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# Phospholipid metabolism activated by v-Src

Song, Jian-guo, Ph.D.

City University of New York, 1993

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# PHOSPHOLIPID METABOLISM

# ACTIVATED BY v-Src

by

**Jian-guo Song**

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

1993

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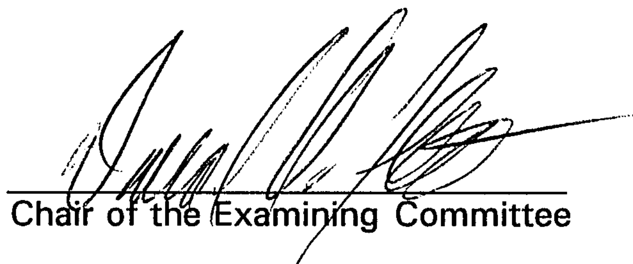

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## Abstract


# PHOSPHOLIPID METABOLISM ACTIVATED BY v-SRC

by

Jian-guo Song

**Adviser: Professor David A.Foster**

Activation of the protein-tyrosine kinase of v-Src in BALB/c 3T3 cells results in the rapid increase in diglycerides (DG), which function as intracellular second messengers. DG is the physiological activator of protein kinase C (PKC). By differential radiolabeling of phospholipids, the major source for the increased DG level in response to v-Src activation was identified as phosphatidylcholine (PC). Increased generation of intracellular choline and phosphatidic acid (PA) in response to v-Src was also observed. Consistent with the above data which suggest the involvement of a phospholipase D (PLD)-mediated PC hydrolysis, v-Src also increased intracellular transphosphatidylation activity, which is characteristic of PLD activity. Thus, the v-Src-induced increase in DG most likely results from the activation of a PLD which generates PA, followed by the hydrolysis of PA to DG by a PA phosphatase.

Phorbol esters that activate protein kinase C (PKC), markedly increase PLD activity in BALB/c 3T3 cells. v-Src- and phorbol ester-induced PLD activities were differentially sensitive to inhibitors of PKC and depleting cells of PKC. Neither depleting cells of PKC nor treatment with the PKC inhibitor staurosporine inhibited v-Src-induced PLD activity; whereas, both PKC-depletion and staurosporine treatment inhibited TPA-induced PLD activity. Additional differences between the increased PLD activity

in v-Src transformed cells and the PLD activity induced by phorbol esters were established using a differential radiolabeling strategy. These data demonstrate that v-Src activates a PLD activity that is independent of PKC. Thus, it is likely that the DG generated by the combined action of PC-PLD and PA phosphatase are responsible for the activation of PKC by v-Src.

Taken together, this data suggest that v-Src activates a distinct PLD activity that is specific for PC. The potential for the generating multiple intracellular signals through complete phospholipid metabolism is described.

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## List of Abbreviations

AA, Arachidonic Acid

Alkyl-lyso-PC, 1-O-Alkyl-lyso-phosphatidylcholine

cAMP, Cyclic Adenylate Monophosphate

DG, Diglyceride

DMEM, Dulbecco's Modified Eagle Medium

EGTA, Ethylenebis-(oxyethylenenitrilo)tetraacetic Acid

fMLP, Formylmethionyl-Leucyl-Phenylalanine

G Protein, GTP-binding protein

GAP, GTPase Activating Protein

GTPγS, Guanosine 5'-O-(thiotriphosphate)

$IP_3$, Inositol-triphosphate

MARCKS, Myristoylated-Alanine Rich C Kinase Substrate

MG, Monoglyceride

PA, Phosphatidic Acid

PC, Phosphatidylcholine

PBu, Phosphatidylbutanol

PE, Phosphatidylethanolamine

PEt, Phosphatidylethanol

PI, Phosphatidylinositol

$PIP_2$, Phosphatidylinositol-4,5-Bisphosphate

PKC, Protein Kinase C

PLA, Phospholipase A

PLC, Phospholipase C

PLD, Phospholipase D

PRO, DL-Propranolol

PTK, Protein Tyrosine Kinase

SRD, Schmidt Ruppin D Strain

TLC, Thin-Layer Chromatography

TPA, 12-O-Tetradecanoylphorbol 13-acetate

# Introduction

Cells regulate their growth, differentiation and other cellular functions through intracellular signals that are initiated and altered in response to extracellular signals. These extracellular signals include growth factors, chemical agonists or antagonists, ions, antibodies and antigens. Cell surface receptors convert extracellular signals into intracellular signals, which cause structural and functional changes in cells. Constitutive alterations of intracellular signaling mechanisms through genetic mutation have a profound impact on cellular structure, function and other properties. v-Src is an oncogenic form of c-Src. It has a constitutively active protein-tyrosine kinase (PTK) activity that can transform cells in vitro and induce tumor in animals (1, review). Cells transfected with the v-Src gene have altered properties in morphology, extracellular matrix, and a variety of other biochemical and molecular events including intracellular signal transduction. These transfected cells lose their contact inhibition and normal growth control and become growth factor independent. The PTK activity of v-Src or c-Src does not always induce cell division signals. In PC12 cells, v-Src induce cellular differentiation instead of cellular transformation (2). In terminally differentiated blood platelets, there is a high levels of c-Src PTK expression (3, 4). Therefore, cells transformed by v-Src, together with

cells transformed by other genetically altered protooncogenes, have been widely used as model systems for the study of the mechanism of oncogenesis, differentiation, and cell division.

PTKs phosphorylate many proteins on tyrosine and it is believed that PTK activities are closely implicated in cellular transmembrane signaling events (5, 6, reviews). Many cell surface receptors have intrinsic tyrosine kinase activity (7) through which extracellular signals are converted to intracellular signals. In the immune system, the T-cell receptor (TCR)-CD3 complexes ($\zeta$-chains) are associated with the PTK Fyn. The major histocompatability (MHC) recognition molecules CD4 and CD8 are physically associated with the PTK Lck. These PTKs connect the signals initiated by antigen and MHC recognition molecules to intracellular signaling pathways which lead to T-cell activation and proliferation (8, 9). One consequence of PTK activation is believed to be the initiation of kinase cascades in which multiple kinases are involved and complex sets of signals are generated leading to cellular response (8, 10).

Many cellular components are utilized in the transduction of intracellular signals. Lipid metabolites, carbohydrates, ions and proteins, all function to transduce intracellular signals. In less than a decade, a large body of evidence has been presented, implying the importance of some of these components. PKC is known to function as a critical

component in the transduction of many intracellular signals (11, 12). PKC has also been implicated in v-Src-induced intracellular signals (13, 14-19). Activation of PKC requires cofactors (12, 20) including phospholipids, diglyceride (DG), and $Ca^{2+}$. DG, which is generated from phospholipids, is an intracellular second messenger that leads to the activation of PKC (11).

It is believed that there are two major pathways in the generation of DG. The first pathway, involves the phospholipase C-$\gamma$ (PLC-$\gamma$) mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) that concomitantly generates the PKC activator DG and the $Ca^{2+}$ mobilizer inositol-triphosphate ($IP_3$). Although, considerable data has been accumulated over the last decade, demonstrating the significance of this pathway (21, 22), it is still not clear that increases in DG levels seen in response to a variety of stimuli can be accounted for by the hydrolysis of this relatively minor phospholipid source (23). While PLC-$\gamma$ can be activated by the PTK activities of epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) receptors, PLC-$\gamma$ is not activated by the PTK activity of the CSF-1 receptor. In addition, it has been shown that mutants of the receptors for EGF, PDGF and FGF that fail to activate PLC-$\gamma$, still induce mitogenesis (24-27). Thus, activating PLC-$\gamma$-mediated $PIP_2$ hydrolysis apparently is not essential for the cell division response to PTK activity.

In this regard, it has recently been shown that a pathway or pathways other than $PIP_2$ hydrolysis contributes to the DG increases upon cellular response to various stimuli (28-46; for review, see 47, 48). This novel pathway involves phosphatidylcholine (PC) hydrolysis that generates DG, either directly, through the action of a PLC or indirectly, through a PLD that generates PA, which is then converted to DG by a PA phosphatase. In the presence of alcohol, PLD catalyzes the transphosphatidylation reaction. In this reaction, alcohol molecules substitute for $H_2O$ and replace the original phospholipid head group, resulting in the generation of phosphatidylalcohol and the free phospholipid head group molecules. PLD catalyzed hydrolysis of different phospholipids including PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), with different specificities, has been reported (49-51). The transphosphatidylation reaction has proved highly valuable in demonstrating PLD activity.

Stimuli leading to the hydrolysis of PC and the subsequent production of DG include phorbol esters, growth factors, $\beta$-adrenergic receptors, $Ca^{2+}$, ionophores, various receptor agonists, and some biologically active peptides. Since PC is a major lipid in the phospholipid pool, it is possible that PC is a major source for sustained increases in DG levels (52).

The structural and biological differences between DG produced via

the $PIP_2$ pathway and the PC pathway are still not clear. DG produced from $PIP_2$ has been reported to be rapidly converted to PA by DG kinase, whereas DG produced from PC is a poor substrate for DG kinase (41, 42). Thus there may be significant functional differences between the DG produced from PC and phosphatidylinositol ($PIP_2$). These differences can only be due to the difference in the aliphatic groups attached to the glycerol backbone. 1-ether-linked glyceride is believed mainly to exist in choline containing lipids. The characteristic of fatty acid compositions of phospholipids has been exploited to study PC-derived DG and other lipids in response to a variety of stimuli (29, 51, 53-59). DG derived from ether-linked PC is able to activate PKC (53). In human neutrophils, PC containing arachidonic acid is predominantly of the SN-1 alkyl ether class of phospholipid and is the major endogenous source of arachidonic acid (29, 51). Since the arachidonic acid and its metabolites (prostaglandin E, leukotrienes) play a pivotal role in inflammatory processes, this evidence may implicate the significance of the hydrolysis of PC and PE, which generate intracellular signals and biologically active molecules, in mediating this immunological function. Agonist stimulated hydrolysis of 1-ether-linked PC by PLD has been reported (54, 56-58). Two studies on phospholipid metabolism and DG production stimulated by vasopressin, bradykinin, and phorbol esters provide evidence of DG production with different kinetics, and suggests a biphasic production

of DG without identifying the source(s) of DG from each phase (56, 57). The first phase is upstream from the second phase, and is required for the occurrence of the second phase (53, 54). Kinetic studies on the sources of DG stimulated by platelet-derived growth factor (PDGF) and phorbol esters in Swiss 3T3 fibroblasts gives further supporting evidence suggesting a pathway in which both PIP-PLC and PC-PLD are activated (60). Studies on K-Ras-transformed and non-transformed NIH-3T3 fibroblasts provide another piece of evidence indicating that both PIP-PLC and PC-PLD pathways are involved in intracellular signaling events (61). Since PIP-PLC, PC-PLD have different sensitivities to various factors, such as $\alpha$-thrombin, PDGF, vasopressin, bradykinin and PKC (56, 62-64), the PI-PLC and PC-PLD pathways may be subjected to differential regulation or controlling mechanisms. With interaction between two second messenger-producing pathways, cells could respond to different stimuli more efficiently.

Although PC-PLD-mediated PC breakdown and DG production has been studied primarily in granulocytes, neutrophils, and platelet systems, this pathway exists in a variety of cell types. PI-PLD activation has been reported (65-67), although the significance of this observation has yet to be established. In the PC-PLD pathway, increased PA production is the initial event in response to stimuli. PA is biologically active itself and is a specific activator of PI-4-Phosphate kinase (68).

PA itself is an important substrate for the regeneration of $PIP_2$ in the PI cycle. PLD also stimulates insulin release(69), suggesting a potential role for endogenous PLD activity in pancreatic islet function.

