

factor than inactivated ones (Boguski & McCormic, 1993), it is likely that the interaction between RalGDS and Ral, which is presumably to form the Ras/RalGDS/Ral signalling complex, was impaired by the activated RalA.

Since RalA displayed its ability to enhance Src transformation, it would be interesting to test whether a dominant negative mutant RalA suppress Src transformation. The expression of a dominant negative mutant RalA (S28N), which is analogous to Ras S17N that has been shown to interfere with Ras exchange factor activation of endogenous Ras (Farnsworth & Feig, 1991), reduced colonies formation (>0.5 cm in diameter) in soft agar by 45% relative to the control empty vector, although the total number of colonies were approximately the same (Table 2). A ΔN11 mutant RalA also reduced colony formation by 20%. The suppression of Src transformation by RalA mutants was reduced when lesser amount of plasmid was used for transfection. Cells expressing dominant inhibitory mutant Ral exhibited slower growth. These results suggested that RalA is involved in Src transformation, and it is mediated by Ras.

Characterization of different Ras effectors has led us to look inside the mechanism of Ras-mediated cell transformation. Raf is a well characterized Ras effector. A point mutation in the Ras effetor domain that is defective for Raf binding, retained weak transformation by oncogenic Ras (White et al., 1995), suggesting that there are other Ras downstream effectors mediating Ras transformation. Distinguishable sites in the Ras effector domain are found to be responsible for the interactions of Ras with Raf and RalGDS (White et al., 1995). The data presented by us suggested that RalA plays a role in modulation of Src transformation. This is believed to be mediated through the interaction of Ras and RalGDS as a result of Src activation. Recently, Ras related superfamily

members, Rac and Rho, were reported to mediate Ras transformation (Qiu et al., 1995; Prendergast et al., 1995), and Ras effector domain was found to be responsible for Rho activation (Khosravi-Faret et al.; Joneson et al., 1995). Thus, it appears that signal pathways downsteam from Ras can be distiguished by interaction with different sites on the effector domain of Ras, and that they contribute to full cell transformation mediated through Ras. In addition, as will be reported (Urano, et al., 1995), RalA enhances both Ras- and Raf-induced transformation in focus formation assay. Thus, RalA may enhance the transforming potential of Src and Ras by increasing PLD activity and lipid second messenger production.

# PART V. SUMMARY AND DISCUSSION

We have elucidated an intracellular signaling pathway, in which Ras and Ral comprise a GTPase signaling cascade that mediates v-Src-induced PLD activity. The activation of PLD in turn generates a variety of phospholipid secondary messengers (Boarder, 1994; Foster, 1993; Exton, 1994). These phospholipid signaling molecules, including phosphatidic acid, lysophosphatidic acid and diacylglycerol have been demonstrated to be mitogenic stimuli or effectors (Liscovitch & Cantley, 1994; Divecha & Irvine, 1994; Exton, 1994).

The implication of G-protein involvement in the activation of PLD by v-Src was first suggested by experiments carried out in a permeabilized cell system. PLD activity induced by v-Src was sensitive to GDP-βS, and enhanced by GTP-γS. Furthermore, GTP-dependent activation of PLD by v-Src was dependent upon the presence of ATP, suggesting that a phosphorylation event may be involved. The PLD activity in v-Src transformed cells was unaffected by either cholera or pertussis toxin, two specific heterotrimeric G-protein effectors (Gilman, 1987), indicating that heterotrimeric G-proteins were unlikely to be involved. Other types of heterotrimeric G-proteins were also ruled out based on the observation that a neutralizing antibody to Gq did not have an inhibitory effect on the activation of PLD by v-Src. Furthermore, treatment of cells with $ALF_4^-$ had no effect on PLD avtivity. Therefore, the identification of G-protein involvement was focused on monomeric types.

It has been well established that Ras is activated upon tyrosine kinase activation, and plays a central role in the mediation of a wide variety of cellular processes (Bourne et al., 1990). Three lines of evidence are presented which

demonstrate that Ras is a mediator for the activation of PLD by the tyrosine kinase v-Src. First, a neutralizing Ras monoclonal antibody (Y13-259) inhibited PLD activity in membranes isolated from v-Src transformed Balb/c3T3 cells. Secondly, the stimulatory effect of cytosol for PLD activity was reduced by preclearing cytosol with immobilized GTP bound Ras. Lastly, expression of a dominant inhibitory Ras mutant in v-Src-transformed cells reduced the PLD activity to the level observed in parental cells. Overexpression of a dominant inhibitory mutant of Raf, a Ras downstream effector (Moodie et al., 1993), in v-Src transformed cells did not abolish PLD activity. Another Ras downstream effector, PI3 kinase (Rodriguez-Viciana et al., 1994), was localized in the membrane fraction of v-Src transformed cells as judged by western blot. In addition, a specific inhibitor of PI3 kinase, Wortmannin, had no effect on the PLD activity in v-Src transformed cells (our unpublished observation). Thus two apparent Ras downstream effectors were ruled out as contributor to PLD activation by v-Src.

Previous experiments showed that cytosol, depleted by GTP bound Ras, was decreased in its ability to stimulate PLD activity in the presence of GTP-γS. This observation strongly suggested that the cytosolic factor(s) depleted by GTP bound Ras was related to the function of a Ras downstream effector(s) in the activation of PLD. RalGDS was recently shown to interact with Ras. The significance of such interaction is to localize RalGDS to the membrane for the activation of Ral (Urano et al., submitted for publication). To determine whether Ral could possibly play a role in mediating PLD activity, we have used a series of mutant forms of RalA. These include an activated mutant (Q72L) that constitutively binds to GTP, a dominant inhibitory mutant (S28N) that preferentially binds to GDP, an effector domain mutant (D49N) that looses its

ability to interact with downstream effectors and an amino terminal deletion mutant (ΔN11) that is a novel Ral-specific amino terminal domain. These mutants were stablely transfected into v-Src transformed cells. Overexpression of a ΔN11 RalA mutant decreased PLD activity. In *in vitro* binding studies, wild type RalA and effector domain RalA mutant proteins were both able to associate with PLD, whereas deletion of the amino terminal domain of Ral decreased its ability to bind to PLD, indicating that the association of PLD and Ral was depended on its amino terminal domain, but not its effector domain. Overexpression of wild type RalA had more pronounced stimulatory effect on PLD activity than active mutant (Q72L) RalA in v-Src-transformed cells. However, neither caused activation of PLD in NIH 3T3 cells by themselves, suggesting that Ral alone is not sufficient for the activation of PLD.

It has been suggested that active GTP binding proteins have a lower affinity for exchange factor than their inactive counterparts (Boguskin & McCormick, 1993; Farnswowth et al., 1991). Therefore, RalGDS , as it interacts with Ras, brings wild type Ral with associated PLD more efficiently than activated Ral to form a Ras/RalGDS/Ral-PLD signalling complex, as shown in Figure 18, where the PLD activation event takes place. Since v-Ras-transformed cells display elevated PLD activity (Carnero et al., 1994; our observation), it is plausible to postulate that Ras might control another effector regulating PLD activity in the signalling complex. A dominant negative (S28N) and an effector domain (D49N) mutants of RalA were both able to block PLD activity in v-Src-transformed cells, strongly suggesting that RalA may also control a downstream effector upon its GTP and GDP binding, which is likely to be another component(s) in the signalling complex in coorperation with an undefined Ras effector in the activation of PLD. A gene for a protein that binds to the RalA

effector domain has been recently identified as a RhoGAP (Cantor et al., 1995). Whether this Rho GAP is involved in the signalling complex for the activation of PLD reminds to be investigated.

As summarized in Figure 19, the activation of PLD by v-Src tyrosine kinase appears to be mediated by two GTP-binding proteins, Ras and Ral. Activated v-Src phosphorylates Shc, which serves as an adaptor for the Grab2/Sos complex. Sos is thus recruited to the membrane for the activation of Ras (Buday and Downward, 1993). The GTP bound Ras interacts with RalGDS and localizes it to the membrane proximal region (Kikuchi et al., 1994). RalGDS in turn activates Ral by interacting with Ral and increasing its GTP binding (Urano et al., 1995). During this process, Ral and associated PLD recruits a downstream effector to the Ras/RalGDS/Ral-PLD signalling complex. This Ral downstream effector presumably coorperates with another Ras effector for the PLD activation. A Ras/Ral GTPase cascade is thus proposed for v-Src induced phospholipid signal pathway.

Recent work on Ras-related GTPases that regulate the cytoskeleton has led to the discover of a new regulatory mechanism involving mutiple GTPases in a cascade. In mammalian cells, a cascade of Cdc42 controlling Rac and Rho coordinates the actin cytoskeleton during cell movement (Nobes and Hall, 1995). In yeast cells, a related cascade of BUD1 (RSR1) controlling CDC42 and possibly Rho proteins coordinates polarization of the cytoskeleton during cell division by budding (Chant, 1994). There are more GTPase links between Rac, Cdc42, and Rho, as well as between Ras and Rho which have been recently reviewed (Chant and Stowers, 1995). However, their biological properties and the mechanisms by which these molecules coordinate their cascades are unknown. It was also

reported that stimulation of lamellipodial outgrowth by oncogenic Ras depends on Rac (Ridley et al., 1992). Rac was recently reported to be essential in Ras transformation, but not in Raf transformation (Qiu et al., 1995). Thus, another Ras and Rac GTPase cascade was suggested to be involved in different cellular processes. We have also found that cell transformation by v-Src was enhanced by overexpression of wild type RalA, and inhibited by a dominant inhibitory mutant RalA based on a soft agar colony formation assay. Thus, RalA is likely to play a role in cell transformation. If the activation of Ras by v-Src leads to the activation of Ral, Ras should be involved in the Ral-mediated v-Src-induced cell transformation. In agreement with others, expression of RalA was found to enhance the transforming activities of Ras and Raf in focus-formation assays, and a dominant negative mutant form of RalA suppresses the transforming activities of Ras and Raf (Urano et al., submitted for publication). Taken together, these observation demonstrate another GTPase cascade, Ras and Ral, in the regulation of cell transformation. It appears that GTPase cascades as sophisticated regulatory mechanisms hold trememdous potential for regulating cellular behavior. Considering the findings that v-Src-induced PLD activity is mediated by the Ras/Ral cascade, cell transformation by oncogenic Src and Ras, at least partially, depends on the activation of PLD that results in an increase of phospholipid second messenger production. Alternatively, the Ras/Ral cascade in the activation of PLD may mediate other cellular behavior, such as cytoskeletal organization, endocytosis, membrane ruffling and trafficking, or an unkown function waiting to be characterized.

# PART VI. TABLES AND FIGURES

**Table 1. PLD activity in membranes isolated from v-Src-transformed and normal BALB/c 3T3 cells[a]**

| | Relative PLD activity | | | | |
|---|---|---|---|---|---|
| | [3H]-myristate | | | [3H]-arachidonate | |
| Components | Src | BALB | Src/Gen | Src | Src/Gen |
| | (cpm/mg membrane protein) | | | | |
| Membrane | 2541 (1.0) | 1470 (1.0) | 1407 (1.0) | 1148 (1.0) | 1008 (1.0) |
| Membrane + Cytosol | 4830 (1.9) | 2345 (1.6) | 2247 (1.6) | 1603 (1.4) | 1680 (1.4) |
| Membrane + GTPγS | 6860 (2.7) | 2254 (1.5) | 2180 (1.6) | 1377 (1.2) | 1209 (1.1) |
| Membrane + Cytosol + GTPγS | 18354 (7.2) | 3381 (2.3) | 4943 (3.5) | 3366 (2.9) | 3192 (2.9) |

a: PLD activity in membranes isolated from v-Src-transformed (Src) and parental BALB/c 3T3 (BALB) cells was determined in the presence and absence of cytosol (150 μg) and the non-hydrolyzable GTP analog GTPγS (10 μM). Membranes were prepared from cells prelabeled with either [$^3$H]-myristate or [$^3$H]-arachidonate as described in Materials and Methods. The effect of the protein-tyrosine kinase inhibitor genistein (100 μM) on PLD activity in membranes from v-Src-transformed cells (Src/Gen) labeled with either [$^3$H]-myristate or [$^3$H]-arachidonate was determined. The reaction is then allowed to proceed for 15 min. PLD activity is presented as the amount of radioactivity (cpm) converted into PEt in the presence of exogenous ethanol per mg of membrane protein. Data are the average of duplicates from a representative experiment where duplicate values varied by less than 10%. The relative values normalized to the basal membrane PLD activity is shown in parenthesis.