The regulation of PLD activity and PC metabolism is not well understood. PC metabolism has been reported to be both dependent upon PKC (17, 20, 26, 28-34, 43), and, independent of PKC (20, 32, 38-40). Overexpression of the $\beta$-isoform of PKC leads to the increased DG production via PC-PLC and PC-PLD mediated pathways (64). Recent in vitro investigations have shown that exogenous PKC activates PLD activity in a phosphorylation-independent manner (70). Results from in vitro studies demonstrated that the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) and non-hydrolyzable GTP analogs activate PLD in neutrophil membranes (71). Regulation of PLD-mediated PC hydrolysis by cyclic adenylate monophosphate (cAMP) in neutrophils has also been reported by two research groups (72, 73). In neutrophils, increasing the concentration of cAMP inhibited the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced DG, PA production and transphosphatidylation activity (72, 73). Muscarinic receptors, which trigger various signal transduction mechanisms including inhibition of adenylate cyclase through Gi$\alpha$ have been shown to be potent stimulants of PC hydrolysis pathway through which intracellular second messengers are generated. Interestingly, it is reported that PLC-

catalyzed hydrolysis of PC inhibits adenylate cyclase. This inhibition is via a mechanism in which a pertussis toxin-sensitive G protein and PKC are involved (30). The effect of $Ca^{2+}$ on PLD activity has also been reported (74, 75). The evidence presented in the literature suggest that agonist stimulated PLD activity is dependent on $Ca^{2+}$ levels. The $Ca^{2+}$ ionophores, A23187 and ionomycin stimulate PLD activities; in contrast, $Ca^{2+}$ depletion or [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA) which chelates $Ca^{2+}$ inhibits the PLD activities (74, 75).

Increased levels of DG have been observed in cells transformed by v-Src (14, 76); however, the source of the increased DG is unclear. The time course for increased inositol phosphate levels in v-Src-transformed cells did not correlate with the time course for increased DG (16, 77), suggesting the DG produced in response to v-Src is generated from a source other than phosphatidylinositol (PI) or phosphorylated derivatives of PI.

In this thesis, evidence is presented demonstrating the following observations:

1. Increases in DG observed in response to the activation of the oncogenic protein-tyrosine kinase v-Src are derived primarily from the hydrolysis of PC by a type D phospholipase and the subsequent hydrolysis of PA to DG by a PA phosphatase.

2. v-Src activates a PLD activity that is independent of PKC and is distinguishable from the PLD activity induced by phorbol esters, implying that the DG generated by the combined action of PC-PLD and PA phosphatase are responsible for the v-Src-induced activation of PKC.
3. v-Src induces MG production in a DG independent pathway, suggesting an involvement of a type A phospholipase (PLA) which may lead to the generation of lyso-PA in response to v-Src.

These data suggest that v-Src activates a distinct PLD which may lead to the generation of multiple intracellular signals through production of a variety of phospholipid metabolites.

# Materials and Methods

**Cells and cell culture conditions.** BALB/c 3T3 cells are obtained from *American Tissue Culture Collection*, Rous sarcoma virus (*Schmidt Ruppin D* strain) (SRD cells) or the temperature-sensitive LA90 strain of Rous sarcoma virus infected BALB/c 3T3 cells (provided by Joan Brugge) were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% bovine calf serum (*Hyclone*) (in some cases, with 10% new born calf serum) (*GIBCO*) until confluent. Mostly, the master plates (100-mm) of temperature sensitive LA90 cells were grown at $34^{o}C$, the experimental 60-mm plates of LA90 cells were maintained at $40^{o}C$ after splitting and seeding. Medium can be changed once depending on the status of the cells and the days of incubation. Confluent cells were incubated in DMEM with 0.5% bovine calf serum for one day before doing experiment.

**Materials.** [$^{3}$H]-myristate, [$^{3}$H]-palmitate , [$^{3}$H]-arachidonic acid , [$^{3}$H]-choline, [$^{3}$H]-inositol, [$^{3}$H]-glycerol, [$^{3}$H]-alkyl-lyso-PC and [$^{32}$P]-ATP were obtained from *New England Nuclear*. Choline, DG, MG, PA, PC, PI, propranolol, staurosporine and TPA were obtained from *Sigma*. Thin-layer chromatography (TLC) plates (Silica gel 60A) were obtained from *American Scientific Products*. Amprep™ anion exchange minicolumns were from *Amersham*. Phosphatidylethanol (PEt) and

phosphatidylbutanol (PBu) were obtained from *Avanti Polar Lipids*. DG mass assay kit was from *Lipidex, Inc*.

**Prelabeling of phospholipid.** Unless otherwise indicated, confluent cells (in 60-mm culture dishes) were prelabeled for 20 hours in 4 ml of media containing 0.5% bovine calf serum. Isotopes were added to the culture medium as follows: [$^3$H]-myristate, 5 $\mu$Ci (40 Ci/mmol); [$^3$H]-palmitate, 5 $\mu$Ci (60 Ci/mmol); [$^3$H]-arachidonate, 2 $\mu$Ci (240 Ci/mmol); [$^3$H]-inositol, 2 $\mu$Ci (15 Ci/mmol); [$^3$H]-choline, 2 $\mu$Ci (85 Ci/mmol); [$^3$H]-glycerol, 20 $\mu$Ci (40 Ci/mmol); and [$^3$H]-alkyl-lyso-PC, 2 $\mu$Ci (45 Ci/mmol).

**Extraction of phospholipase products. (i) Lipids.** Extraction of lipids was performed according to the procedures described by Billah et al. (78) with modifications. Prelabeled LA90 or BALB/c 3T3 cells were shifted from 40$^0$C to 34$^0$C for the indicated times. Cells were then washed with isotonic tris-glucose saline buffer and rapidly treated with 0.65 ml of methanol (MeOH):6N HCl (50:2). Lipids were extracted by the addition of 0.65 ml of $CHCl_3$. Phase separation was obtained by adding 200 $\mu$l of 1M NaCl. The organic phase was re-extracted with 0.65 ml of 0.3M NaCl and 0.2 ml of MeOH:6N HCl (50:1). All procedures described above were performed at 4$^0$C unless otherwise indicated. Lipids were dried under a $N_2$ stream and redissolved in $CHCl_3$:MeOH (95:5). **(ii) Choline and phosphorylcholine.** Cells were

washed and extracted as described above. $H_2O$ (200 $\mu$l), instead of 1M NaCl, was added, the aqueous phase was recovered and dried by *Speed-Vac* concentrator. The lipids was redissolved in 50% MeOH.

**Characterization of phospholipid metabolites by TLC.** Extracts of phospholipid metabolites were characterized by TLC as described by Billah et al. (78), with modifications. About 5$\mu$g of lipid standards were chromatographed along with the sample lipids and were visualized by treating TLC plates with iodine vapor in a glass tank with iodine crystals for few minutes. To quantitate the metabolically labeled compound, the appropriate regions of TLC plates determined by autoradiogram were scraped and the radioactivity was counted by liquid scintillation. The following solvent systems were used to separate lipids: for DG, n-hexane:diethyl ether:MeOH:acetic acid (90:20:3:2) (v:v:v:v) (solvent system I); for PC and PI, $CHCl_3$:MeOH:$CH_3COOH$:$H_2O$ (50:25:8:4) (v:v:v:v) (solvent system II); for PA, PEt and PBu, the upper phase of ethylacetate:trimethylpentane:$CH_3COOH$:$H_2O$ (9:5:2:10) (v:v:v:v) (solvent system III); for choline and phosphorylcholine, 0.6% NaCl:MeOH:$NH_3$ (50:50:1) (v:v:v) (solvent system IV). The following procedures were used to separate PA, PEt or PBu, MG, and DG on the same plate. Plates were chromatographed by the solvent system III, half way, two times with intermittent drying, for fully separating PA, PEt or PBu on the bottom half of the plates. The plates were then further

chromatographed by the solvent system I to separate DG and MG on the top half of the plates.

**DG mass assay.** Lipid extracts prepared as described above were labeled with bacterial DG kinase using [$\gamma$-$^{32}$P]-ATP, extracted and resolved as described by the vendor's instruction which is based on the description of Preiss et al. (79). The following solutions are included in the vendor's DG mass assay kit. Solution A, sn-1,2-dioleoylglycerol (DAG); solution B, $\beta$-octylglucoside (detergent) + 1,2-dioleoyl-sn-3-phosphatidylglycerol (activator) ($\beta$-OG+PG); solution c, assay buffer; solution D, DG kinase; solution E, adenosine triphosphate (ATP). **(i). DG kinase assay.** To each assay tube, add 10 $\mu$l of solution D, 20 $\mu$l of solution B, 60 $\mu$l of solution C, 8 $\mu$l of solution E (ATP, to final concentration of 1.0 mM) and 1.2 $\mu$l of $H_2O$. 0.8 $\mu$l of [$^{32}$P]-ATP (~10 mCi/ml) was added after incubating at room temperature for 30 minutes. The above mixtured solution were then added to tubes containing sample lipids or standard DAG (0 to 200 pmol/tube). **(ii). Re-extraction of radiolabeled lipids.** Add 3 ml of MeOH:$CHCl_3$ (2:1, v:v) to stop the reaction. Add 0.7 ml 1% HCl and votex. Add 1ml of $CHCl_3$ and 1ml 1% HCl. Spin at top speed for 2-5 min in table-top centrifuge after votex. Aspirate off upper layer. Re-extract twice the organic phase with 2 ml 1% HCl. Remove half (1 ml) of the organic phase and count by autoradio-liquid scintillation. **(iii). Characterization of [$^{32}$P]-**

**labeled lipids.** The other half of the lipids were dried and separated by TLC as described above. PA was separated by using the solvent system of $CHCl_3$:acetone:MeOH:acetic acid:$H_2O$ (10:4:3:2:1, v:v:v:v:v). Plot the standard curve (cpm versus pmol DG). The mass of DG was determined from the standard curve.

**Inositol phosphate analysis.** Amprep™ anion exchange minicolumns were used to separate phosphatidylinositol and Inositol phosphates. Experiments were performed as described by Paris and Pouysseuer (80).

**Transphosphatidylation assay.** Cells were cultured and labeled as described above. Usually, cells were labeled with 5 $\mu$ci $^3$H-myristic Acid or 5 $\mu$ci $^3$H-palmitic acid for overnight (or for 5 hours in some cases). Ethanol (to final concentration of 1%, unless otherwise indicated) or butanol (to final concentration of 0.4%) was added to the cell culture plates 10 minutes before doing temperature shift or adding other compound(s). Lipids were then extracted and characterized as described above.

# Results

**Activation of kinase activity of v-Src leads to increased levels of DG.** Previous studies demonstrated that v-Src activates PKC in both avian and murine fibroblasts (17, 19). Since PKC is activated by DG, increases in DG levels in response to the protein-tyrosine kinase of v-Src were examined. The effect of increased v-Src kinase activity was examined using BALB/c 3T3 cells transfected with a temperature-sensitive strain of Rous sarcoma virus [LA90 cells, described by Gray and Macara (81), and Qureshi et al. (19)]. Phospholipids of LA90 cells and the parental BALB/c 3T3 cells were metabolically prelabeled with [$^3$H]-glycerol for 24 hours. Quiescent LA90 cells maintained at the non-permissive temperature for v-Src (40°C) were shifted to the permissive temperature (35°C) to activate the protein-tyrosine kinase activity of v-Src, and levels of [$^3$H]-labeled DG were examined by TLC. As shown in Fig.1a, [$^3$H]-glycerol-labeled DG levels rapidly increased in LA90 cells after temperature shift. This treatment had no effect on [$^3$H]-labeled DG levels in the parental BALB/c 3T3 cell line (Fig.1a). To establish that the increase in [$^3$H]-labeled DG reflected real increases in DG levels and not simply increases in [$^3$H]-glycerol incorporated into DG in response to v-Src, we looked at DG levels using a DG mass assay (79). As shown in Fig.1b, temperature shift of LA90 cells led to an increase in DG as

detected by phosphorylation of DG to PA by bacterial DG kinase. This effect was not seen in BALB/c 3T3 cells similarly treated. Our data (unpublished) also shows that the total incorporated $^{3}$H-lipids in cells does not change in response to v-Src, whereas, $^{3}$H-DG presented as percentage of total $^{3}$H-lipids isolated from organic phase increased in response to v-Src, indicating that the increases in DG are unlikely the consequence of increased incorporation of $^{3}$H-isotopes into DG. The rapid increase in DG level suggests an early signaling event activated by v-Src. The time course for the generation of DG was consistent with the time course for v-Src-induced PKC activity observed previously (17, 19).

**Differential labeling of phospholipids identifies the source of the v-Src-induced generation of DG.** Although increased levels of DG in response to v-Src have been reported (16), there have been conflicting reports on concomitant increases of inositol phosphates. Chiarugi et al. (82) reported a rapid increase in inositol phosphates in response to v-Src in rat fibroblast; on the other hand, increased levels of inositol phosphates were not detected by Martins et al. (76) in chicken embryo fibroblast or by Han et al. (83) in LA90-transformed BALB/c 3T3 cells upon activation of v-Src. In our experiments, we did not find reproducible changes in inositol phosphate levels in response to v-Src in either avian or murine fibroblast over the time period where increased

levels of DG were observed (data not shown). Thus, it is unlikely that the increases in DG observed in response to v-Src are derived from PI or PI phosphorylated derivatives of PI.

Phospholipids have characteristic fatty acid compositions (84, 85) that permit differential labeling with different radioactively-labeled fatty acids (31-37, 84-86). For example, PI is preferentially labeled with arachidonate, which is the predominant fatty acid esterified to the SN-2 position of the glycerol backbone of PI (87, 88). In contrast, myristate is predominantly incorporated into PC (35, 84). To determine the source of DG produced in response to v-Src, we exploited the differential fatty acid composition of phospholipids. In Table 1, the percentage of [$^3$H]-labeled myristate, arachidonate, palmitate, and glycerol incorporated into PC and PI in LA90 cells grown at 40°C is shown. Myristate is incorporated predominantly into PC with only about 2% of myristate being incorporated into PI. Arachidonate is incorporated equally into both PC and PI; however, since PC is far more abundant (approximately 35% of the total phospholipid) than PI (approximately 3.5% of the total phospholipid) (23), the equivalent incorporation of arachidonate into PC and PI indicates that arachidonate is incorporated with high efficiency into PI. Glycerol and palmitate are incorporated into PC and PI with efficiencies reflecting the relative abundance of PI and PC.

The differential efficiency of labeling phospholipids with fatty acid

precursors (as shown in Table 1) was used to identify the source of v-Src-induced increases in DG. When phospholipids were labeled with [$^3$H]-arachidonate, no increase in labeled DG was detected in response to v-Src (Fig.2). However, when either [$^3$H]-myristate or [$^3$H]-palmitate was used to prelabel phospholipids, increased DG levels in response to v-Src were observed (Fig.2). Since [$^3$H]-myristate and [$^3$H]-palmitate are predominantly incorporated into PC (35, 84), the data suggest that PC is the source of v-Src-induced DG production. The lack of increased DG in [$^3$H]-arachidonate-labeled cells suggests that the source of v-Src-induced DG is not PI.

**v-Src induces production of choline with kinetics similar to those observed for DG production.** DG can be generated by either type C or type D phospholipase. If v-Src-induced DG is derived from PC, then phosphorylcholine would be produced if a type C phospholipase is activated, and choline would be produced if a type D phospholipase was activated. To observe changes in the level of choline metabolites, LA90 cells were metabolically labeled with [$^3$H]-choline and levels of intracellular choline and phosphorylcholine were measured by TLC after shifting from the non-permissive to the permissive temperature. As shown in Fig.3, increased levels of choline were detected in response to v-Src. Importantly, the time course of the increase was similar to that observed for DG production. There was no change in phosphorylcholine

levels in response to v-Src (Fig.3). Although these data could also be explained by the activation of a phosphorylcholine phosphatase, to our knowledge no such activity has been reported in mammalian cells. These data provide additional evidence that PC is the source of the v-Src-induced increases in DG levels. Furthermore, since choline rather than phosphorylcholine was increased in response to v-Src, the data suggest that hydrolysis of PC results from the action of a type D phospholipase.

**v-Src-induced increases in PA levels are detectable in the presence of PA phosphatase inhibitors.** If, as suggested above, a type D phospholipase is activated by v-Src, then both choline and PA should be generated. However, consistent increases in the level of PA were not detected in response to v-Src, possibly due to the rapid metabolic conversion of PA to DG by a PA phosphatase. We therefore examined the effect of propranolol, which has been shown to inhibit PA phosphatase activity (78, 89), on v-Src-induced DG, PA, and choline. Propranolol treatment of LA90 cells prior to activating v-Src by temperature shift inhibited v-Src-induced increases in DG (Fig.4a). Concomitant with the loss of DG production was the detection of an increase in PA levels in response to activating v-Src (Fig.4b). Propranolol treatment had little effect on choline production induced by v-Src (Fig.4c). Although the brief treatment with propranolol may

induce effects other than inhibiting PA phosphatase activity, the data demonstrate that v-Src is able to induce the production of both PA and choline, the expected products of a type D phospholipase-mediated hydrolysis of PC.

**v-Src induces transphosphatidylation activity.** Type D phospholipases catalyze the transphosphatidylation of PC to PEt or PBu in the presence of ethanol or butanol (90, 91). This assay has been used to distinguish between type C and type D phospholipase (35). Activation of v-Src by temperature shift of LA90 cells in the presence of ethanol or butanol led to substantial increases in PEt and PBu (Fig.5a). No change in PEt was seen in BALB/c 3T3 cells upon temperature shift (Fig.5b). These data demonstrate that v-Src induces type D phospholipase activity and further suggest that v-Src-induced increases in DG result from type D phospholipase-mediated hydrolysis of PC.

In BALB/c 3T3 cells transformed with the SRD strain of Rous sarcoma virus (SRD cells), the addition of exogenous EtOH leads to a substantial increase in PEt (fig.6a); whereas, the increases in PEt observed in the parental BALB/c 3T3 cells in the presence of 1.5% EtOH is much smaller. Addition of 100 ng /ml TPA in BALB/c 3T3 cells induce significant increases in PEt production (fig.6b). These data suggest that the protein-tyrosine kinase activity of v-Src leads to a constitutive induction of PLD activity.

To determine whether the protein-tyrosine kinase activity of v-Src was responsible for the constitutive PLD activity in SRD cells, the cells were treated with increasing concentrations of herbimycin A. Herbimycin A is benzoquinoid ansamycin antibiotic that has been reported to reverse oncogenic transformation induced by v-Src by inhibiting both tyrosine phosphorylation and increasing v-Src turnover (92-94). We previously found that treating SRD cells with 0.5 $\mu$g/ml herbimycin A led to a reduction in v-Src protein and in vitro v-Src kinase activity that was detected within 4 and 6 hours (95). v-Src-transformed BALB/c 3T3 cells were treated with herbimycin A for six hours. In Fig. 6c, it is shown that with increased herbimycin A, transphosphatidylation could be reduced by as much as 60%. Herbimycin A had no effect on transphosphatidylation induced by TPA (fig.6c). These data suggest that the constitutive PLD activity in v-Src-transformed BALB/c 3T3 cells is a direct consequence of the protein-tyrosine kinase activity of v-Src. These data also distinguish v-Src-induced PLD activity from TPA-induced PLD activity in BALB/c 3T3 cells.

**v-Src-induced PLD activity can be distinguished from phorbol ester-induced PLD activity by a differential substrate utilization.** We demonstrated that v-Src-induced DG production was not detected in cells prelabeled with [$^3$H]-arachidonic acid, (Fig.2). v-Src-induced DG was found to be derived from PC via a PLD/PA phosphatase mechanism

as shown above. Although [$^3$H]-arachidonic acid is incorporated preferentially into PIs (55, 87, 88), [$^3$H]-arachidonic acid is also incorporated into the PC of murine fibroblast (Table 2). We therefore examined whether transphosphatidylation could be detected in SRD cells prelabeled cells with [$^3$H]-arachidonic acid. PEt levels were examined in the presence of 1.5% EtOH in both SRD cells and BALB/c 3T3 cells that had been treated with TPA. As shown in Fig. 7a, transphosphatidylation was not detected in v-Src-transformed cells prelabeled with [$^3$H]-arachidonic acid. In contrast, TPA-induced transphosphatidylation was readily detected in both BALB/c 3T3 and SRD cells prelabeled with [$^3$H]-arachidonic acid (Fig.7a). The inability to detect transphosphatidylation in SRD cells prelabeled with [$^3$H]-arachidonic was not due to a reduced incorporation of arachidonic acid into the phospholipids of these cells. As shown in Table 2, [$^3$H]-arachidonic acid was incorporated efficiently into the PC of both BALB/c 3T3 and SRD cells. In addition, the induction of PEt production by TPA in SRD cells was easily detected when [$^3$H]-arachidonic acid was used to prelabel phospholipids (fig.7a). Transphosphatidylation of phospholipids to PEt was readily detected in v-Src-transformed cells when either [$^3$H]-myristic acid (Fig. 7b) or [$^3$H]-palmitic acid (Fig.7c) were used to prelabel the cells. TPA also induced transphosphatidylation of phospholipids that had incorporated [$^3$H]-myristic acid and [$^3$H]-palmitic acid (Figs.7b and 7c). We next examined

the ability of v-Src and TPA to induce transphosphatidylation of phospholipids with ether linkages in the SN-1 position of PC. Ether linkages are common in the SN-1 position of PC and can account for as much as 70% of PC in some cells (51, 59). BALB/c 3T3 and SRD cells were prelabeled with [$^3$H]-alkyl-lyso-PC. As shown in Fig.7d, no PEt was detected in the v-Src-transformed SRD cells; whereas, PEt was detected in both BALB/c 3T3 and SRD cells treated with TPA. These observation can not be explained by differential incorporation of the labeled phospholipid precursors into SRD cells and BALB/c 3T3 cells since both cell lines incorporate the labeled precursors into phospholipids with approximately the same efficiency (Table 2). Thus, the data in Fig. 7 suggest that the PLD activated by v-Src has a more selective substrate range than the PLD(s) activated by TPA. The data also suggest that the substrate utilized by the PLD activated by v-Src does not contain arachidonic acid or SN-1 ether linkages.

**v-Src- and TPA-activated PLD activity are differentially sensitive to depleting cells of PKC and the protein kinase inhibitor staurosporine.** Since the data presented above suggested that the PLD activated by v-Src may be different from that activated by TPA, we examined whether v-Src could activate PLD activity in cells that had been depleted of PKC. Prolonged treatment (20 to 48 hours) with the phorbol ester TPA (100 ng/ml) leads to the down regulation PKC and this treatment has been

used extensively to demonstrate PKC requirements in a variety of systems (11, 5, 17, 96). BALB/c 3T3 and SRD cells were depleted of PKC as described previously (97). In Fig.8a, it is shown that the EtOH-dependent transphosphatidylation activity in v-Src-transformed cells was not inhibited by depleting cells of PKC activity, whereas TPA-induced transphosphatidylation in BALB/c 3T3 cells was sensitive to PKC depletion. In fact, there was a reproducible, increase in transphosphatidylation in v-Src-transformed cells that had been depleted of PKC (Fig.8a).

We next examined the effect of the protein kinase inhibitor staurosporine on v-Src- and TPA-induced transphosphatidylation. Staurosporine has been reported to inhibit PKC in vivo (98) and we have found that at 500 nM, staurosporine blocks TPA-induced phosphorylation of the PKC substrate (C. Joseph and D.A. Foster, unpublished data). As shown in Fig.8b, staurosporine, like PKC depletion, had some stimulatory effect upon transphosphatidylation in v-Src-transformed cells, while inhibiting TPA-induced transphosphatidylation.