**TABLE 2. Effects of RalA and its mutants on v-Src-induced colony formation in soft agar [a]**

| | Number of Colonies | |
|---|---|---|
| Cell Line | Transfection I | Transfection II |
| Src | 79 ± 3 | |
| Src–(ZIP-neo) | 73 ± 7 (100%) | 88 ± 4 (100%) |
| Src–(Ral wt) | 101 ± 16 (138%) | 120 ± 5 (136%) |
| Src–(Ral Q72L) | 82 ± 8 (112%) | 108 ± 5 (122%) |
| Src–(Ral S28N) | 40 ± 2 (55%) | 54 ± 8 (61%) |
| Src–(Ral ΔN11) | 56 ± 4 (77%) | 68 ± 3 (78%) |

a: 2 X $10^3$ v-Src-transformed NIH cells transfected with pZIPNeo, pZIPNeo-RalA(WT), pZIPNeo-RalA(Q72L), pZIPNeo-RalA(S28N) and pZIPNeo-RalA(ΔN11) were suspended in 0.3% soft agar containing 400 μg/ml G418, and seeded onto Φ60 mm plate with grid as described in Materials and Methods. Colonies were grown at 37°C for three weeks with adding fresh top agar every week. Larger than 0.5 cm colonies were counted. Data are the mean of triplicate plates for each type of cell lines. The values indicating percentage increase of colony number relative to the control are shown in parenthesis.

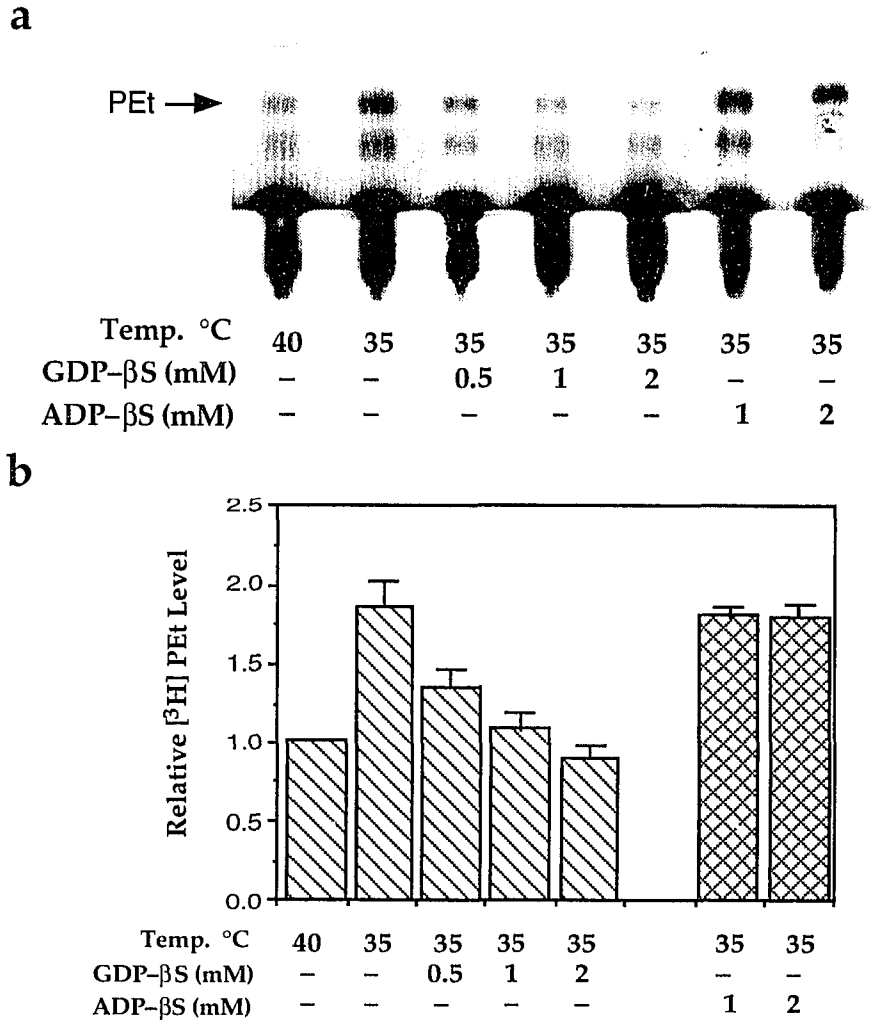


**Figure 1. v-Src-induced PLD activity is inhibited by GDPβS, but not ADPβS.** Induction of PLD activity was measured by the production of [$^3$H] PEt as described in Materials and Methods. (a) Autoradiograph of TLC from a representative experiment. (b) Quantitative analysis of PEt production data from (a). The mean value for [$^3$H]-PEt in LA90 cells maintained at the non-permissive temperature for v-Src were assigned a value of 1. The means for duplicate samples obtained at the permissive temperature for v-Src in the presence of either GDPβS or ADPβS were then normalized to the control value. The data represent the means of [$^3$H]-PEt from duplicate dishes relative to the control values with standard error for at least three independent experiments. The mean baseline cpm value was 2254 ± 170.

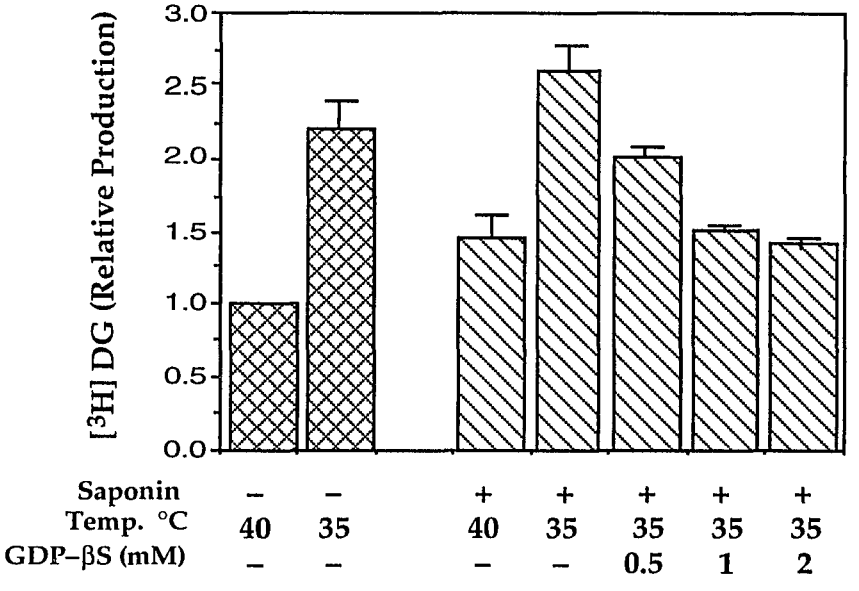


**Figure 2. DG production is inhibited by GDPβS.** The production of DG was examined in the presence or absence of GDPβS as indicated. The mean value for [$^3$H]-DG in LA90 cells maintained at the non-permissive temperature for v-Src were assigned a value of 1. The means for duplicate samples obtained at the permissive temperature for v-Src in the presence of GDPβS were then normalized to the control value. The data represent the means of [$^3$H]-DG from duplicate dishes relative to the control values with standard error for at least three independent experiments. The mean baseline cpm value 9528 ± 720.

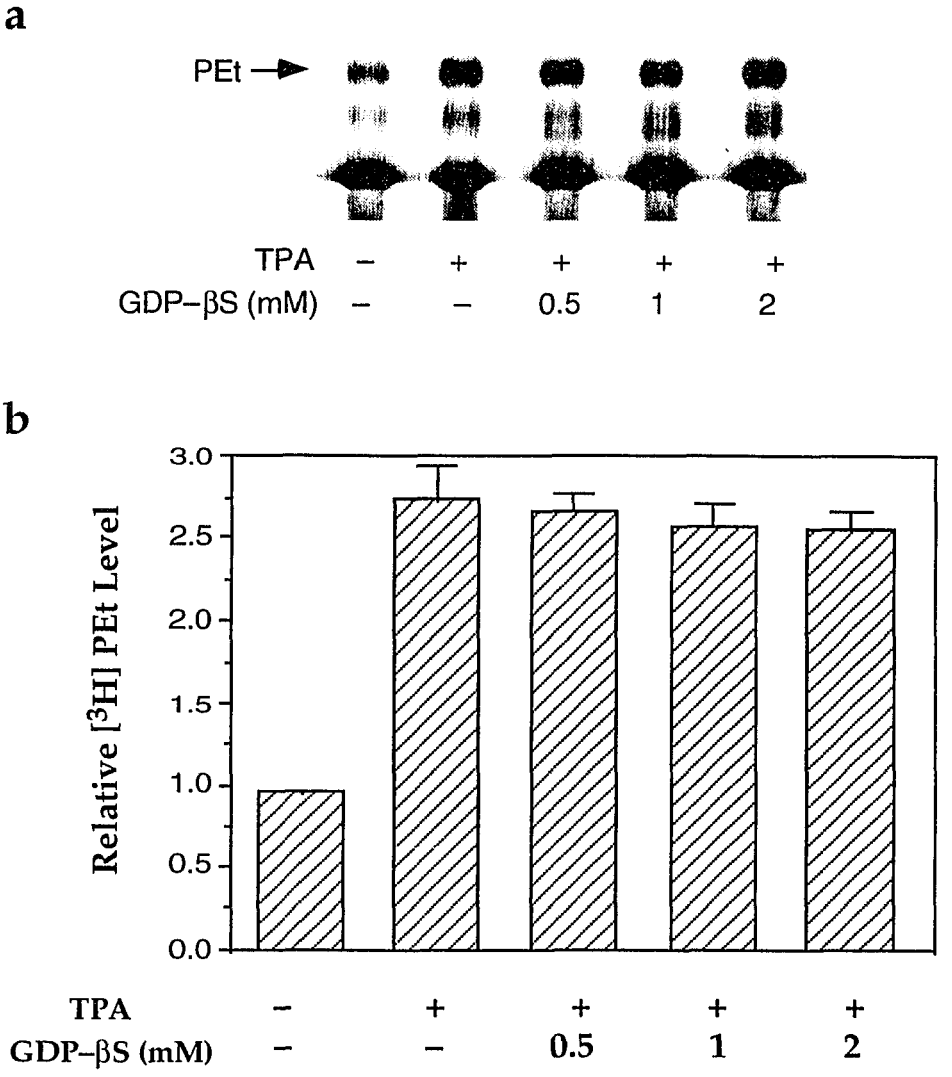


**Figure 3. The effect of GDPβS on TPA-induced PLD activity.** Induction of PLD activity by TPA was examined in the presence of GDPβS. (a) Autoradiograph of PEt production as monitored by TLC. (b) Quantitative analysis of the data from (a). The mean value for [$^3$H]-PEt without TPA stimulation was assigned a value of 1. The means of duplicate samples obtained with treatment of TPA and GDPβS were normalized to the control. The data represent the means of [$^3$H]-PEt from duplicate dishes relative to the control values with standard error for at least three independent experiments. The mean baseline cpm value was 2140 ± 198.

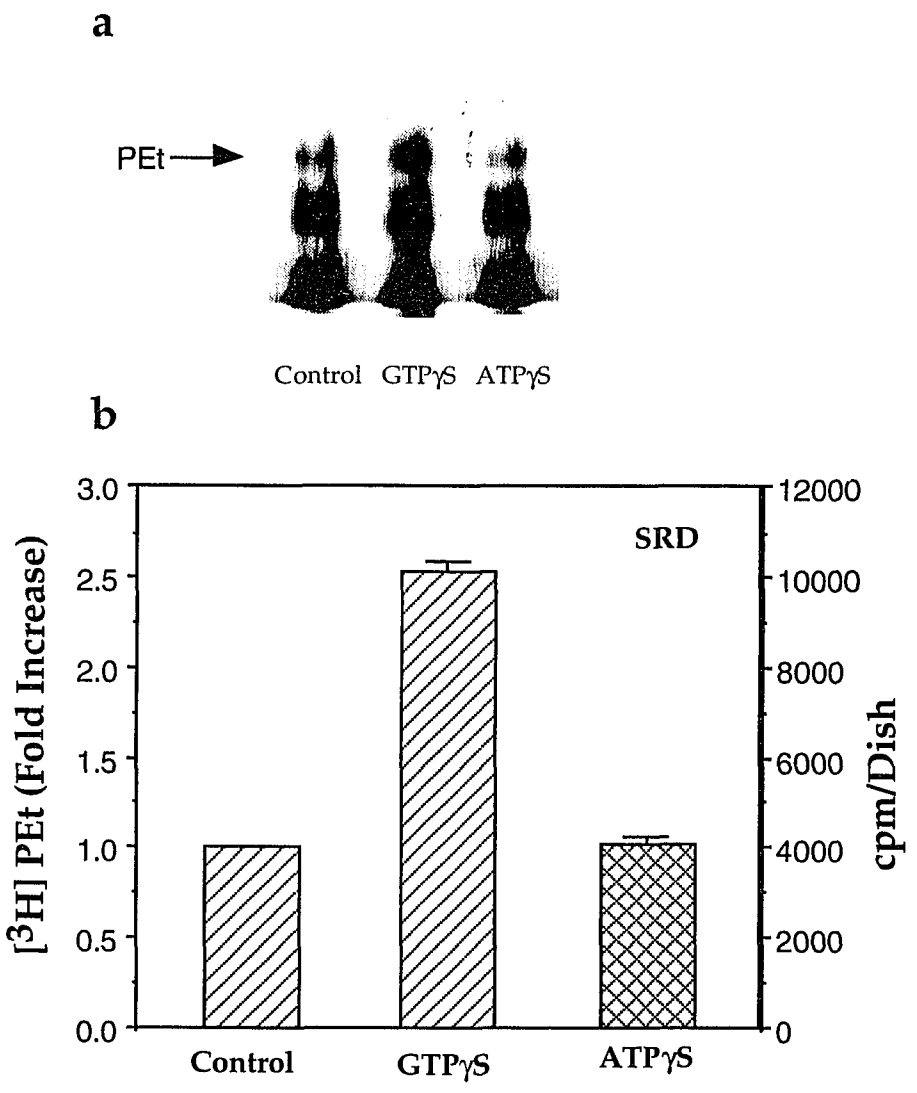


**Figure 4. The non-hydrolyzable GTP analog, GTPγS, stimulates PLD activity in v-Src-transformed cells.** v-Src-transformed SRD cells were examined for PLD activity in the presence or absence of either GTPγS or ATPγS (100 μM). (a) Autoradiograph of PEt production as mornitored by TLC. (b) Quantitative analysis of the data from (a). [$^3$H]-PEt levels were determined as described in Materials and Methods. The [$^3$H]-PEt value from cells permeabilized in the absence of either GTPγS or ATPγS were used as controls. The data are the means of duplicates determined from a representative experiment that was performed at least three times.

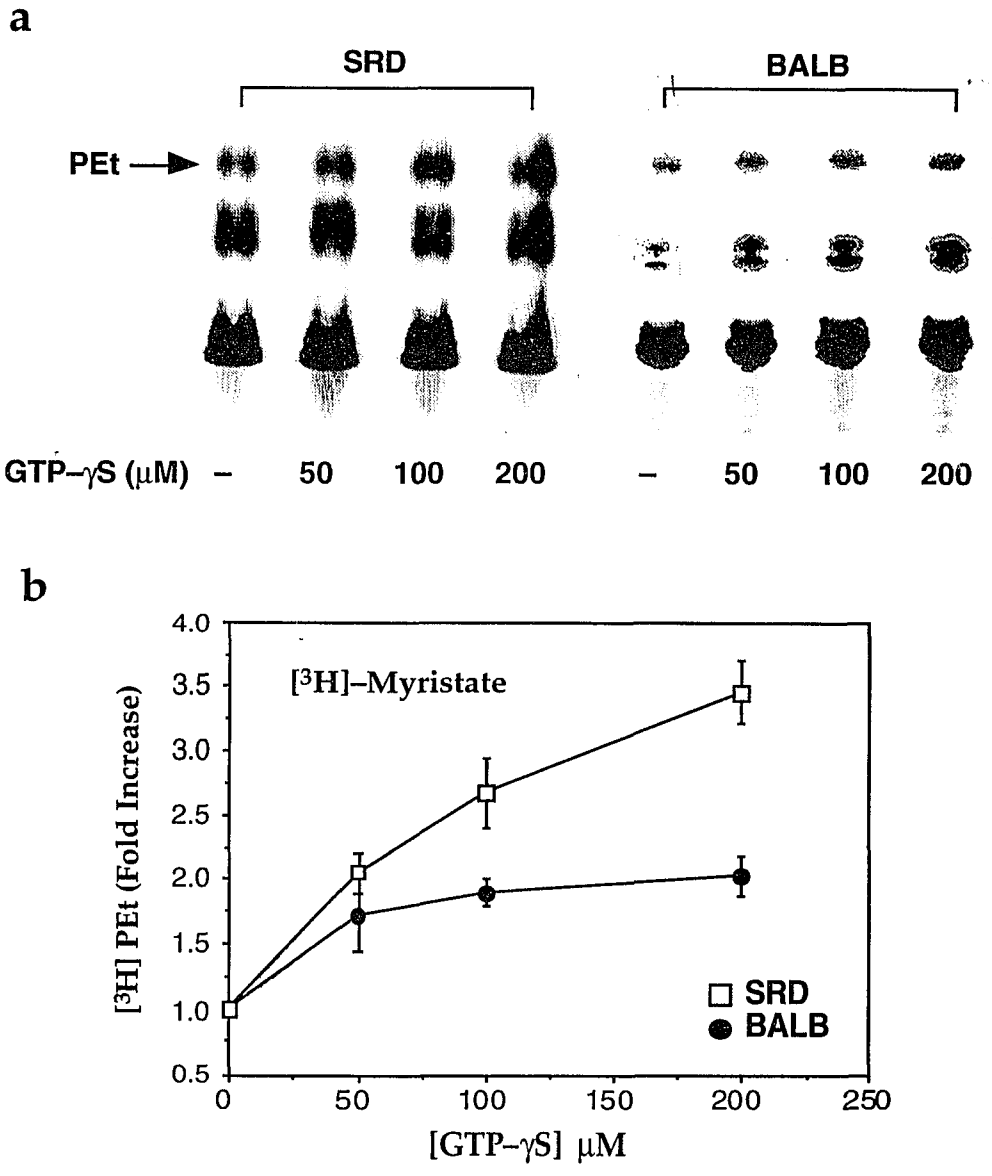


**Figure 5. GTPγS preferentially enhances PLD activity in v-Src-transformed cells relative to the parental BALB/c 3T3 cells prelabeled with [$^3$H]-myristate.** (a) Autoradiograph of PEt production as monitored by TLC, in SRD and BALB/c 3T3 cells. (b) Quantative analysis of the data from (a). v-Src-transformed SRD cells and its parental BALB/c 3T3 cells were examined for PLD activity in GTPγS dose-dependent manner as described in Materials and Methods. The data represent the means of [$^3$H]-PEt values relative to the control values from at least two independent experiments. Baseline cpm values for [$^3$H]-myristate were 4882 ± 715 and 1860 ± 216 for SRD and BALB/c 3T3 cells respectively.

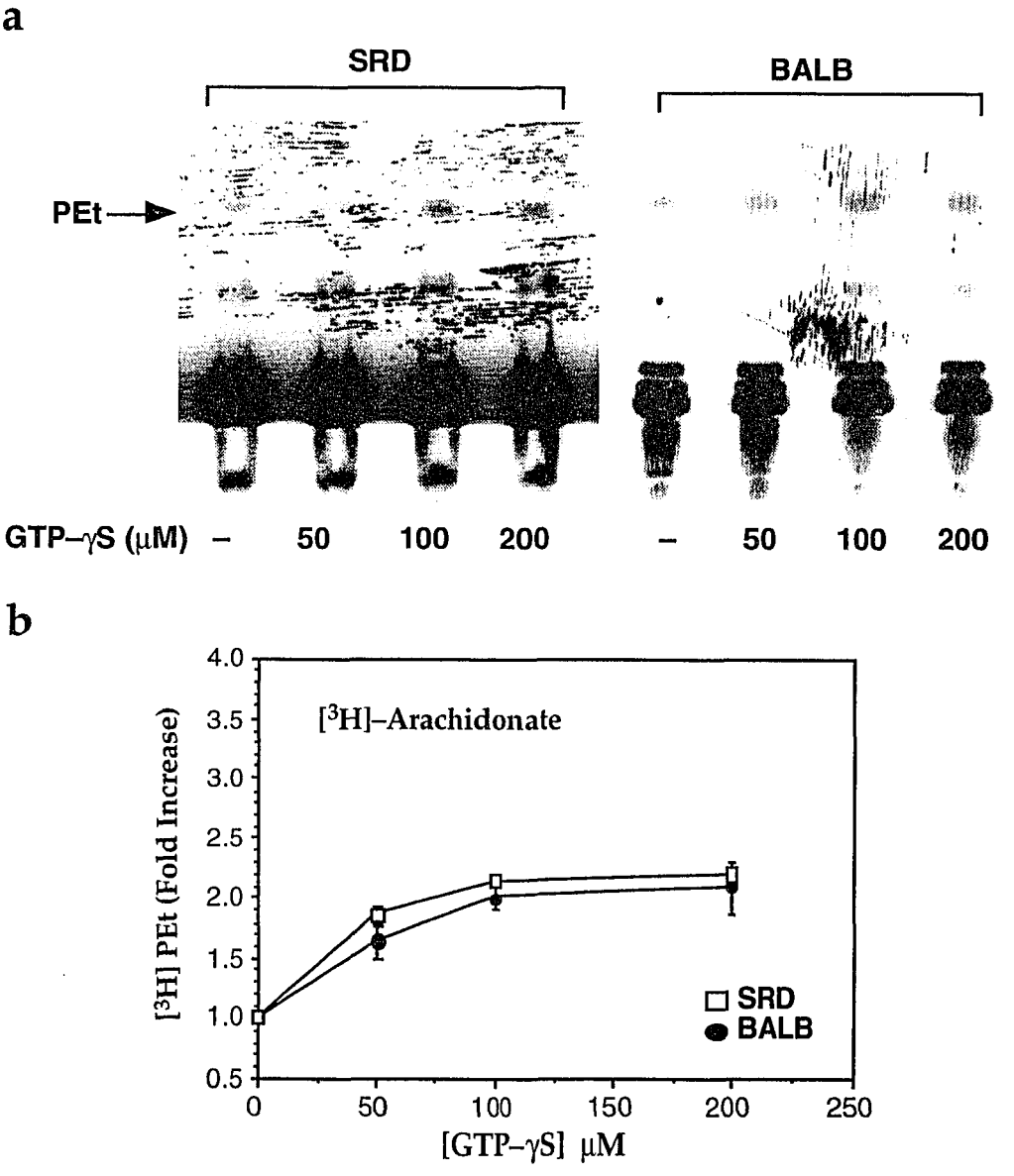


**Figure 6. GTPγS enhancement of PLD activity in v-Src-transformed cells relative to the parental BALB/c 3T3 cells prelabeled with [$^3$H]-arachidonate.** (a) Autoradiograph of PEt production as monitored by TLC, in SRD and BALB/c 3T3 cells. (b) Quantative analysis of the data from (a). v-Src-transformed SRD cells and its parental BALB/c 3T3 cells were examined for PLD activity with increasing concentrations of GTPγS. The data represent the means of [$^3$H]-PEt values relative to the control values from at least two independent experiments. Baseline cpm values for [$^3$H]-arachidonate were 2160 ± 382 and 1684 ± 235 for SRD and BALB/c 3T3 cells respectively.