To establish that the lack of inhibitory effect on v-Src-induced PLD activity to staurosporine and PKC-depletion was not simply a reflection of a difference in the different cell lines used, we examined the effect of PKC depletion and staurosporine on v-Src-induced PLD activity in

LA90 cells (99). As shown in Table 3, activating the kinase activity of v-Src induced increased PEt levels within 30 min as described previously (55). The v-Src-induced increase in PEt levels was insensitive to both PKC depletion and staurosporine. If the LA90 cells are labeled with [$^3$H]-arachidonic acid, v-Src-induced increases in PEt were not observed. However, TPA-induced increases in PEt in LA90 cells can be detected and these increases were sensitive to PKC depletion (Table 3). The data in Figs.8, 3b and Table 3 suggest that the increased PLD activity in v-Src-transformed cells is independent of PKC. This further distinguishes the PLD activity induced by v-Src from that induced by phorbol esters that activate PKC and strongly suggests that the activation of PLD activity by v-Src is independent of PKC.

**v-Src-induced increases in DGs are independent of PKC.** We previously demonstrated that DG produced in response to v-Src is derived from PC via a PLD/PA phosphatase-mediated mechanism rather than the better characterized PLC-mediated hydrolysis of PI-4,5-bis-phosphate (55). The primary metabolite of PLD activity is PA, which can be converted to compounds other than DG, such as lyso-PA via a $PLA_2$. Therefore, it was possible that the PA produced from the PKC-independent PLD activity observed in Fig.8. was not metabolized to the DG produced in response to v-Src. We therefore examined whether v-Src-induced increases in DG were also insensitive to PKC depletion and

staurosporine. To examine v-Src-induced DG levels, we used LA90 cells as described above. As shown in table 4, v-Src-induced increases in DG were not significantly affected by either PKC depletion or staurosporine. In contrast, TPA induced increases in DG were inhibited by these treatments (table 4). Thus, it is likely that the PKC-independent PLD activity detected in v-Src-transformed cells generates PA that is then further metabolized to generate the increases in DG seen in response to v-Src.

**v-Src induces an increase in MG levels.** Activating the protein-tyrosine kinase activity of v-Src also leads to rapid increases in the level of MG. The kinetics for the increased generation of MG is similar to that of the DG (fig.9). MG is a primary metabolite of both DG and lyso-phosphatidic acid (lyso-PA) through the action of a DG lipase and a lyso-PA phosphatase respectively. Increased DG observed in response to v-Src is produced by the combined action of PLD and PA phosphatase. The conversion of PA to DG is blocked by propranolol and results in the accumulation of PA at the expense of DG levels as described above. In contrast, propranolol treatment enhanced the effect of v-Src on MG levels (fig.10a), suggesting that MG was generated from PA by a mechanism that was independent of DG production. Treatment of Balb/c 3T3 cells with propranolol does not produce the same effect (fig.10b). This may imply that v-Src-induced PA is converted to lyso-PA

which is a $Ca^{2+}$ mobilizer and is mitogenic (100, 101) and then metabolized to MG by a lyso-PA phosphatase. Therefore, we tried to separate lyso-PA to see if v-Src activation leads to an increase in lyso-PA levels. However, we have not been able to get a clear cut result because of some technical difficulties.

# Discussion

We have shown that v-Src-induced-increases in DG derive from a cellular lipid that is preferentially labeled with myristate. Myristate is incorporated almost exclusively into PC, marking PC as the source of v-Src-induced DG. Consistent with this conclusion, we found that free choline levels were elevated in response to the kinase activity of v-Src. The generation of choline rather than phosphorylcholine suggests a type D phospholipase/PA phosphatase mechanism for generating DG. Consistent with this hypothesis, we found that blocking PA phosphatase with propranolol inhibited v-Src-induced increases in DG levels and led to concomitant v-Src-induced increases in PA. Moreover, activating the protein-tyrosine kinase activity of v-Src increased transphosphatidylation activity which is catalyzed by PLD. Finally, the protein tyrosine kinase activity of v-Src leads to a constitutive induction of PLD activity. PLD activated by v-Src and by phorbol esters can be distinguished by using different phospholipid precursors to prelabel cellular phospholipids. The PLD activated by v-Src was unable to catalyze the detectable transphospatidylation of cellular phospholipids prelabeled with either arachidonic acid or alkyl-lyso-PC; whereas TPA was able to induce the transphosphatidylation of phospholipids prelabeled with both of these compounds. Thus, the PLD activated by v-Src likely has a more

restricted substrate range than the PLD(s) activated by phorbol esters with regard to the aliphatic composition of the phospholipid substrates. We also demonstrated that v-Src-induced PLD activity was not inhibited but enhanced by, staurosporine and depleting cells of PKC.

Additionally, increased MG levels in response to v-Src were observed. v-Src-induced MG was generated from PA by a mechanism independent of DG production, suggesting involvement of lyso-PA generation via a $PLA_2$-mediated hydrolysis of PA. These data are consistent with the hypothesis that v-Src-induced increases in DG derive from PC via a type D phospholipase and PA phosphatase; v-Src activates a PLD that is specific for a subpopulation of PC lacking both arachidonic acid and ether linkages.

Increased levels of inositol phosphates in transformed rat cells have been reported (77, 82). Although increased levels of inositol phosphates in response to v-Src were not detected in LA90 cells concomitant with the production of DG, it is still possible that low levels of DGs could be derived from PI. However, if some DG is generated from PI, the contribution to total DG produced in response to v-Src is small relative to that generated from PC since the DG we observe in response to v-Src is derived primarily from a phospholipid into which myristate is efficiently incorporated. Myristate is not incorporated into PI (see Table 1). In addition, v-Src-induced DG was not detected when

cells were labeled with a fatty acid (arachidonate) which is preferentially incorporated into PI (see Fig.2).

The transphosphatidylation activity induced by v-Src suggests that a PC-specific type D phospholipase is the signaling component that is activated by v-Src; however, PA phosphatase could also be activated by v-Src. The mechanism by which v-Src induces a type D phospholipase/PA phosphatase mechanism for increasing DG levels is not yet clear. Data from our laboratory suggest that a G-protein is required for v-Src to activate PKC and PLD (102, 103). Thus, v-Src-induced PC hydrolysis may be indirect and require the activation of a G-protein which activates a PC-specific type D phospholipase to generate the DG that in turn activates PKC.

To generate DG via a type D phospholipase requires a second enzyme (a PA phosphatase) to convert PA to DG. This raises a question as to what advantage there is in using an indirect pathway to increase DG levels when DG levels can be increased directly using type C phospholipase. Other protein-tyrosine kinases have been demonstrated to be associated with phospholipase C-$\gamma$, which hydrolyses PI-4,5-bis-phosphate (104, 105). The induction of a type D phospholipase instead of a type C phospholipase by v-Src to increase DG levels may generate a more complex or specific biological signal. In this regard, the generation of phosphatidic acid may be important. PA has been

reported to induce protein phosphorylation (106). It has also been suggested that PA is a potential mitogenic signal (107). Recent evidence indicates that PA is a specific activator of phosphatidylinositol-4-phosphate kinase, suggesting that v-Src-induced signals may be enhanced by influencing the PI metabolism. PA or a metabolite of PA inhibits the H-Ras GTPase activating protein, GAP (108, 109). Thus, the production of PA might enhance v-Src signals going through H-Ras, which has previously been implicated in v-Src signaling (102, 103 110, 120). Another rationale for generating PA would be to provide the metabolic precursor for the mitogenic phospholipid lyso-PA, which is the product of type $A_2$ phospholipase-mediated hydrolysis of PA (101). Consistent with the possibility that lyso-PA may be generated from v-Src-induced PA, increases in the levels of MG, the primary metabolite of lyso-PA, in response to v-Src have been detected (Fig.9, 10). This increase was not blocked in the presence of propranolol which prevented DG production in response to v-Src, suggesting that the source of MG was not DG. This observation implicates lyso-PA production in response to v-Src. Thus, an indirect pathway for increasing DG levels may be important for the induction of more complex biological signals which could include the positive stimulation of H-Ras-mediated signals and the production of the mitogenic phospholipid lyso-PA and create the cross-talk between the PC pathway

and the PI pathway in signaling events.

Since v-Src activates intracellular signals that are dependent upon PKC (17-19), we suggested that the DG produced by this mechanism might be responsible for the activation of PKC (55). The kinetics of v-Src-induced DG production correlates with v-Src-induced phosphorylation of the PKC substrate MARCKS (17, 19). The DG produced in response to v-Src increases gradually to a maximum level approximately 30 min after activating the kinase activity of v-Src (55). This gradual increase is slower and longer lived than that reported for the DG produced from PLC-mediated hydrolysis of PIs (45, 111-113). DG produced in response to a variety of stimuli can vary considerably in their aliphatic components (84, 85, 114). It has also been shown that different classes of DG, based on aliphatic composition, are differentially metabolized by DG kinase to PA (115). DGs produced from PC have been reported to be poor substrates for DG kinase relative to the DG produced from PI (111). Thus, it may be important that the DG produced in response to v-Src is derived from PC. It may also be important in this regard that v-Src-induced increases in both DG and PEt (see Fig.7a) were not observed when cells are prelabeled with arachidonic acid. Although arachidonic acid is incorporated preferentially into PI (87, 88), we demonstrated that arachidonic acid is also incorporated into the PC of these cells (Table 2). In human

neutrophils, PC containing arachidonic acid is predominantly of the SN-1 alkyl ether class of phospholipid (29). Thus, the observation that PEt production was not observed in SRD cells when alkyl-lyso-PC was used to prelabel cellular PC, may be related to the lack of observable PEt production when cells were prelabeled with arachidonic acid. The activation by v-Src of a PLD that is specific for PC lacking arachidonic acid and ether linkages may be important for producing DGs that are poor substrate for DG kinase or other enzymes that metabolizes DG, and thus lead to a more prolonged DG signal. In this regard, it may be important that, although TPA induced higher levels of transphosphatidylation than v-Src (see Fig.7b and 7c), v-Src induced higher levels of DG than TPA (data not shown). Thus, the PA produced in response to TPA is likely metabolized differently than the PA generated in response to v-Src, which is rapidly hydrolysed to DG (Fig.4). This further implicates the aliphatic composition of phospholipid as being biologically important since without the head group, the only structural differences are the aliphatic components.

TPA was able to induce PLD activity when alkyl-lyso-PC was used to prelabel cells. Therefore, TPA is capable of activating a PLD that recognizes PC. Since v-Src activates a PLD activity that is specific for PC, but does not recognize the alkyl-lyso-PC, the data presented here suggest that there are PLDs with substrate specificities for PC with

different aliphatic composition. The TPA-activated PLD recognizes PC containing ether linkages in the SN-1 position and possibly arachidonic acid-containing PC, whereas v-Src-activated PLD does not recognize PC containing arachidonic acid or ether linkages, but does recognize PC containing myristate and palmitate. The extent to which the specificity of PLD for the aliphatic components of PC exists remains to be determined; however, the data presented here demonstrate that there are PLDs that distinguish between PCs on the basis of their aliphatic makeup.

PKC has been implicated in the transduction of intracellular signals activated by v-Src (14-19). If v-Src activates PKC, it might be predicted that v-Src would activate the PLD(s) that are activated by PKC in response to phorbol esters and that the transphosphatidylation products produced in response to TPA should also be produced in response to v-Src. This was clearly not the case since TPA produced a greater variety of transphosphatidylation products than v-Src (see Fig.7). Thus, TPA is likely activating additional PLD activity not activated by v-Src. Phorbol esters have been demonstrated to activate a PLD that hydrolyzes PI (54, 55), which preferentially incorporates labeled arachidonate (55, 91, 92). Thus, TPA might be activating a PLD that is specific for PI that are efficiently labeled with arachidonic acid. The lack of induction of this TPA-induced PLD by v-Src might be the result of additional signals,

activated by v-Src, that down regulate the PLD(s) activated by direct activation of PKC(s) with phorbol esters. In this regard, it may be important that v-Src also activates PKC-independent as well as PKC-dependent intracellular signals (19). The PKC-independent signals activated by v-Src could modify or restrict the PLD activity that is activated in response to PKC. It is also possible that, since the DG produced in response to v-Src differs from the DG produced in response to phorbol esters that activate PKC, the subspecies of DG produced in response to v-Src do not induce the same effects that phorbol esters induce. Kolesnick and Paley (116) previously demonstrated that phorbol esters and DG can induce differential effects. Molecular details of this apparently complex intracellular signaling phenomena remain to be determined.