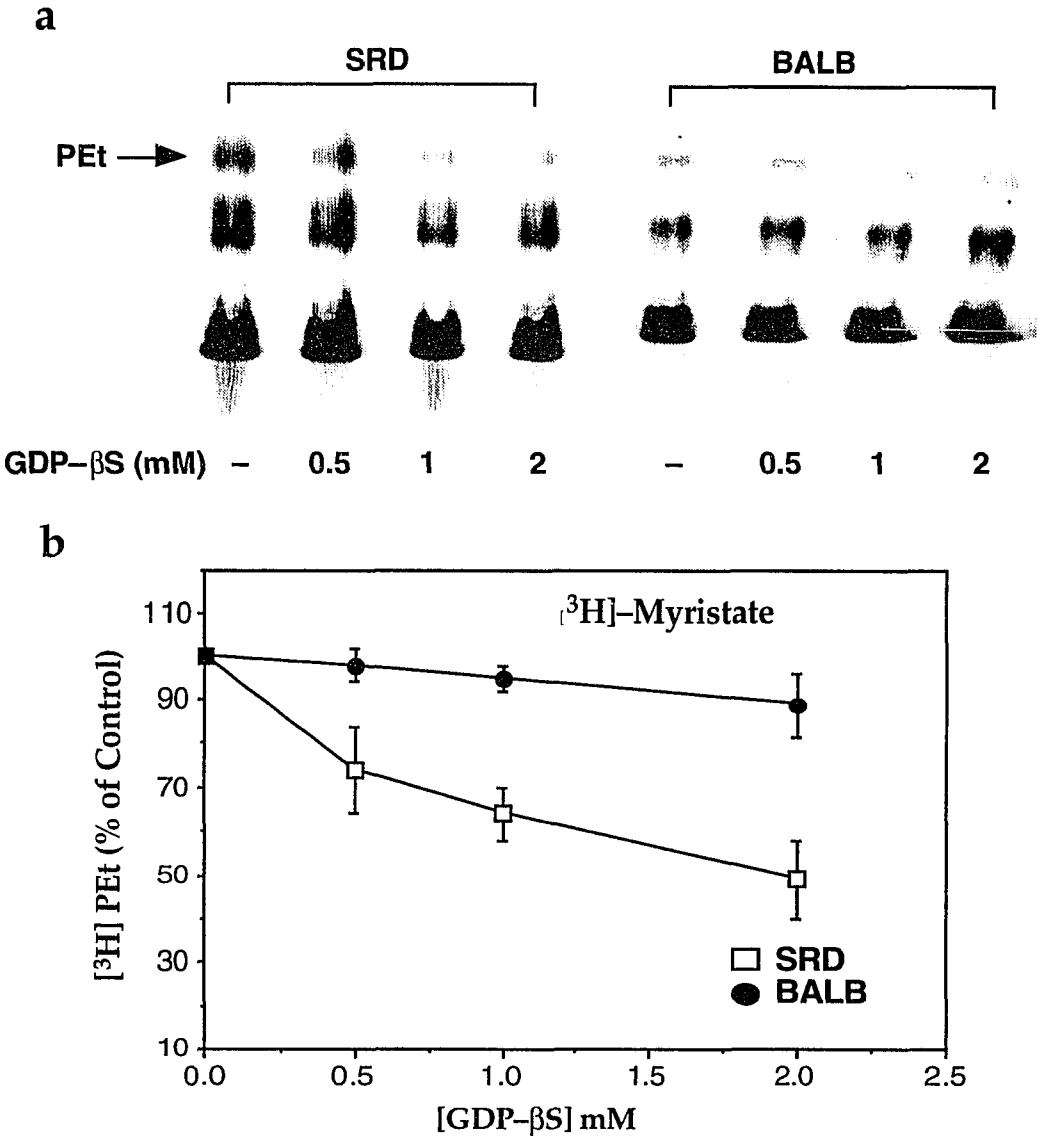


**Figure 7. GDPβS inhibition of v-Src-induced PLD activity in v-Src-transformed cells prelabeled with [$^3$H]-myristate.** (a) Autoradiograph of PEt production as monitored by TLC, in SRD and BALB/c 3T3 cells. (b) Quantative analysis of the data from (a). v-Src-transformed SRD cells and BALB/c 3T3 cells were examined for PLD activity with increasing concentrations of GDPβS. The data represent the means of [$^3$H]-PEt values relative to control values from at least two independent experiments. Baseline cpm values for [$^3$H]-myristate were 4882 ± 715 and 1860 ± 216 for SRD and BALB/c 3T3 cells respectively.

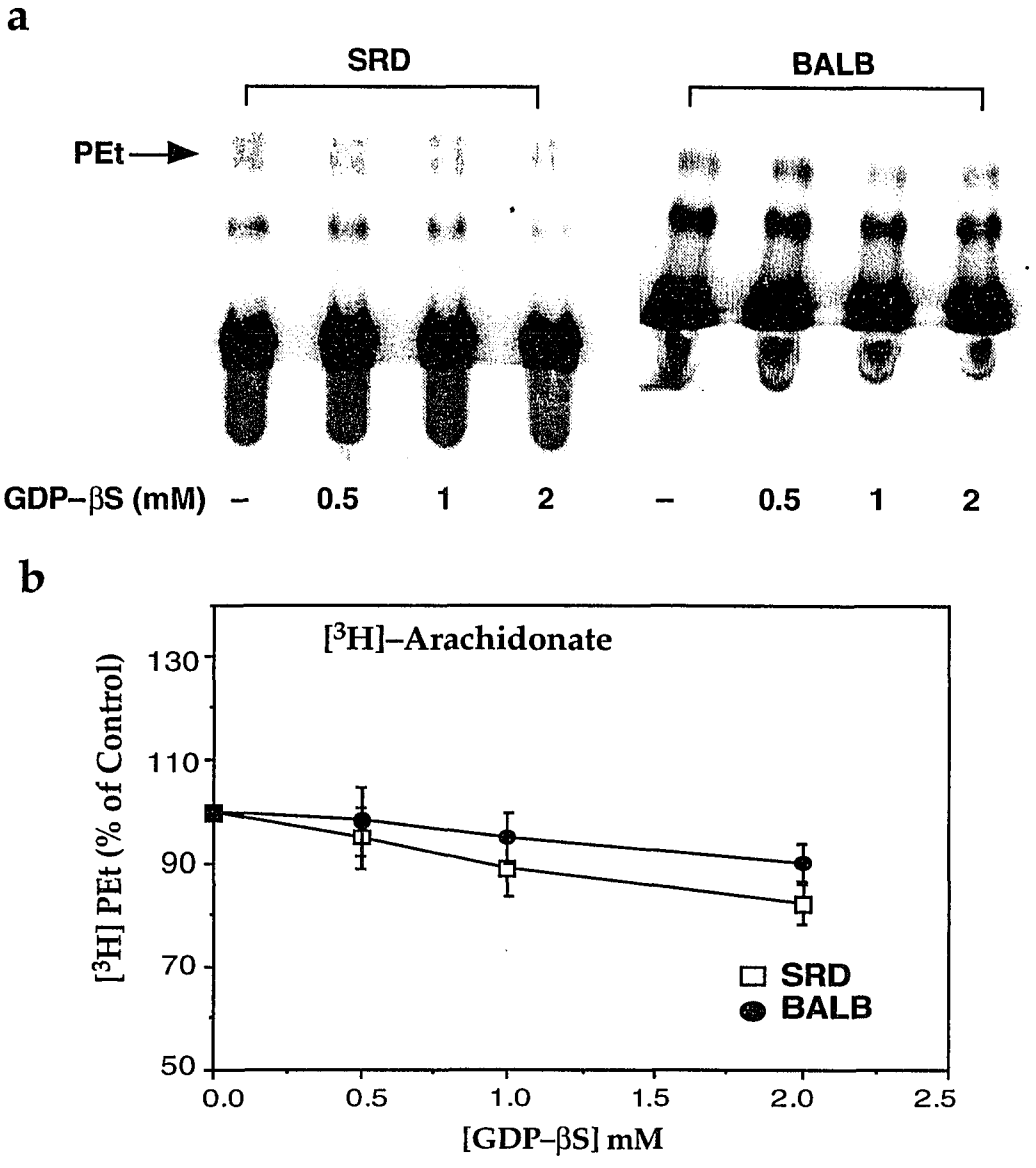


**Figure 8. GDPβS inhibition of v-Src-induced PLD activity in v-Src-transformed cells prelabeled with [$^3$H]-arachidonate.** (a) Autoradiograph of PEt production as monitored by TLC, in SRD and BALB/c 3T3 cells. (b) Quantative analysis of the data from (a). v-Src-transformed SRD and BALB/c 3T3 cells were examined for PLD activity in increasing concentrations of GDP–βS. The data represent the means of [$^3$H]-PEt values relative to control value from at least two independent experiments. Baseline cpm values for [$^3$H]-arachidonate were 2160 ± 382 and 1684 ± 235 for SRD and BALB/c 3T3 cells respectively.

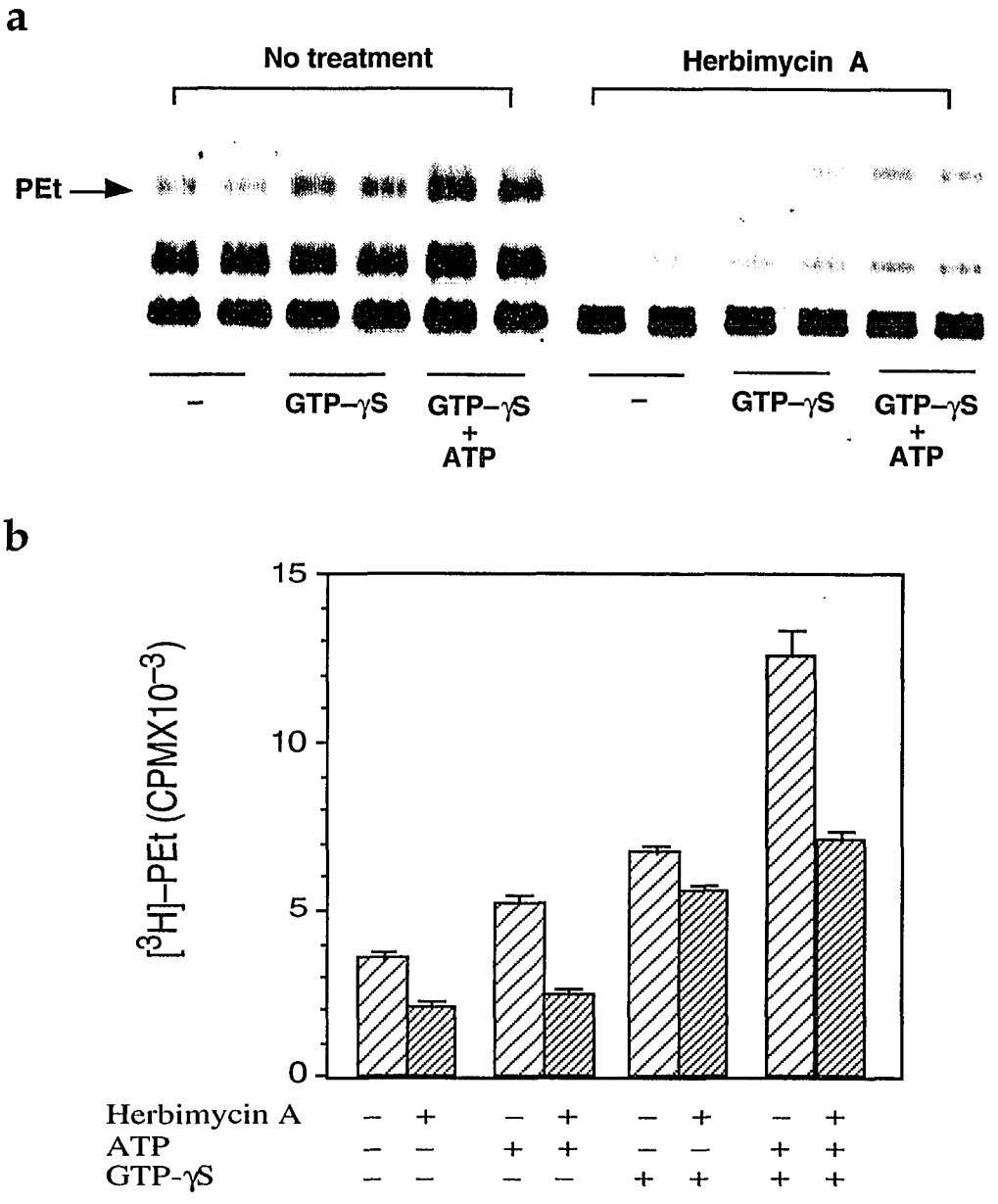


**Figure 9. ATP dependence and herbimycin A sensitivity of PLD activity in SRD cells.** (a) Autoradiograph of PEt production as monitored by TLC, in SRD cells. (b) Quantitative analysis of the data from (a). PLD activity was determined in [$^3$H]-myristate-prelabeled SRD cells in the presence and absence of herbimycin A (1 μg/ml, 16 hr), ATP (1 mM), GTPγS (100 μM) as shown. The data are mean cpm values ± standard error for duplicate samples from a representative experiment that was performed twice.