The mechanism by which v-Src induces a PLD/PA phosphatase mechanism for increasing DG levels is not yet clear. We recently demonstrated that a G-protein is required for the tyrosine kinase v-Fps to activate PKC (102). We have evidence suggesting that v-Src also requires a G-protein to activate PKC (102, 103). G-proteins have been implicated previously in the activation of PLD activity (71, 117-119). Thus, the activation of a PLD activity by v-Src may be indirect and require the activation of a G-protein which activates a PC-specific PLD to generate PA, which is then hydrolyzed by a PA phosphatase to DG,

that in turn, activates PKC. Moreover, the tyrosine phosphorylation of G Protein $\alpha$ subunits by c-Src has been reported recently, suggesting the possibility that The G-protein required for the v-Src-induced activation of PLD may be a direct substrate of v-Src (120).

The data presented in this thesis is summarized as the following:

1. v-Src increases DG production via a pathway other than PI-PLC.
2. v-Src also increases choline production and activates transphosphatidylation that is the characteristic of PLD.
3. Increased PA production in response to v-Src are detectable in the presence of the PA phosphatase inhibitor propranolol.
4. v-Src-activated PLD activity is independent of PKC and is distinguishable from the PLD activity activated by phorbol esters.
5. Increased MG levels in response to v-Src is occurs via a mechanism independent of DG production activated by v-Src.

All this data demonstrates that v-Src increases DG levels primarily through a distinct type D phospholipase-mediated hydrolysis of PC. Since PLD activity and DG production in response to v-Src is via a mechanism which is upstream of PKC, it is thus most likely that DG induced by v-Src is responsible for the activation of PKC by v-Src. In addition to providing sustained increase in DG, the PC-PLD/PA phosphatase pathway activated by v-Src also produce a variety of phospholipid metabolites which may lead to the generation of multiple

intracellular signals including DG, PA, PI, arachidonic acid and lyso-PA, and diversify the intracellular response to the protein tyrosine kinase activity of v-Src.

**Table 1**

Differential labelling of phospholipids in LA90 cells

| Label | Label incorporated (% cpm ± SEM) into: PC | PI |
|---|---|---|
| [$^3$H]-myristate | 57.5(+/-0.7) | 2.3(+/-0.4) |
| [$^3$H]-arachidonate | 17.0(+/-4.2) | 19.8(+/-1.9) |
| [$^3$H]-palmitate | 51.5(+/-3.5) | 6.2(+/-0.1) |
| [$^3$H]-glycerol | 52.0(+/-5.6) | 7.0(+/-5.0) |

LA90 cells grown at the non-permissive temperature were prelabeled with the indicated phospholipid precursor. The percentage of label incorporated into PI and PC was calculated by determining the radioactivity incorporated into either PC or PI and dividing this value by the total cpm present in the organic phase. Total cpm were determined prior to TLC and the cpm for PC and PI were determined after TLC. After lipid drying, chromatography and lipid spots scrapping, there were some loss of the lipid samples. Therefore, the absolute percentages for each specific lipid are higher than the values in the table, but the relative values represent real differences in the efficiency of incorporation of the phospholipid precursors into PI and PC. The data above is the average of two separate experiments.

**Table 2**

Relative incorporation of phospholipid precursors into cellular phospholipids of BALB/c 3T3 and SRD-transformed BALB/c 3T3 cells

Percentage of total $^{3}$H-lipids incorporation

| | BALB/c 3T3 | | | SRD Cells | | |
|---|---|---|---|---|---|---|
| | $^{3}$H-AA | $^{3}$H-MA | $^{3}$H-PA | $^{3}$H-AA | $^{3}$H-MA | $^{3}$H-PA |
| PC | 33.2 | 32.1 | 35.0 | 32.0 | 49.0 | 45.0 |
| PI | 11.0 | 0.9 | 2.3 | 32.0 | 0.29 | 4.5 |
| PE | 8.7 | 3.5 | 5.0 | 13.8 | 4.9 | 22.0 |
| PS | 2.3 | 0.6 | 1.8 | 12.7 | 0.5 | 1.3 |

BALB/c 3T3 cells and SRD cells were prelabeled with the indicated phospholipid precursor (AA, arachidonic acid; MA, myristic acid; PA, palmitic acid). The percentage of label incorporated into PC, PI, phosphatidylethanolamine (PE), and phosphatidylserine (PS) was calculated by determining the radioactivity incorporated into PC, PI, PE, and PS and dividing this value by the total cpm present in the organic phase extracted as described previously (52). Total cpm were determined prior to TLC and the cpm for PC, PI, PE, and PS were determined after TLC. Alkyl-lyso-PC already has the choline headgroup and is incorporated exclusively into PC (data not shown). After lipid

drying, chromatography and lipid spots scrapping, there was some loss of the lipid samples. Therefore, the absolute percentages for each specific lipid is higher than the values shown. The data above are the averages of two separate experiments where values did not vary by more than 20%.

**Table 3**

v-Src-induced increases in PLD are independent of PKC

Fold Induction (PEt)

| | Control | PKC depl. | Staurosporine |
|---|---|---|---|
| $^3$H-Myristate | | | |
| v-Src | 1.7 | 1.6 | 2.3 |
| TPA | 12.0 | 1.4 | 4.8 |
| $^3$H-Arachidonate | | | |
| v-Src | 1.0 | 1.1 | 1.3 |
| TPA | 7.6 | 1.3 | ND |

LA90 cells, which express a temperature sensitive mutant of v-Src (Qureshi et al., 1991), maintained at the non-permissive temperature for v-Src (40$^0$C) were either shifted to the permissive temperature for v-Src (34$^0$C), or treated with TPA (100 ng/ml) for 30 min and DG levels were determined as described previously (46). The fold increase in DG in response to either v-Src or TPA in control LA90 cells, LA90 cells that had been depleted of PKC by prolonged exposure to TPA (100 ng/ml, 26 hours), or LA90 cells that had been treated with staurosporine (500 nM, added 15 minutes prior to stimulation was calculated. The average of at least three experiments is reported.

**Table 4**

v-Src-induced increases in DG are independent of PKC

Fold Induction (DG)

| | Control | PKC depl. | Staurosporine |
|---|---|---|---|
| v-Src | 2.08 +/- 0.27 | 1.86 +/- 0.10 | 1.89 +/-0.90 |
| TPA | 2.12 +/- 0.13 | 1.25 +/- 0.16 | 1.38 +/- 0.06 |

LA90 cells maintained at the non-permissive temperature for v-Src (40°C) were either shifted to the permissive temperature for v-Src (34°C), or treated with TPA (100 ng/ml) for 30 minutes as in Table 3. The fold increase in DG in response to either v-Src or TPA in untreated LA90 cells, LA90 cells that had been depleted of PKC, or LA90 cells that had been treated with staurosporine was calculated. LA90 cells were prelabled with [$^3$H]-myristic acid, which is incorporated almost exclusively into PC, as described previously (52). The average of at least three experiments is reported.