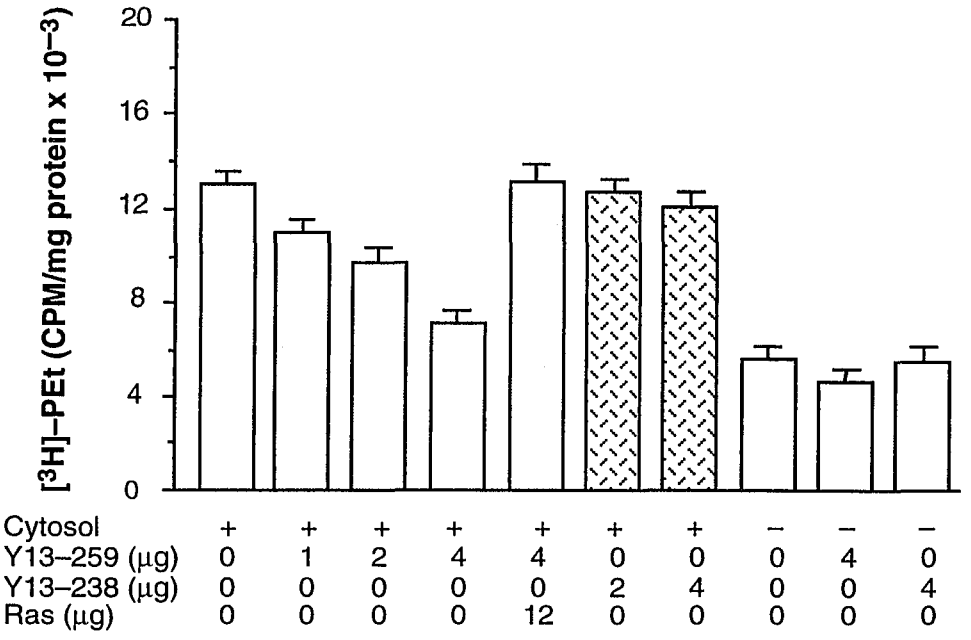


**Figure 10. A neutralizing Ras antibody inhibits PLD activity in membranes from v-Src-transformed cells.** Membranes from v-Src-transformed cells, prelabeled with [$^3$H]-Myristate were prepared as described in Materials and Methods. Membranes were incubated with indicated antibodies for 45 min at 22 °C. Then 150 μg of cytosol, 10 μM GTPγS and 1% ethanol were added as indicated and the reaction proceeded for 15 min. PLD activity was determined as described in Materials and Methods. The data represent the average of duplicate determinations ± range from a representative experiment that was repeated at least three times.

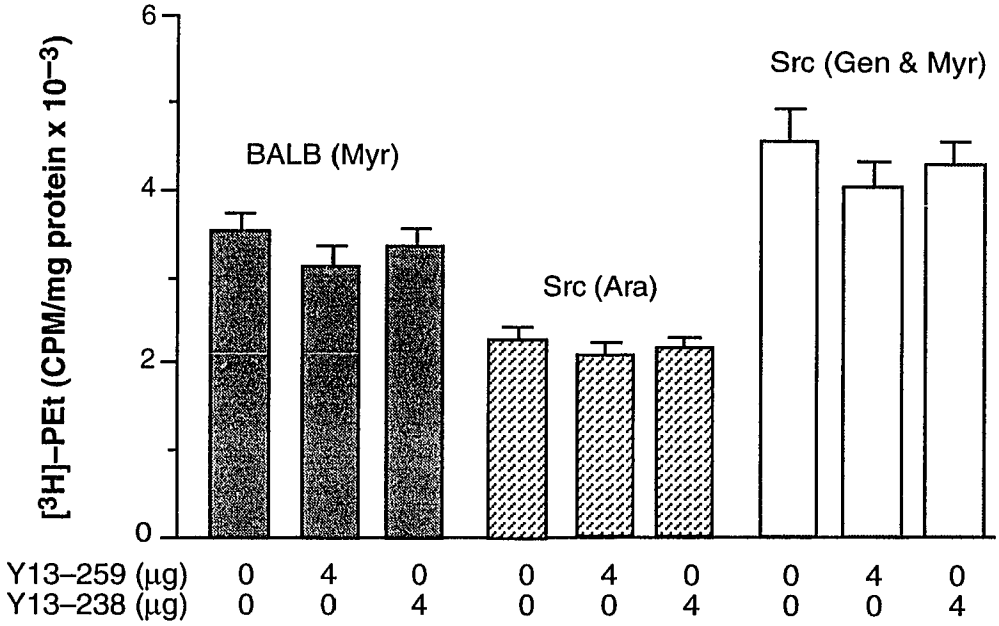


**Figure 11. Effect of Ras antibodies on PLD activity in membranes isolated from BALB/c 3T3 cells, v-Src-transformed cells prelabeled with [$^3$H]-arachidonate, and v-Src-transformed cells treated with genistein.** Cell membranes were isolated and incubated with Ras antibodies (Y13-259 and Y13-238) as indicated. After 45 min of incubation at 22 °C, 150 μg of cytosols and 10 μM GTPγS were added and the reaction was allowed to proceed for 15 min in the presence of 1% ethanol. The data represent the average of duplicate determinations ± range from a representative experiment that was repeated at least three times.

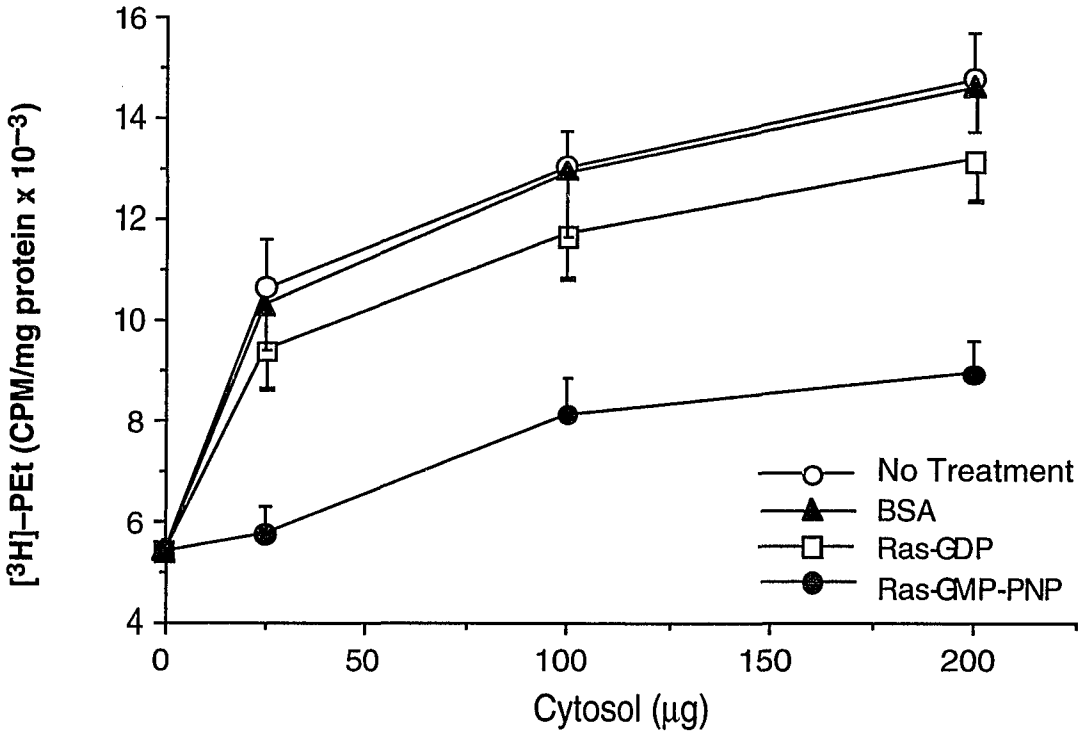


**Figure 12. Ras depletes the cytosol of its stimulatory potential in a GTP-dependent manner.** Membranes from v-Src-transformed cells, prelabeled with [$^3$H]-Myristate, were prepared and PLD activity was determined in the presence of increasing amount of cytosol and 10 µM GTPγS. The cytosolic fractions were either untreated or pretreated with immobilized bovine serum albumin (BSA), Ras-GMP-PNP, or Ras-GDP. The data represent the average of duplicate determinations ± range from a representative experiment that was repeated at least three times.

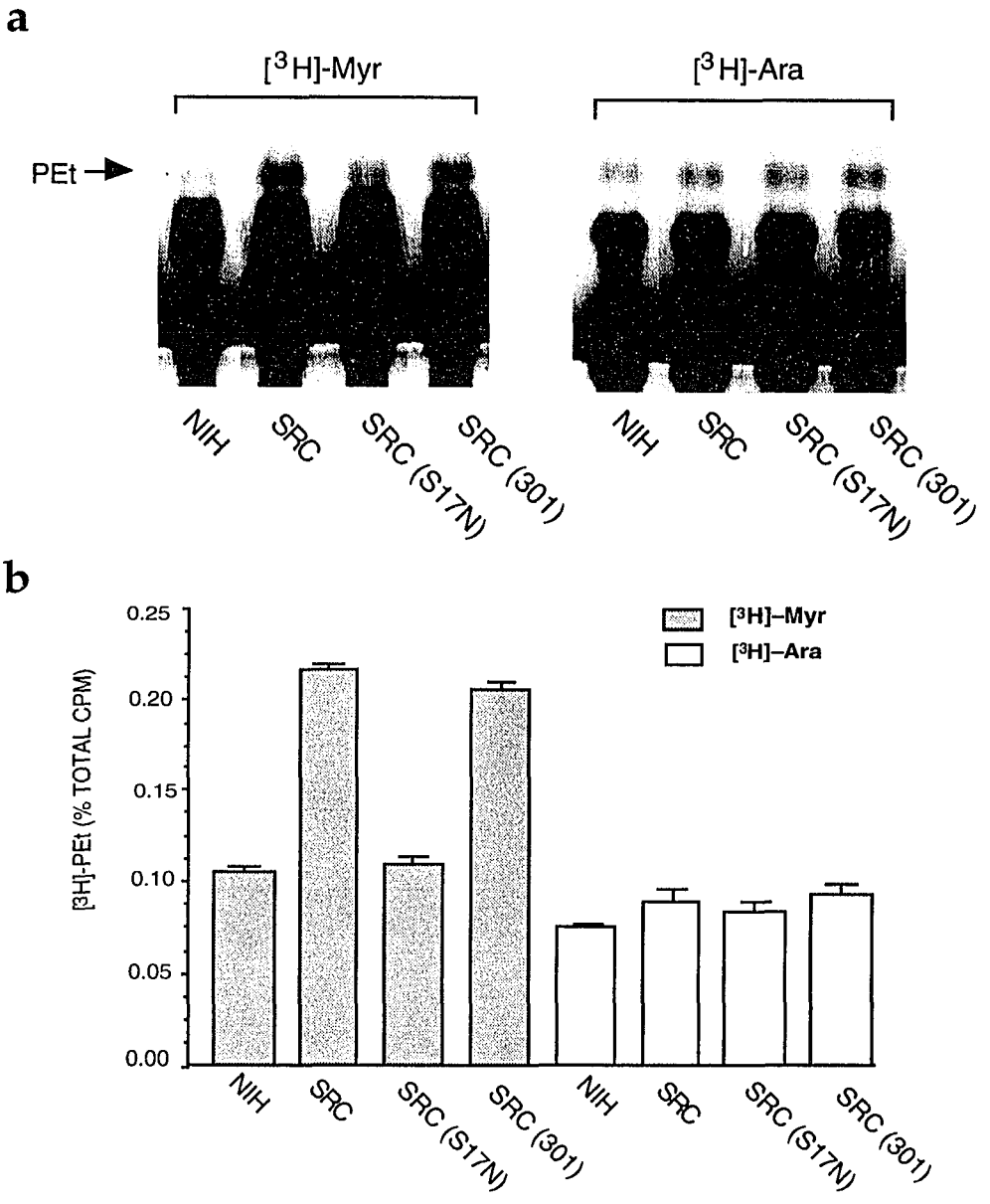


**Figure 13. A dominant negative Ras mutant blocks PLD activity in v-Src-transformed cells.** (a) Autoradiograph of PEt production as monitored by TLC. (b) Quantitative analysis of the data from (a). NIH 3T3 cells, v-Src-transformed NIH 3T3 cells and v-Src-transformed NIH 3T3 cells stably transfected with plasmids expressing control vectors, dominant negative mutant of Ras (pZIP S17N) and Raf-1 (pMNC K301W) were prelabeled with either [$^3$H]-myristate or [$^3$H]-arachidonate as shown. PLD activity in these cells was then assayed as described in Materials and Methods. The data are the average of duplicates ± range from a representative experiment repeated three times.

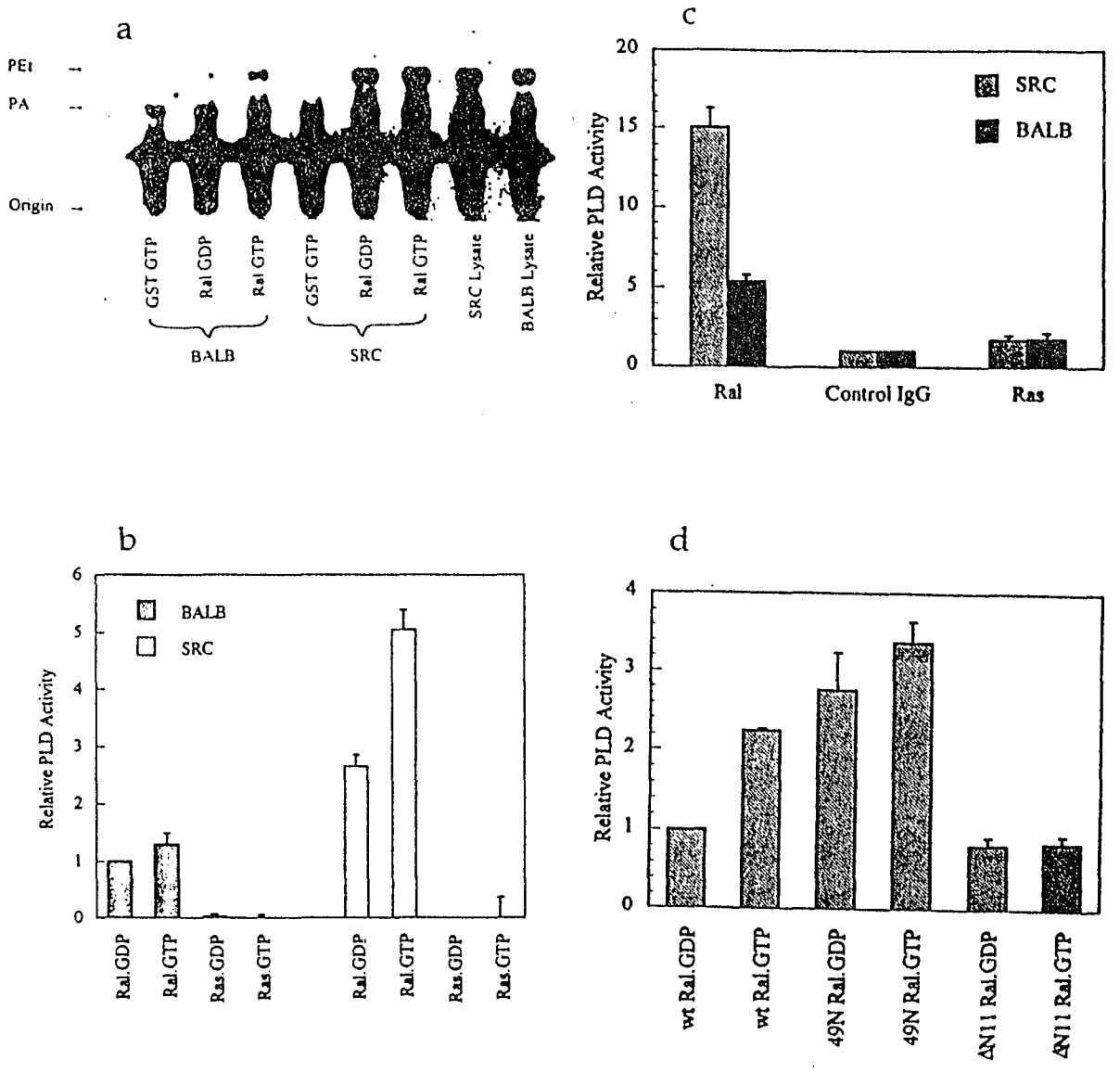


**Figure 14. Association of Ral with PLD activity in v-Src-transformed and parental Balb cells.** Cell lysates were prepared and incubated with immobilized wild type or various mutants of Ral proteins (with GTPγS or GDPβS bound), or antibody for Ral, Ras and control IgG. Immobilized or immuno complexes were then subjected to PLD activity assay as described in Materials and Methods. (collaboration results)

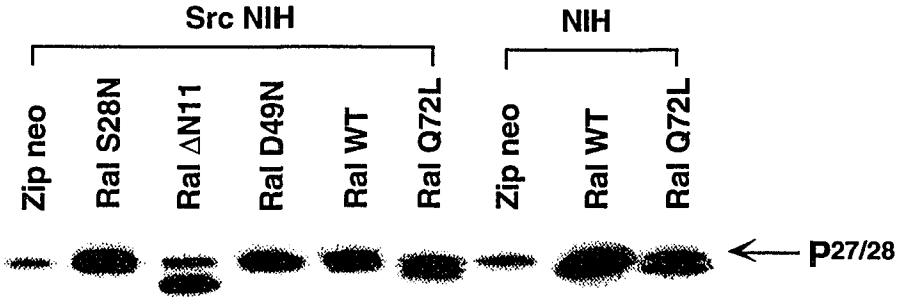


**Figure 15. Overexpression of various mutant and wild type Ral proteins in v–Src transformed and parental cells.** v–Src transformed and parental NIH cells were stably transfected with plasmid vectors containing various mutant and wild type Ral genes as described in Materials and Methods. The cells were grown to 80% confluence and 25 μg of total proteins of lysates were loaded in each lane for 12% SDS–PAGE. Western analysis was performed as described in Materials and Methods. Ral protein appeared to have the mobility of a 27/28 kDa protein as indicated by an arrow. Ral (ΔN11) has a smaller molecular weight (lower band), whereas Ral (Q72L) appeared as doublet bands.

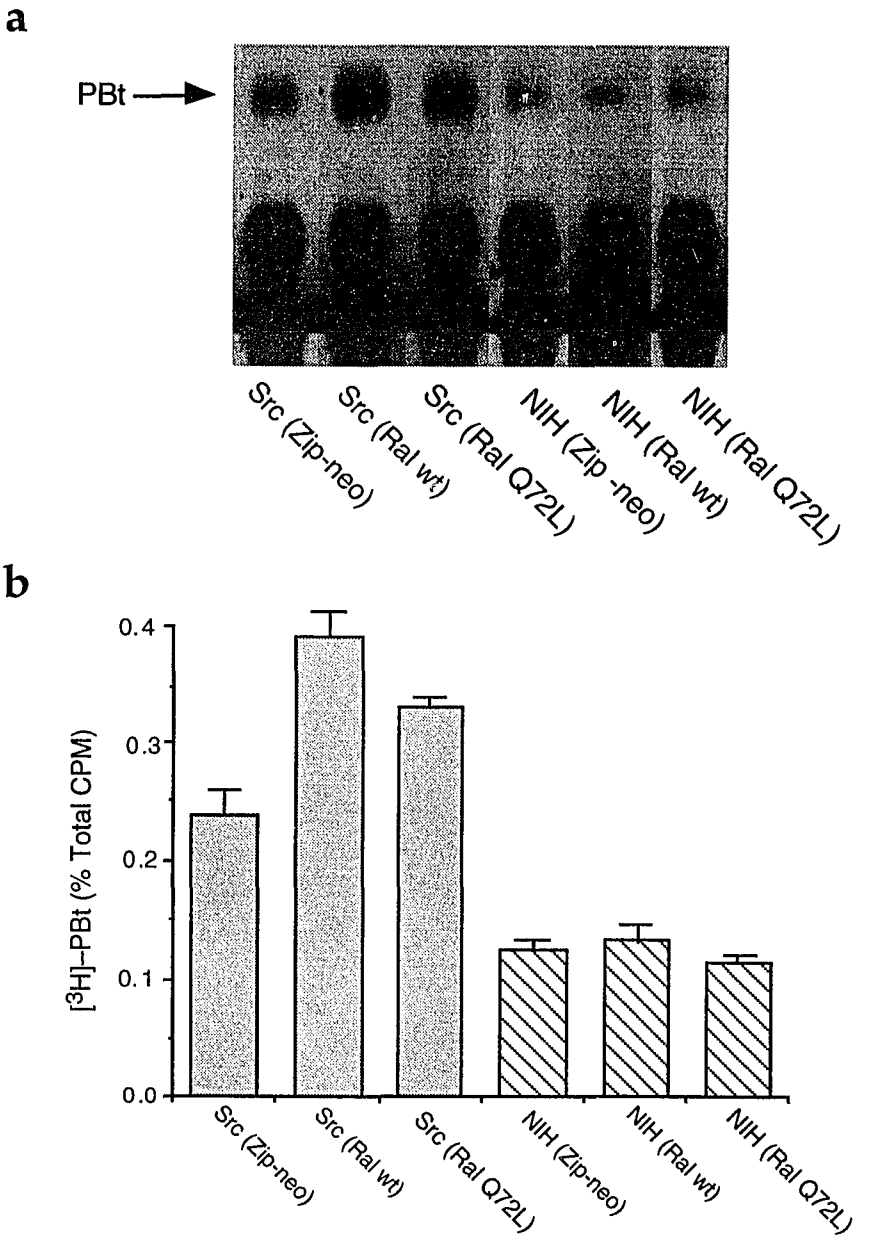


**Figure 16. The effect of wild type or activated mutants of Ral on PLD activity in v-Src-transformed and parental NIH 3T3 cells.** (a) Autoradiograph of PBt production as monitored by TLC. (b) Quantitative analysis of the data from (a). v-Src-transformed and parental NIH 3T3 cells were stably transfected with vectors expressing wild type Ral and a Ral gene containing an activating mutation (Q72L). PLD activity in these cells was then assayed as described in Materials and Methods. The data are the average of duplicates ± range from a representative experiment repeated three times.