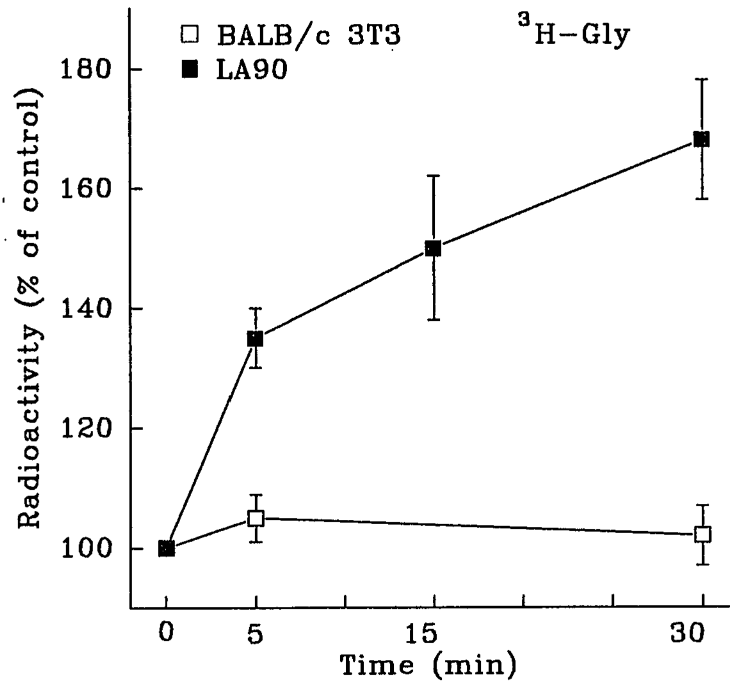



Fig.1a

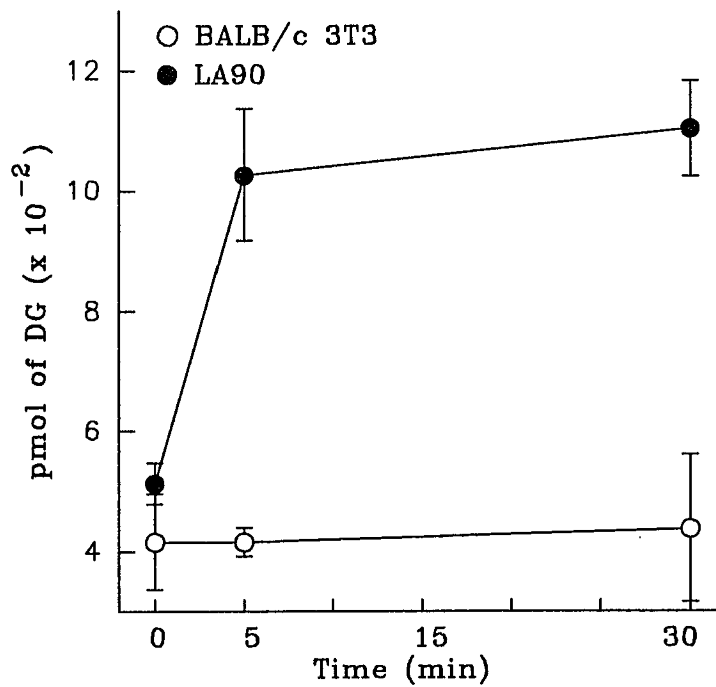



Fig.1b

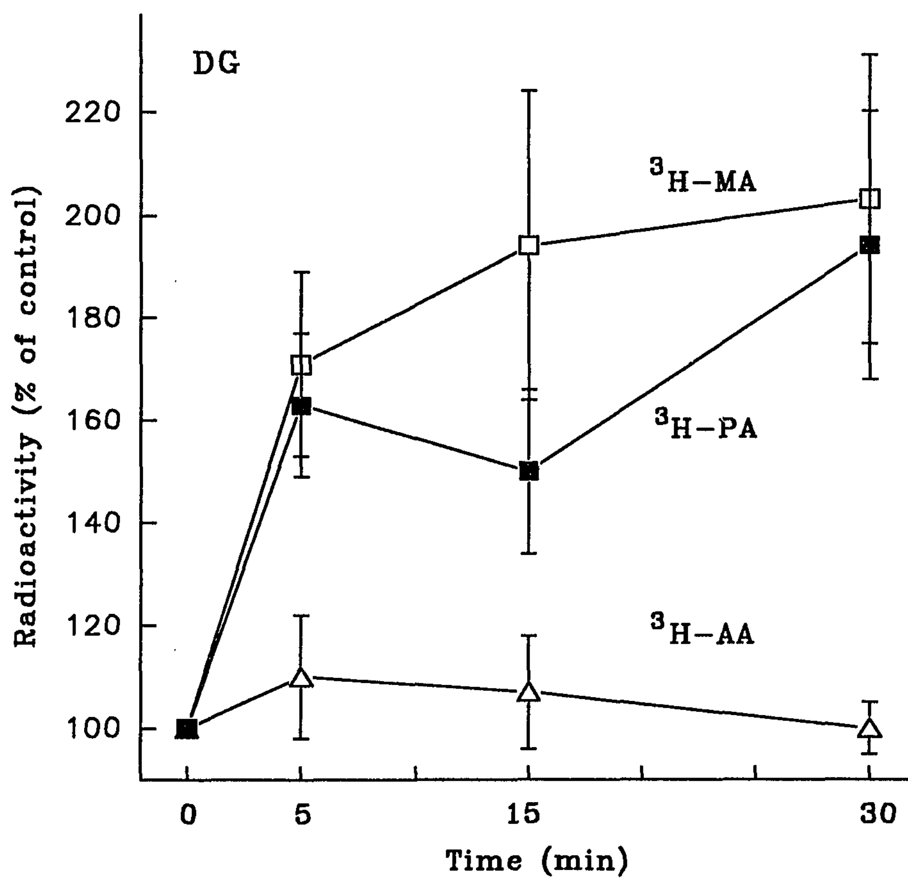



Fig.2

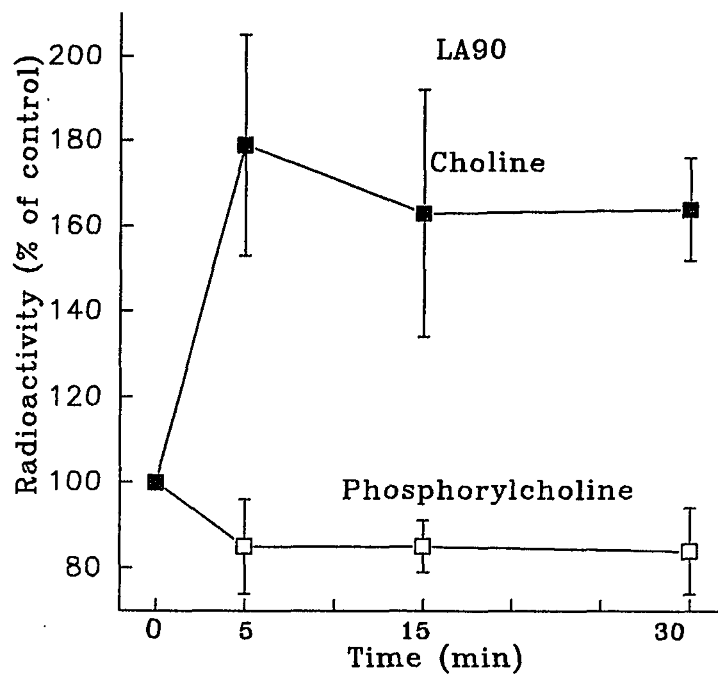



Fig.3a

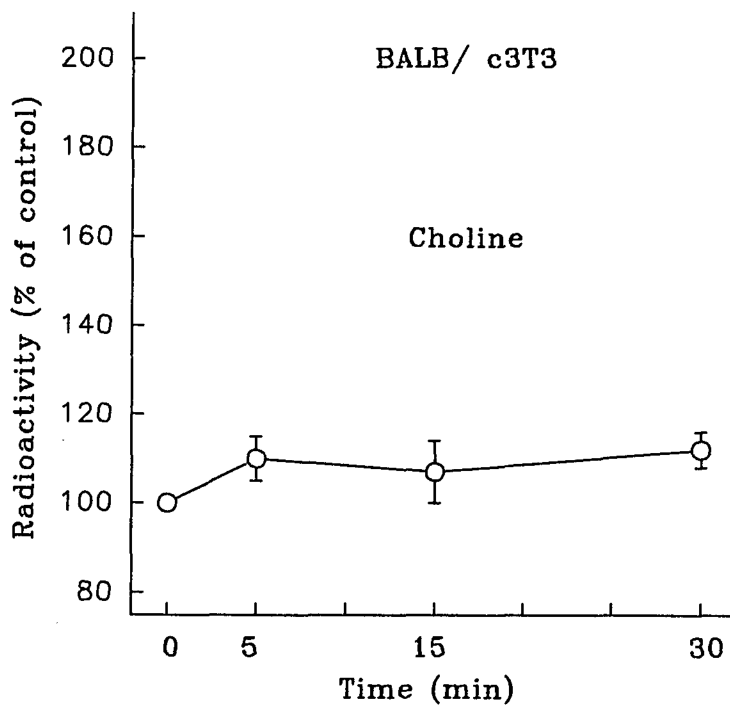



Fig.3b

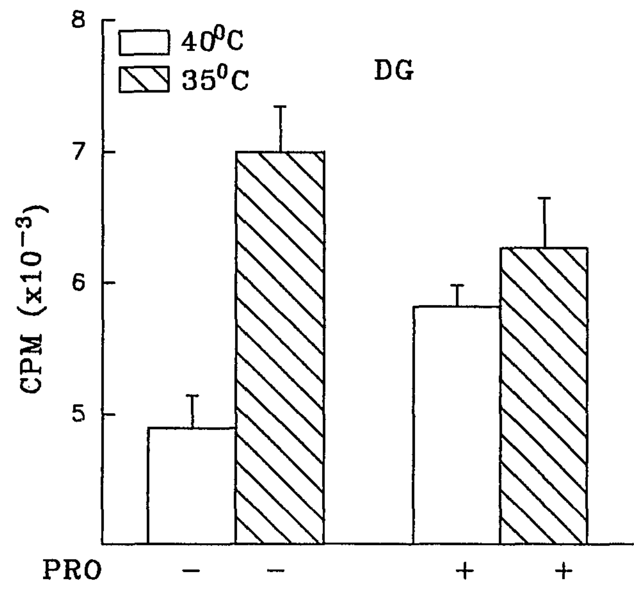



Fig.4a

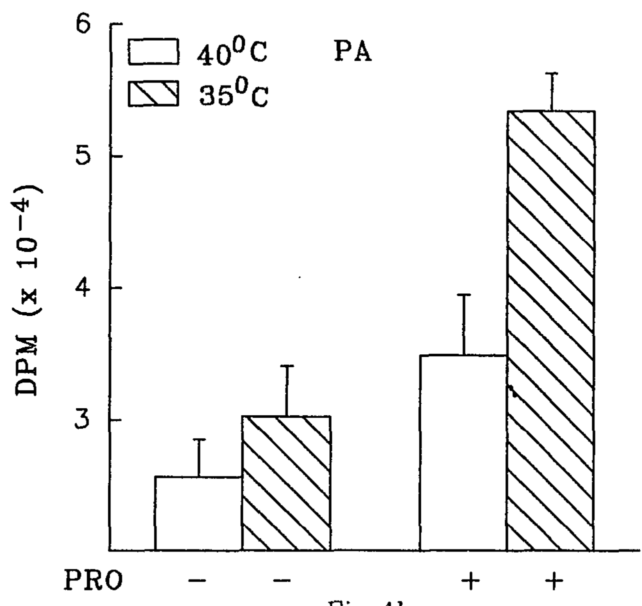



Fig.4b

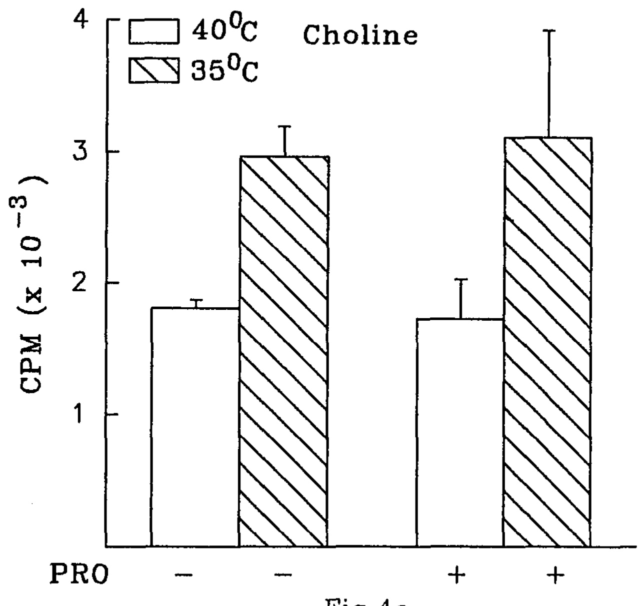



Fig.4c