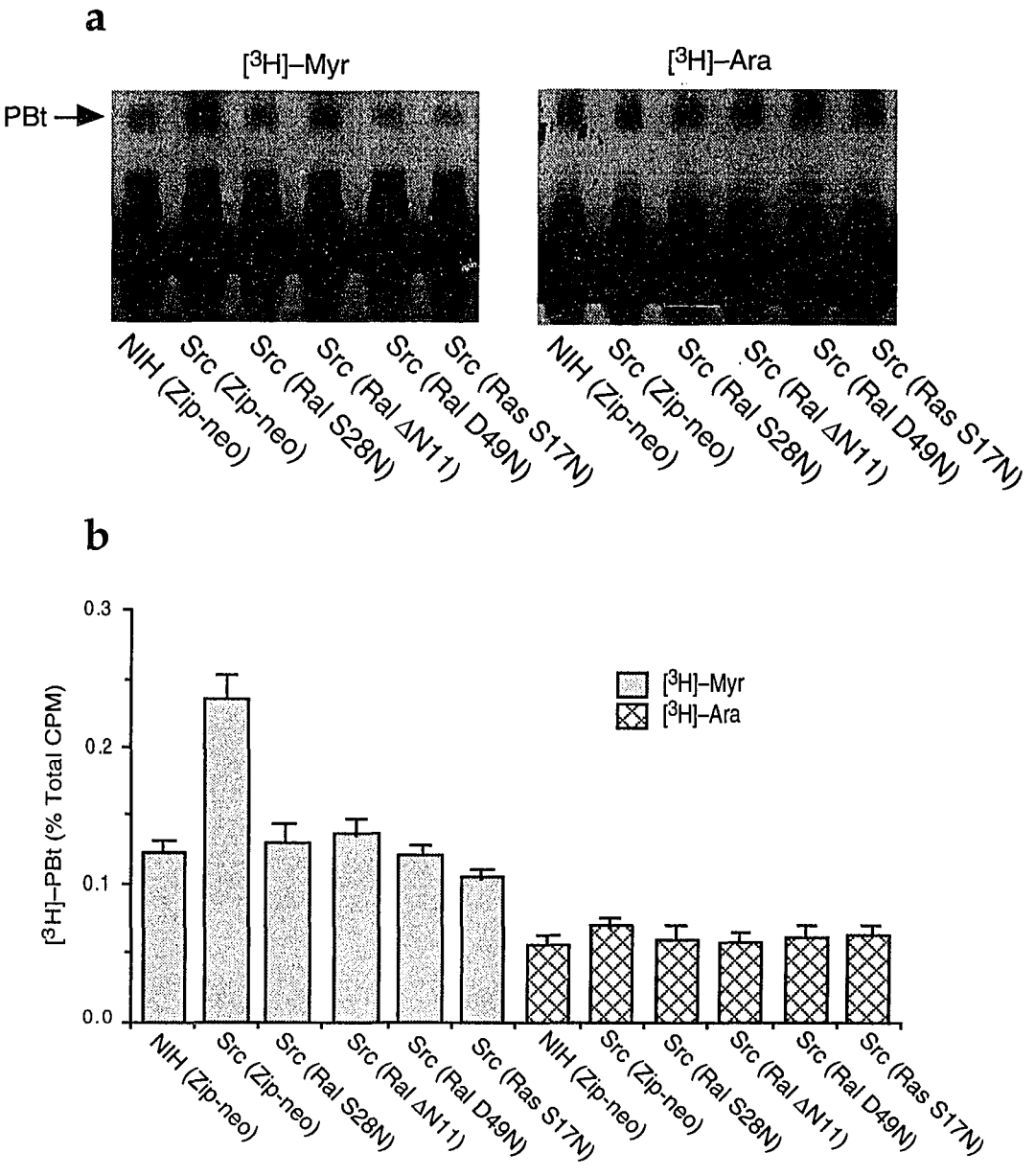


**Figure 17. The effect of Ral and Ras mutants on PLD activity in v-Src transformed NIH 3T3 cells.** (a) Autoradiograph of PBt production as monitored by TLC. (b) Quantitative analysis of the data from (a). v–Src–transformed cells were stably transfected with vectors containing mutants of Ral and Ras as shown. PLD activity in these cells was examined as described in Materials and Methods. The Data represent the average of duplicates ± range from a representative experiment repeated three times.

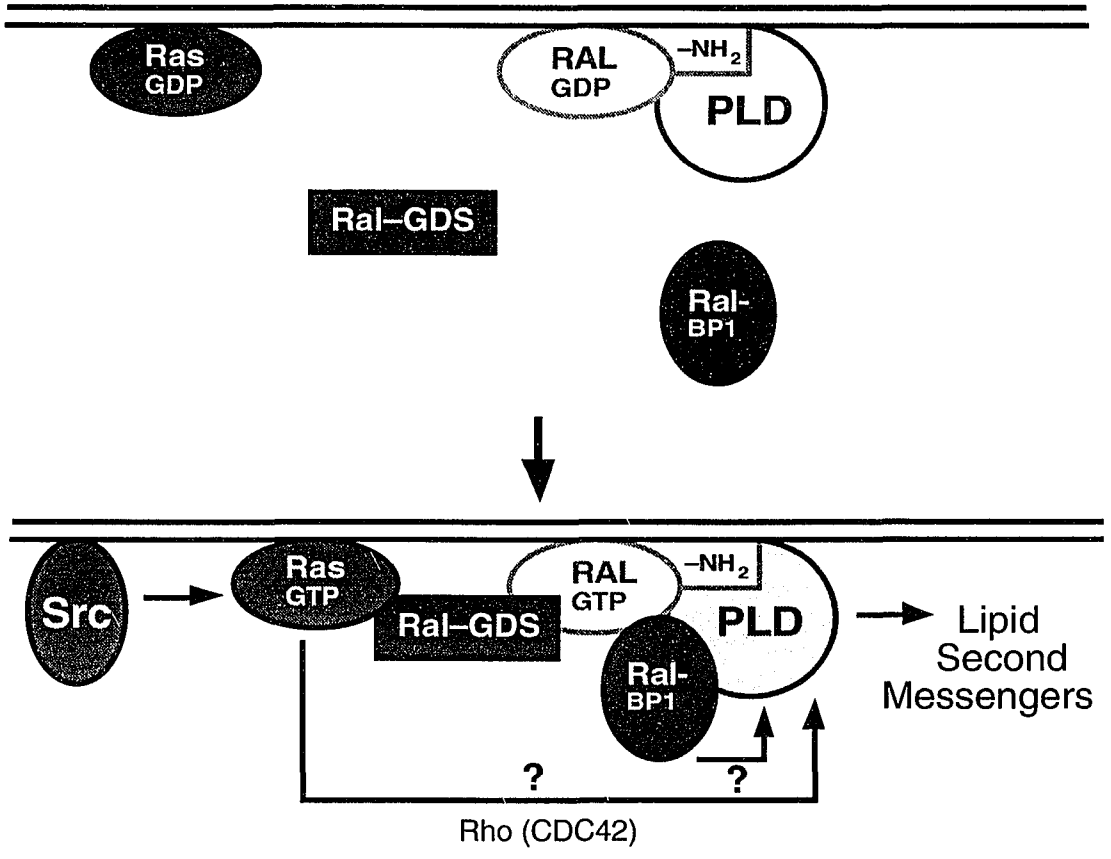


**Figure 18. Proposed model for RalA involvement in v-Src-induced PLD activation.** RalA, which is a membrane-bound protein, serves as an anchor for PLD. Ral-GDS serve as a conduit for bringing PLD into a complex with activated Ras. A protein associated with the effector domain of RalA designated Ral binding protein 1 (Ral-BP1) as described by Cantor et al. (1995) could also play a role in PLD activation. A factor(s dependent upon another downstream effector molecule of Ras is postulated to account for the ability of activated Ras to increase PLD activity in NIH 3T3 cells and the inability of RalA or activated RalA to increase PLD in these non-transformed cells.

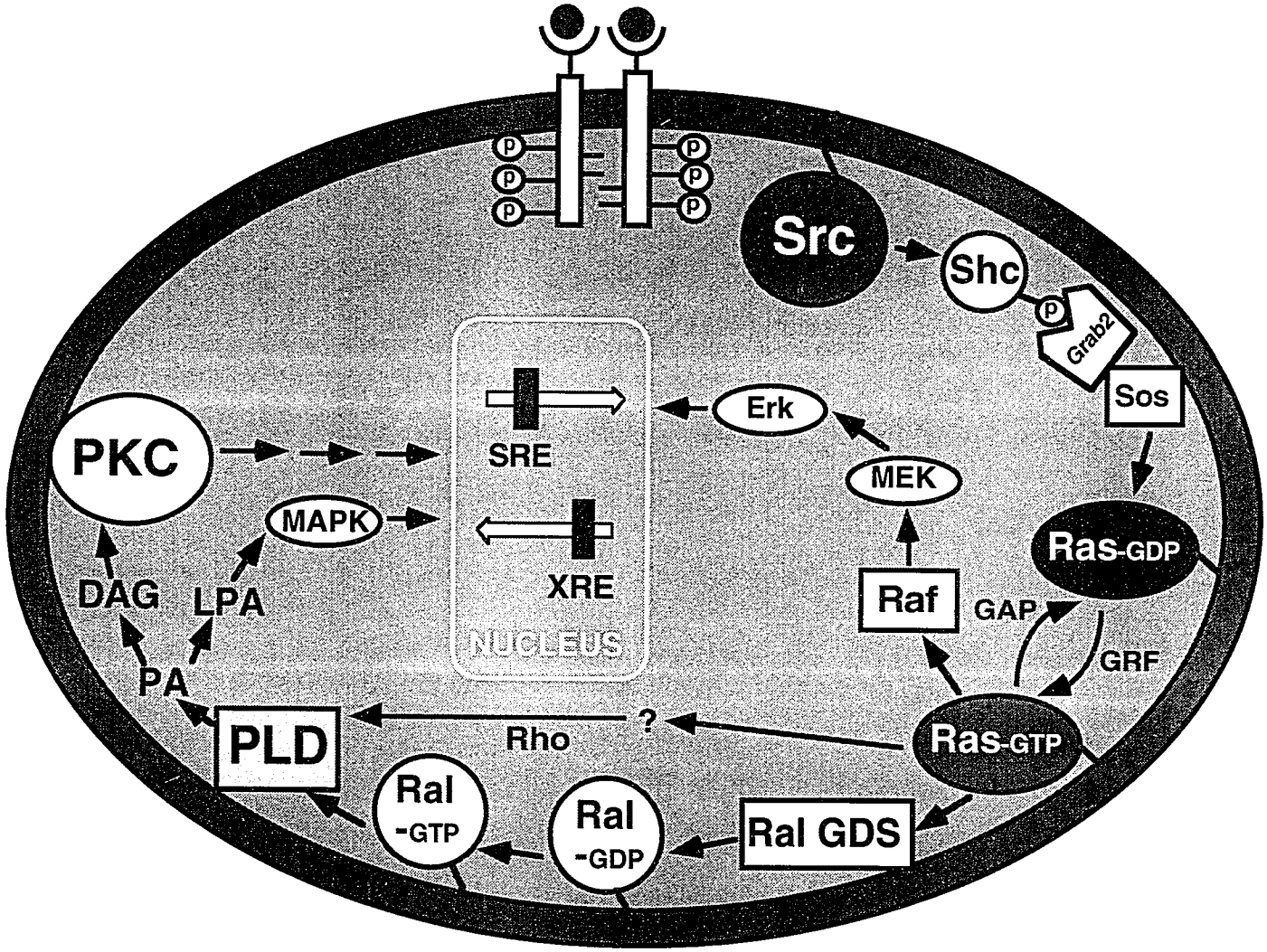


Figure 19. Proposed Ras/Ral GTPase cascade in v–Src–induced PLD activation and its role in intracellular signalling.

# PART VII. MATERIALS AND METHODS

**Materials.** GTPγS, GDPβS, ATPγS and ADPβS were purchased from Boehringer Mannheim. Saponin, cholera and pertussis toxins, apoprotinin, Leupeptin, MgATP, GST-agarose and IPTG were obtained from Sigma. Genistein was purchased from Upstate Biotechnology Inc (UBI). [$^3$H]-Myristate (NET-830), and [$^3$H]-arachidonate (NET-2982) were obtained from New England Nuclear. Antibodies to Ras (Y13-259 and Y13-238) and Src (327) were from Oncogene Scientific Inc. Antibodies to antiphosphotyrosine (PY20), Ral, PI3 kinase ($p^{85}$, $p^{110}$) and Sos were from Transduction Laboratory. Transfection materials, Lipofectamine and G418, were from Gibco. PEt, PBt and DG standards were obtained from Avanti Polar Lipids. Precoated silica 60A thin layer chromatography (TLC) plates were from Scientific Products. SDS electorphoresis and western blot materials were from Hoest and Bio-Rad, respectively.

**Cells and cell culture conditions.** BALB/c 3T3 and BALB/c 3T3 cells infected with either Rous sarcoma virus (Schmidt Rupin D strain) (SRD cells) or the temperature-sensitive LA90 strain of Rous sarcoma virus (Maroney et al., 1992) (LA90 cells) were maintained in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum (HyClone). NIH 3T3 and NIH 3T3 transfected by v-Src cells (obtained from Richard Jove, University of Michigan) were maintained in Dulbecco's modified Eagle medium supplemented with 5% 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.

**Prelabeling of phospholipids.** For in vivo studies, cells in 35 mm culture dishes were prelabeled for 4 to 6 h in 2 ml of Dulbecco's modified Eagle media containing 0.5% newborn calf serum. Isotopes were added to the culture media as follows: for [$^3$H]-Myristate, 3 μCi (40 Ci/mmol); for [$^3$H]-arachidonate, 2 μCi (240 Ci/mmol). For in *in vitro* studies, cells in 150 mm culture plates were prelabeled for 12-16 hours in 20 μCi of [$^3$H]-Myristate or 15 μCi of [$^3$H]-arachidonate in o.5 % serum.