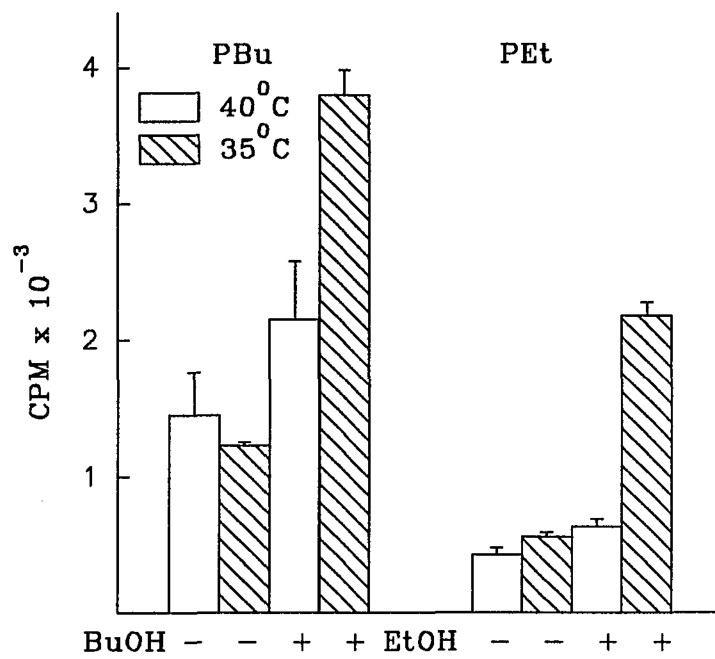



Fig.5a

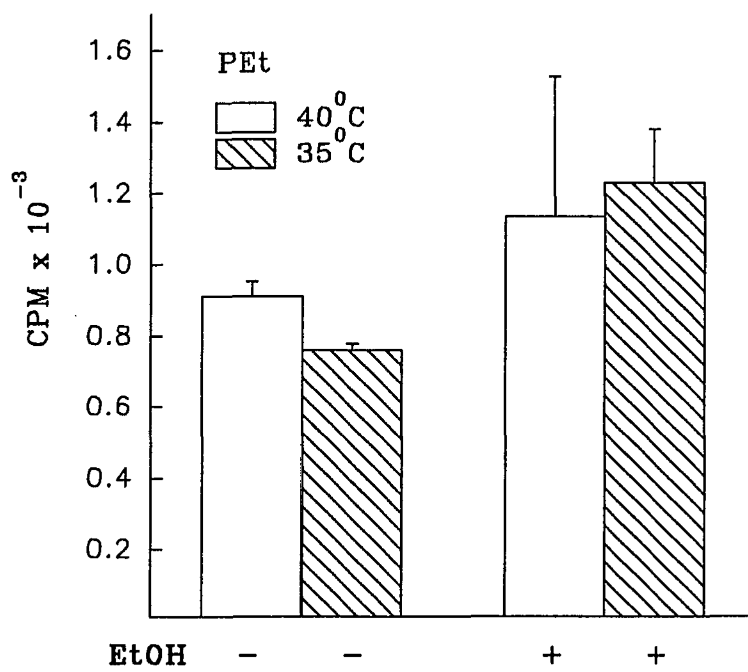



Fig.5b

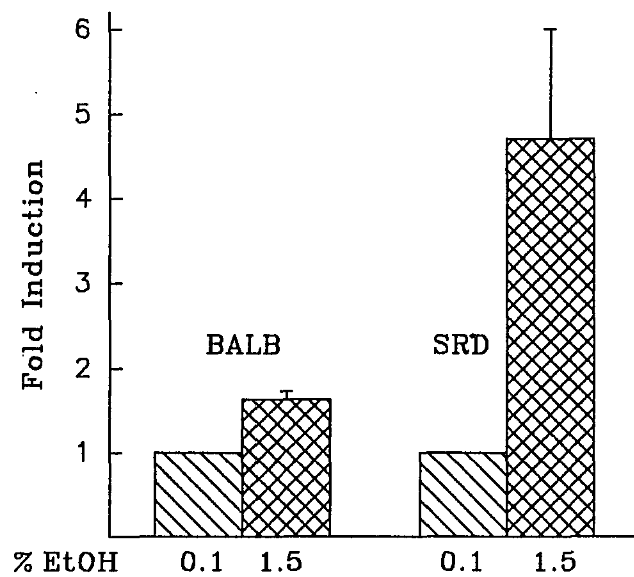



Fig.6a

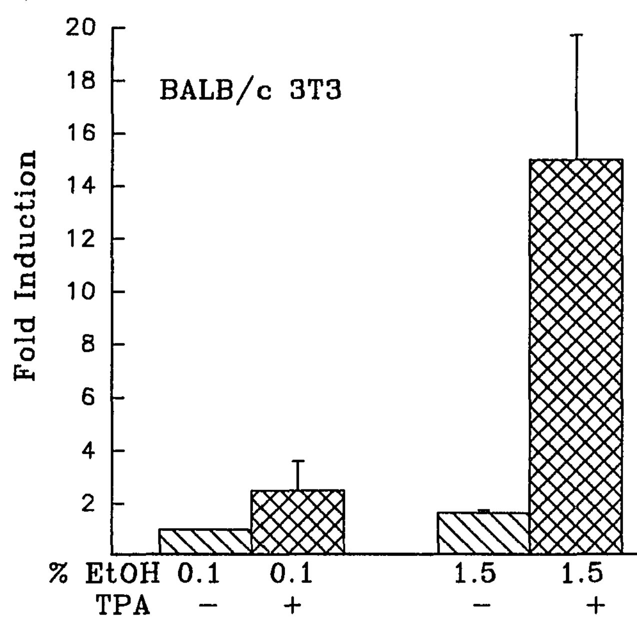



Fig.6b

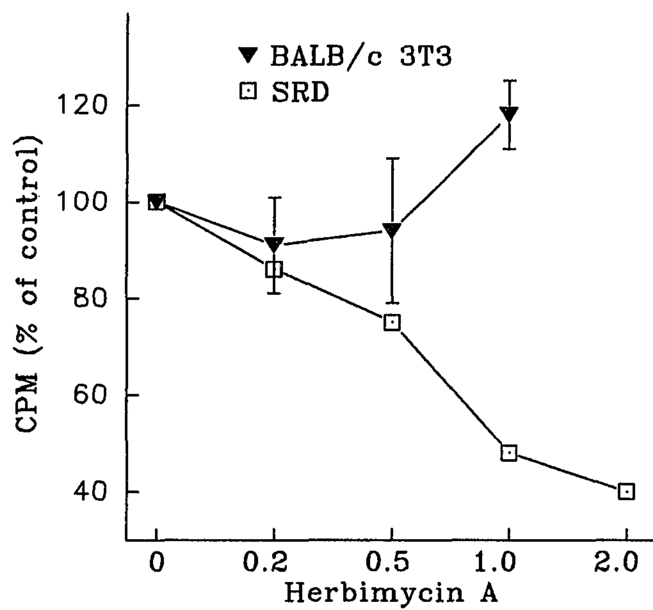



Fig.6c

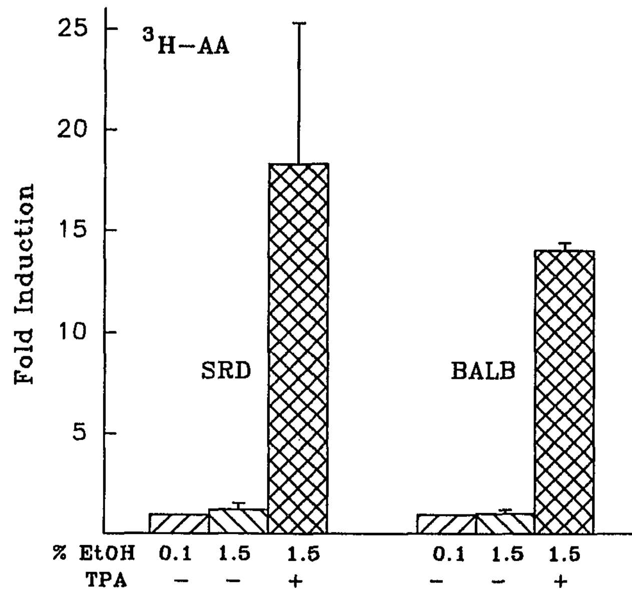
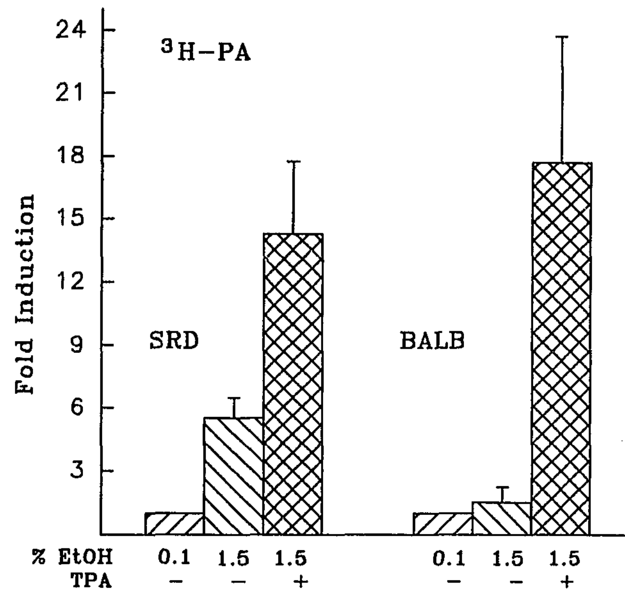



Fig.7a

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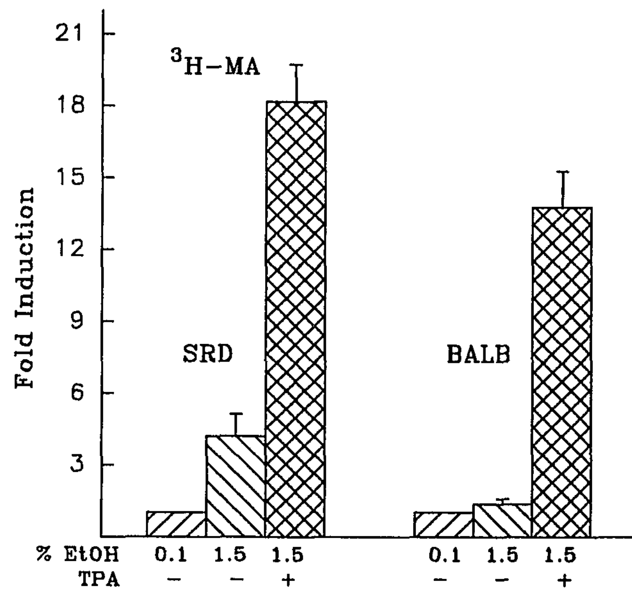