**Extraction of lipids.** Extraction of lipids was performed according to the procedures described by Billah and Song (Billah et al., 1989; Song et al., 1991) with minor modifications. After conducting PLD activity assay, media were aspirated out of culture plates and cells were immediately chilled on ice. Ice cold stopping solution (0.50 ml of MeOH:6N HCl=50:1) was added to cells and cells were scraped into 1.5 ml Eppendorf tubes. Lipids were extracted by the addition of 0.50 ml of chloroform. Phase separation was obtained by adding 0.15 ml of 1M NaCl. The organic phase was reextracted with 0.10 ml of 1M NaCl, 0.10 ml of MeOH and 0.35 ml $H_2O$, recovered, dried under $N_2$, and redisolved in $CHCl_3$:MeOH (9:1).

**Characterization of phospholipid metabolites by TLC.** Extracts of phospholipid metabolites were characterized by TLC (silica gel 60A plates) as described previously (Billah et al., 1989). Lipid standards were visualized by treating TLC plates with iodine vapor. TLC plates were sprayed with En$^3$HANCE (Dupont) and exposed to Kodak XAR-5 film at -70 °C for 3 days. The developed films were scanned by using a densitometer. To accurately quantitate metabolically labeled PEt and DG, appropriate regions of TLC plates corresponding to PEt or DG were scraped, counted in a scintillation counter and

normalized to total cpm incorporated into cellular lipid. Total cpm was determined by taking an aliquot of the initial chloroform extract. The following solvent systems were used: For DG, hexane:diethylether:MeOH: glacial acetic acid (90:20:3:2); for PEt or PBt, the organic phase of ethylacetate:trimethylpentane:acetic acid:$H_20$ (110:50:20:100).

**Cell permeabilization and PLD activity assay in permeabilized cells.** Permeabilization to allow entry of guanine nucleotides was performed according to the procedures of Alexandropoulos et al., (1991) with modifications. Prelabeled cells were washed once with serum-free medium and incubated for 10 min; the cells were then washed with permeabilization medium (110mM KCl, 10mM NaCl, 1mM $KH_2PO_4$, 4mM $MgCl_2$, 1mM EGTA, 0.32mM $CaCl_2$, and 1mM $Na_2ATP$, 5mM creatine phosphate, 3 units/ml creatine kinase, 20mM HEPES, pH 7.0) without saponin; followed by a 3 min treatment with permeabilization buffer containing saponin (70 μg/ml). Guanine or adenine nucleotides were included in the permeabilization buffer as indicated in the text and figures. The permeabilized cells were rinsed twice with permeabilization buffer lacking saponin, but containing the indicated guanine or adenine nucleotides, and the cells were incubated for 10 min in the presence of guanine or adenine nucleotides and 1 % ethanol prior to activating the kinase activity of v-Src by temperature shift in LA90 cells, or prior to stimulation by TPA. The permeabilized SRD and BALB/c 3T3 cells were incubated for 15 min at 37 °C with the indicated guanine or adenine nucleotides. Reactions were terminated as described above. Permeabilization was monitored by trypan blue uptake.

**Bacterial toxin and aluminum fluoride treatment.** The effect of cholera toxin was examined over a concentration range of 20 to 150 ng/ml as described

previously (Qureshi et al., 1991). Pertussis toxin was used over a concentration range of 10 to 250 ng/ml. Pertussis and cholera toxin were administered 10 h prior to harvesting cells. Aluminum fluoride (10 mM NaF, 20 μM $AlCl_3$) was added with the permeabilization medium with and without saponin.

**Isolation of membrane and cytosol.** Cell cultures were grown to confluence at which time the media was replaced with fresh media containing 0.5% newborn calf serum for one day. This treatment reduced background PLD activity presumably due to serum stimulation. Cells in 150 mm culture dishes were then prelabeled overnight for 14 to 16 hr in 20 ml of Dulbecco's modified Eagle media containing 0.5% newborn calf serum. Isotopes were included as follows: 20 μCi [$^3$H]-Myristate, (40 Ci/mmol); 15 μCi [$^3$H]-arachidonate, (240 Ci/mmol). PLD activity in isolated membranes was determined using conditions established by Olson et al. (1991) and Conricode et al. (1992) with modifications. Prelabeled cells were washed twice with cold isotonic phosphate buffered saline, suspended in hypotonic buffer (25 mM Hepes pH = 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1mM dithiothreitol, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride), allowed to swell for 10 min, and then broken by Douncing (30 strokes with type B pestle). The disrupted cells were centrifuged at 500 x g for 5 min to clear nuclei and unbroken cells and the supernatant was then centrifuged 44,000 rpm for 45 min in an SW50 rotor. The supernatant was saved as the cytosolic fraction (approximately 1.5 mg/ml protein). the membrane fraction was recovered from the pellet be resuspending in hypotonic buffer and adjusted to a final protein concentration of 4 mg/ml. The resuspended membranes were put on ice for 30 min and then passed through a 25 gauge needle to break up membrane fragments.

***In vitro* assay of PLD activity.** The 100 μg membrane protein and 150 μg cytosolic protein (if included) were combined by dilution with assay buffer (25 mM Hepes, pH = 7.5; 0.5 mM EDTA; 0.5 mM EGTA; 5 mM $MgCl_2$; 100 mM KCl; 10 mM NaCl; 0.16 mM $CaCl_2$; 1 mM DTT; 5 μg/ml leupeptin; 10 μg/ml aprotinin; 0.5 mM phenylmethylsulfonyl fluoride; 0.1 mM sodium vanidate; 1 mM ATP; 3 mM creatine phosphate; 4 U/ml creatine phosphate kinase) to 500 μl. The PLD activity assay is then initiated by addition of ethanol to a final concentration of 1.0% and 10 μM GTPγS. The reaction was allowed to preceed for 15 Min and terminated by the addition of organic solvent (Methanol : chloroform : $H_2O$ : acetic acid = 100 : 50 : 40 : 4). The lower organic phase were collected and subjected to TLC analysis. Genistein, if included, was added 4 hr prior to harvesting of cells at 100 μM and was maintained in the buffers at 5 μM. PLD activity was determined by the transphosphatidylation of labeled membranes or exogenous phospholipids to phosphatidylethanol (PEt).

For *in vitro* assays of PLD activity by using exogenous liposome substrates, the immobilized Ral, Ras or immunocomplexes prepared as described in "Immunoprecipitation" were added to liposomes containing [$^3$H]-PC and PLD activity assays were conducted as described above.

For determing the effects of Ras antibodies, Y13-259 and Y13-238 on PLD activity in membranes from v-Src-transformed and Balb/c 3T3 cells, Ras monoclonal antibodies Y13-259 (neutralizing) and Y13-238 (non-neutralizing) were examined by added in increasing amounts to the membrane-buffer reaction mixture. Where indicated, purified Ras protein (12 μg) was included to compete with Y13-259. The mixture were incubated with antibodies for 45 min at 22 °C

prior to addition of the 150 μg cytosol (if included), 10 μM GTPγS and 1% ethanol at 37 °C for 15 Min.

Preclearing cytosol with immobilized Ras and its effect on PLD activity was performed as following. Membranes from v-Src-transformed cells were prepared and PLD activity was determined in the increaseing amounts of cytosol. The cytosolic fractions (800 μg) were incubated with immobilized bovine serum albumin (BSA), Ras-GMP-PNP, or Ras-GDP (20-30 μG Ras protein). After a 1 hr incubation at 4 °C, the immobilized BSA and Ras proteins were spun out and the cytosolic fractions were added back to the membranes and PLD activity was determined in the presence of 10 μM GTPγS and 1% ethano at 37°C for 15 Min. Immobilized Ras and BSA were prepared as described in "Immunoprecipitation".

***In vivo* assay of PLD activity.** Cell were seeded at 35 mm dishes and grown to confluence for two days, and then replaced by fresh medium with 0.5% newborn calf serum for one day. The cells were prelabeled with [$^3$H]-Myristate (40Ci/mmol) for 3 μCi, and [$^3$H]-Arachidonate (240Ci/mmol) for 1.5 μCi, respectively, for 4-6 hours. PLD substrates, ethanol or butanol, were then added to the medium for the final concentration of 1%, and cells were incubated for 30 Min. The reaction were stopped by draining out medium and chilling tissue culture dishes onto ice followed by the addition of stopping solution (100:2 of methanol:6N HCl). The extraction of phospholipids and characterization of PLD product were described above. Equal amount of radioactivity (cpm) were loaded onto the TLC plate. The level of PEt (or PBt) was presented as a percent of the total cpm incorporated into phospholipids.

**Transfection and establishment of cell lines.** The coding region (~700 -base-pair) for wild type and vaious mutant forms of RalA genes (Q72L, S28N, D49N, and ΔN11) were subcloned into the BamHI site of the mammalian expression vector pZIPNeoSV(X). Expression of RalA and Neo genes were both under the control of the viral long terminal repeat. NIH 3T3 cells and NIH 3T3 cells transformed by v-Src were plated at a density of $1x10^5$ cells/35mm dish 16-18 hours prior to transfection. Transfections were performed by using lipofectamine reagent (Gibco) according to the vendor's instructions. Transfected cultures were split two days after transfection and selected in 400 μg/ml G418 with changing to fresh medium every other day. After 10-12 days selection, G418-resistant colonies were pooled and expanded for further analysis. For NIH 3T3 cells, individual colonies were picked up and expanded for further studies. The transfaction for each plasmid were done at least twice. Proteins expression levels were verified by western blot.

**Western blot and immunoprecipitation.** For western blot to detect protein expression, cells were lysed in lysis buffer (50 mM Tris-HCL, 1 mM EDTA, 100 mM NaCL, 10 mM NaF, 0.1 mM $Na_3VO_4$ and protease inhibitors, 10 mg/ml apoprotinin, 10 mg/ml leupeptin, 1 mM PMSF ) at 4 $^oC$ for 1 hour on the rocket platform. Total protein concentration was measured by the Bio-Rad assay kit. Protein samples were subjected to SDS PAGE electrophoresis and transfered to nitrocellular membrane. The western blot procedure was followed as described by Amersham (ECL). For immunoprecipitation, lysates were adjusted to a protein concentration of 0.8-1 μg/ml. Immobilized Ras or Ral proteins or antibodies were then incubated with lysates for at least 1 hour at 4 $^oC$. Proyein A- or G- agarose beads were added for antibody immunoprecipitation, and

incubated for an additional 1 hour. The agarose beads were recovered and washed with lysis buffer and PBS.

**Expression and purification of Ras and Ral proteins from bacterials** The procedures for purification of Ras and Ral proteins were followed the protocol from *Current Protocols In Molecular Biology*. Bacteria transformed by pGEX-Ras and pGEX-Ral, which expressing GST fusion proteins, were inoculated into 100 ml LB in the presence of 50 μg/ml Ampicilin for overnight incubation with agitation at 37°C. The culture was then diluted 10 times and incubated in the presence of 50 μg/ml Ampicilin for 3 hours. IPTG (100 mM) were then added to induce GST-fusion protein expression. After 4 hours induction, cells were harvested by centrifuge at 2000 rpm , the cell pellets were suspended in PBS supplymented with protease inhibitors (10μg/ml leupeptin, 10 μg/ml aprotinin,1 mM phenylmethylsulfonyl fluoride). The suspension were then subjected to thaw and freeze for four times, and further sonicated for 2 Min. The broken cells were further lysed by adding 1% Triton X-100. Centrifuge lysates and supernatants were incubated with GST-agarose. The agarose besds were washed three times with PBS and stored at 4 °C.

**Preloading GMP-PNP, GTPγS and GDPβS on Ras and Ral proteins** Reconbinant GTP-binding proteins were preloaded with GMP-PNP, GTPγS or with GDPβS by incubating with 40 mM HEPES (pH7.5), 4mM EDTA, 2mM DTT, and 1 mM GMP-PNP, GTPγS or GDPβS at 30 °C for 10-15 Min. The reactions were stopped by raising the concentration of free $Mg^{2+}$ to 10 mM, and free nucleotide was removed by microcon centrifugation (Amicon).

**Soft agar colony formation assay.** $2 \times 10^3$ cells were suspended in soft agar (0.3 %) and spreaded onto bottom agar (0.6 %) which included 20 % newborn calf serum and 2% antibiotics. 400 μg/ml G418 was also included. Colonies were grown for three weeks with adding fresh top agar every week. Colonies larger than 0.5 cm in diameter were then counted. Φ60mm plates with grid (Nunc) were used for performing experiment.

## PART VIII. REFERENCES


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