Fig.7b

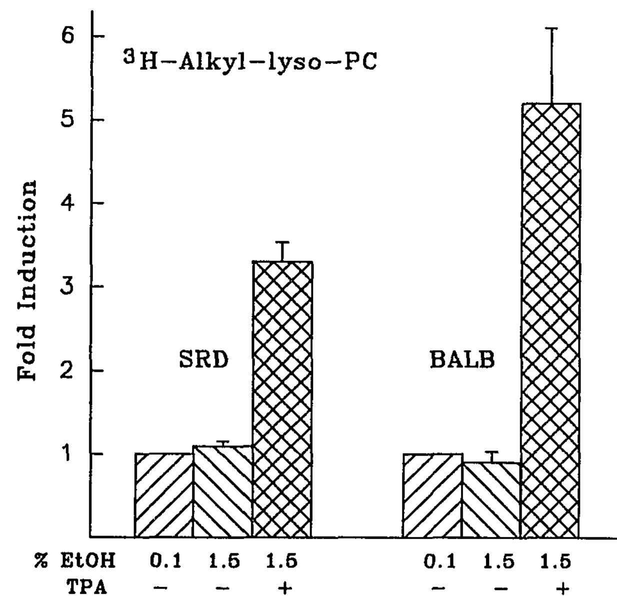


Fig.7d

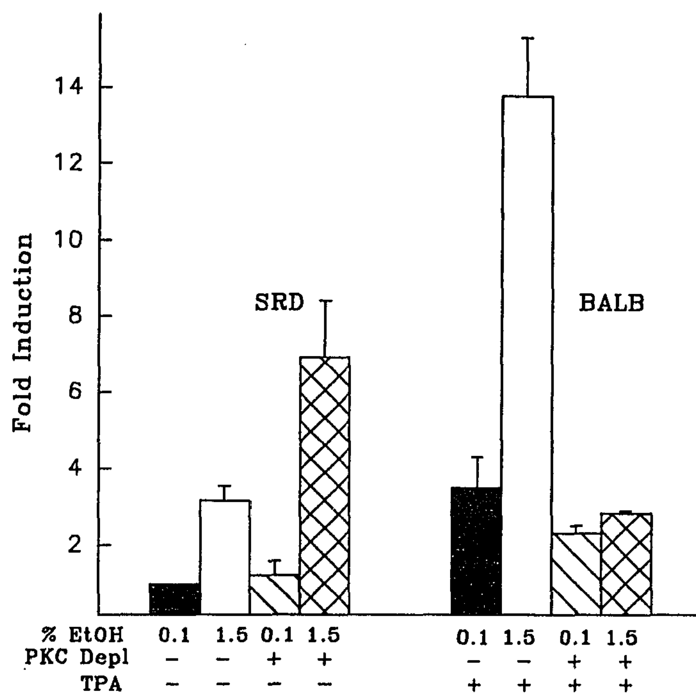


Fig.8a

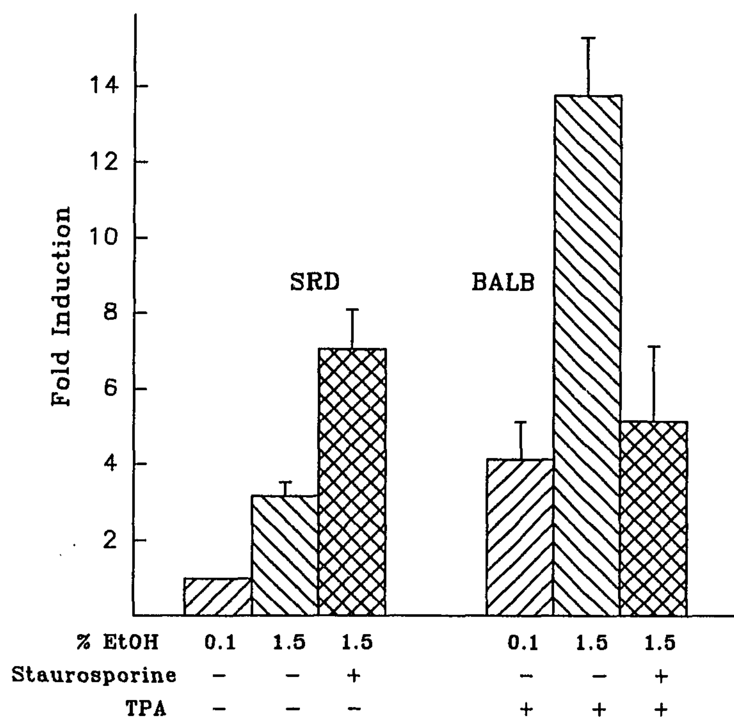


Fig.8b

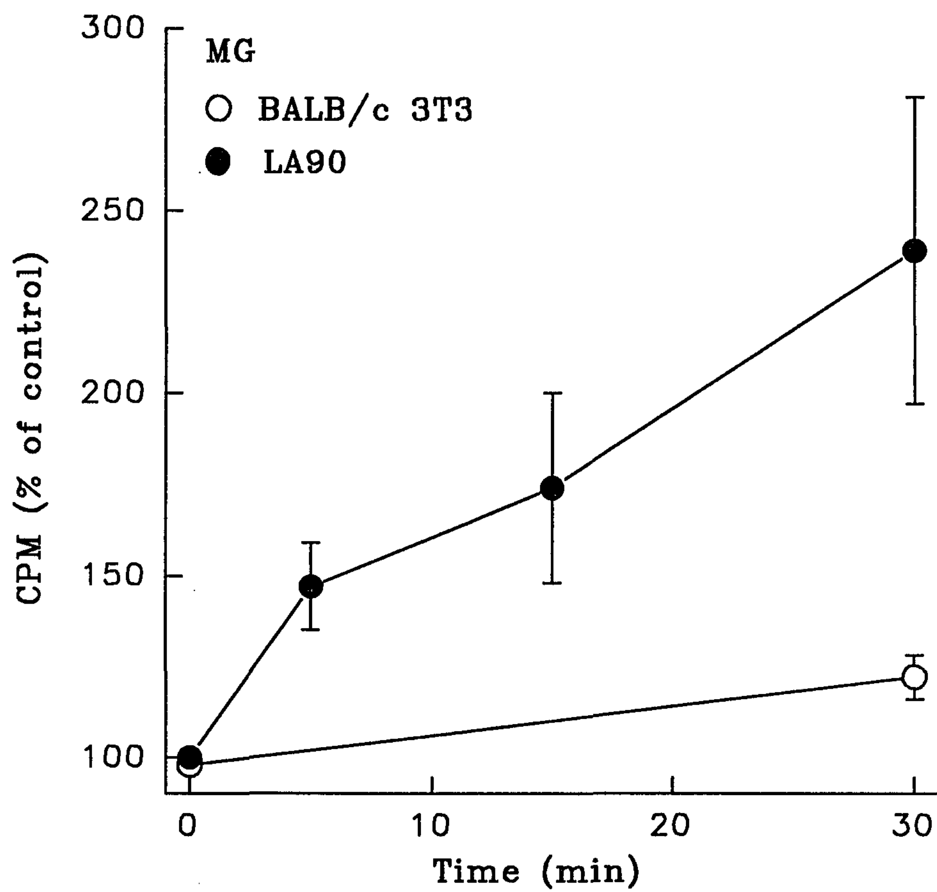


Fig.9

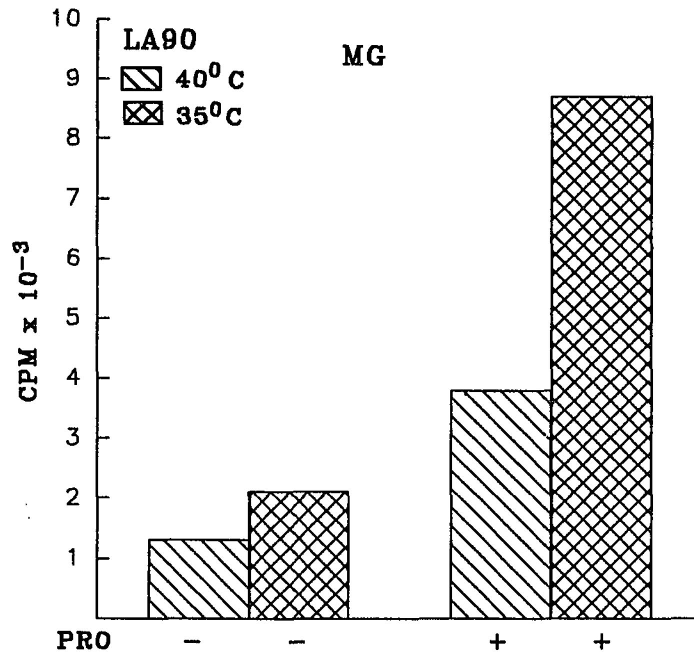


Fig.10a

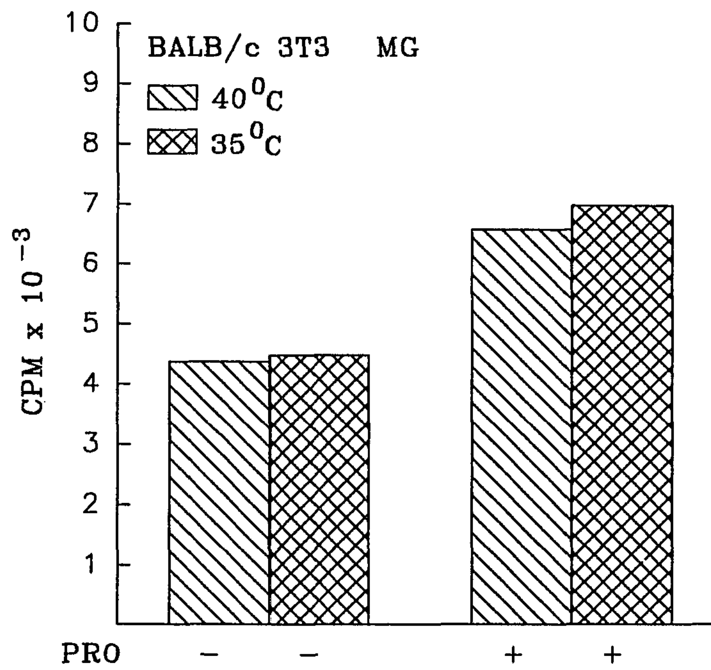


Fig.10b

# FIGURE LEGENDS

**FIG. 1. v-Src-induced DG.** a. LA90 and BALB/c 3T3 cells were prelabeled for 24 hours with [$^3$H]-glycerol. Cells were shifted from the non-permissive (40°C) to the permissive (34°C) temperature for v-Src and levels of DG were determined at the times shown. Data are presented as the average increase in cpm (% of control) incorporated into DG in three separate experiments with standard deviation. b. DG mass was determined using the method of Preiss et al. (72) in LA90 and BALB/c 3T3 cells after temperature shift at the times shown. Data are presented as the average increase in PA labeled with DG kinase (% of control) in two separate experiments.

**FIG. 2. DG production induced by v-Src detected after differential prelabeling with fatty acid precursors.** LA90 cells were prelabeled with either [$^3$H]-myristic acid (MA), [$^3$H]-palmitic acid (PA), or [$^3$H]-arachidonic acid (AA) for 24 hours. Cells were then shifted from the non-permissive to the permissive temperature for v-Src and DG levels were determined as in Fig.1. Data are the averages of at least three independent experiments with standard deviation.

**FIG. 3. Activation of v-Src leads to increased choline production.** Cells

were prelabeled with [$^{3}$H]-choline for 24 hours. Choline and phosphorylcholine were examined at the indicated times after temperature shift from 40°C to 34°C in (a) LA90 cells and (b) BALB/c 3T3 cells. Data are the averages of three independent experiments in LA90 cells and two independent experiments in BALB/c 3T3 cells.

**FIG. 4. The effect of propranolol on v-Src-induced DG, PA, and choline.** LA90 cells were prelabeled with either [$^{3}$H]-palmitic acid or [$^{3}$H]-choline. Radioactivity (cpm) in (a) DG, (b) PA , and (c) choline was examined before, and 30 minutes after, shifting to the permissive temperature. The experiments were carried out in the presence (+) or absence (-) of propranolol (300 $\mu$M) which was added 5 minutes prior to temperature shift. Cells were harvested 30 minutes after temperature shift. The data are the averages of duplicate cultures from a representative experiment using [$^{3}$H]-palmitic acid for DG and PA measurements. Similar data were obtained using [$^{3}$H]-myristic acid as a label.

**FIG. 5. v-Src stimulates type D phospholipase as determined by transphosphatidylation activity.** a. LA90 cells were prelabeled with [$^{3}$H]-palmitic acid and shifted from the non-permissive (40°C) to the permissive (34°C) temperature for v-Src in the presence (+) and absence (-) of either butanol (0.5%) or ethanol (1%). Radioactivity

incorporated into phosphatidylbutanol (PBu) and phosphatidylethanol (PEt) separated by TLC was counted. b. BALB/c 3T3 cells were prelabeled with [$^{3}$H]-palmitic acid and shifted from 40°C to 35°C in the presence and absence of ethanol (1%) and levels of radioactivity incorporated into phosphatidylethanol was determined as in panel 5a.

**Fig. 6. Phospholipase D activity is elevated in v-Src-transformed cells.** (a) BALB/c 3T3 cells (BALB) and BALB/c 3T3 cells treated with TPA (100 ng/ml, 30 minutes) were prelabeled with [$^{3}$H]-myristate in 0.1% EtOH. EtOH (1.5%) (v/v) was added as shown. Cellular lipids were extracted 30 minutes after addition of either EtOH or TPA. Transphosphatidylation products were separated by TLC and quantified by measuring the radioactivity incorporated into PEt. The data are presented as fold increase over the baseline cpm values for PEt in the presence of 0.1% EtOH. Increases were normalized to this baseline value. (b) BALB/c 3T3 cells treated with TPA (100 ng/ml, 30 minutes) and SRD cells were prelabeled with [$^{3}$H]-myristate, EtOH to 1.5% (v/v) was added as shown and PEt levels were determined as in (a). (c) BALB/c 3T3 cells treated with TPA (100 ng/ml, 30 minutes) and SRD cells were prelabeled with [$^{3}$H]-myristate. 1.5% EtOH (v/v) was added 30 minutes prior to harvesting of cellular lipids as in (a). Increasing concentrations of herbimycin A were added 6 hours prior to the

harvesting of cellular lipids and PEt levels were determined as in (a). The cpm values for PEt in the presence of 1.5% EtOH and in the absence of herbimycin A were assigned a value of 100% and all other values (except for BALB/c 3T3 in 6c) were normalized to this value. In 6c, the cpm values of PEt for BALB/c 3T3 cells in the presence of 1.5% EtOH, 100 ng/ml of TPA and the absence of herbimycin A were assigned a value of 100%. The data above are the averages of at least two experiments.

**Fig. 7. v-Src and phorbol esters activate distinguishable PLD activities.** PEt levels in SRD and BALB/c 3T3 cells in the presence (+) and absence (-) of TPA and EtOH were examined after prelabeling with different fatty acid precursors. SRD-transformed and untransformed BALB/c 3T3 cells were prelabeled with either a) [$^3$H]-arachidonic acid (AA), b) [$^3$H]-myristic acid (MA), c) [$^3$H]-palmitic acid (PA), or d) [$^3$H]-alkyl-lyso-PC as described in experimental procedures. EtOH (1.5%) or TPA (100 ng/ml) were added as shown 30 minutes prior to extraction cellular lipids. The cpm values for PEt in the absence of either exogenous EtOH or TPA was assigned a value of one and all other values were normalized to this. Data are the averages of at least two independent experiments.

**Fig. 8. v-Src- and phorbol ester-induced PLD activity are differentially**

**sensitive to PKC depletion (a) and staurosporine (b).** The effects of PKC depletion (PKC depl.) and staurosporine on PEt production induced by TPA and the basal level PEt production in SRD and BALB/c 3T3 cells were examined after prelabeling with [$^3$H]-palmitic acid. PEt production in response to EtOH (1.5%) was compared in cells that were either treated (+) or not treated (-) with staurosporine or with prolonged exposure to TPA (100 ng/ml, 26 hours) to deplete cells of PKC as shown. EtOH (1.5%) or TPA (100 ng/ml) were added 30 minutes and 35 minutes prior to extracting of cellular lipids respectively. Staurosporine was added 15 minutes before adding the TPA. The cpm values for PEt in the absence of exogenous EtOH was assigned a value of one and all other values were normalized to this value. Data are the average of at least three independent experiments.

**Fig. 9. MG production induced by v-Src.** LA 90 cells and BALB/c 3T3 cells were prelabeled overnight with $^3$H-myristic acid. Lipids were extracted at the indicated time after cells were shifted from non-permissive temperature to permissive temperature. MG was separated and counted by liquid scintillation. Data presented are average of at least three separate experiments.

**Fig. 10. Propranolol enhances the v-Src-induced production of MG.**

Cells were prelabeled overnight with 4 $\mu$ci of myristic acid and lipids were extracted. Temperature shift was made in the presence (+) or absence (-) of propranolol. (a). LA90 cells; (b). BALB/c 3T3 cells. Data presented are from a representative experiments that were repeated at least two times.

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