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EGF and v-Fps-induced Diglyceride Production: Regulation via Phospholipase D and Phosphatidic Acid Phosphatase

by

Youwei Jiang

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.

1996

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Date

Chair of Examining Committee

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Date

Executive Officer

Prof. Laurel Eckhardt

Prof. Thomas Haines

Prof. Thomas Schmidt-Glenewinkel

Prof. Lu-hai Wang

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ABSTRACT

EGF and v-Fps-induced Diglyceride Production: Regulation via Phospholipase D and Phosphatidic Acid Phosphatase

by

Youwei Jiang

Adviser: Professor David A. Foster

Activating the protein-tyrosine kinase v-Fps results in a rapid increase in diglyceride (DG) in cells expressing a temperature-sensitive v-Fps (ts v-Fps). The v-Fps-induced increase in DG is detected only when cellular phospholipids are prelabeled with [^{3}H]-myristate, which is incorporated primarily into phosphatidylcholine (PC). PC is predominantly a substrate for phospholipase D (PLD). The primary metabolite of PLD is phosphatidic acid (PA) which can be converted to DG by phosphatidic acid phosphatase (PAP). The cells expressing ts v-Fps have elevated levels of PA and PLD activity at both permissive and non-permissive temperatures for v-Fps relative to the parental 3Y1 cells. These data suggest that PLD is constitutive activated in v-Fps-transformed cells, and the activated PLD alone is not sufficient to regulate v-Fps-induced increase in DG. If we inhibit the conversion of PA to DG, the v-Fps-induced increase in DG is blocked. Upon shifting from the non-permissive to the permissive temperature for ts v-Fps, the membrane PAP activity is increased. These data suggest that PAP regulates v-Fps-induced

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increase in DG. All above evidences indicate that v-Fps induces activation of PLD/PAP signaling pathway to generate DG.

Epidermal growth factor (EGF), like v-Fps, also activates PLD/PAP signaling pathway to generate DG. To further establish PAP as a regulatory site for DG production, we investigated the effect of EGF on PAP activity in A431 cells, which overexpress EGF receptors (EGFR). Upon EGF stimulation, PAP activity in cell lysates is elevated. Protein Kinase C (PKC) inhibitors block the EGF-induced increase in PAP activity. Consistent with a role for PKC, EGF induces a sustained increase in PAP activity in PKC ε immunoprecipitates, but not in PKC α or PKC ζ immunoprecipitates. Concomitant with the increased PAP activity in PKC ε, there is a corresponding decrease in PAP activity in EGFR immunoprecipitates. AG1478 induces a dramatic decrease in PAP activity in both EGFR and PKC ε immunoprecipitates. It correspondingly induces a decrease of PAP activity in the membrane fractions and an increase of PAP activity in the cytosol fractions. AG1478 also blockes EGF-induced DG production. Based on these data, we propose a novel mechanism for PAP activity in regulating EGF-induced DG production, in which EGF induces PAP to dissociate from membrane EGFR and associate with PKC ε to localize PAP to PA substrate.

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PREFACE

This thesis is divided into five sections. Section I is a general introduction. Section II describes my research work. It is composed of two parts. Part I discusses the v-Fps-induced increase in DG production via activating PLD/PAP signaling pathway. Most work in this part has been published in ***Biochemical and Biophysical Research Communications*** (1994) 203, 1195-1203. It is reprinted by permission of the publisher. Part II focuses on the regulating mechanism of PAP activity in response to EGF stimulation. Section III is a brief summary. Section IV contains experimental procedures. Section V is the bibliography for the entire document.

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List of Abbreviations

AA	arachidonic acid
ARF	ADP-Ribosylation Factor
cAMP	cyclic AMP
$cPLA_2$	cytosolic phospholipase A_2
DG	diglyceride
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GIP	Ras GTPase inhibitory protein
IP_3	inositol-1,4,5-trisphosphate
LSM	lipid second messengers
lyso-PA	lysophosphatidyl acid
lyso-PC	lysophosphatidyl choline
MAPK	mitogen activating protein kinase
NEM	N-ethylmaleimide
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PC-PLC	phosphatidylcholine specific phospholipase C
PBt	phosphatidylbutanol

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PI	phosphatidylinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PKA	protein kinase A
PLC-γ	phospholipase C-gamma
PLD	phospholipase D
PS	phosphatidylserine
RTK	receptor tyrosine kinases
Ras-GAP	Ras GTPase activating protein
SH2	Src homology 2
Stau.	Staurosporin

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SECTION I

Introduction

Lipids have been viewed primarily as structural elements of the cell membrane. Lipids are now recognized as precursors of signaling molecules. Upon stimulation of cell-surface receptors, lipids are hydrolyzed to generate lipid second messengers (LSM) which can interact with target proteins to transduce signal across the cellular plasma membrane. The epidermal growth factor (EGF)-induced hydrolysis of phosphatidylinositol (4,5) bisphosphate (PIP_2) is the best studied lipid signal transduction pathway. Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinases (RTK). Upon binding of EGF, EGFR dimerizes and activates its tyrosine kinase activity in the cytoplasmic domain leading to autophosphorylation (Rozengurt, 1986; Hunter and Cooper, 1985; Fantl, et al. 1992). Following autophosphorylation, phosphorylated tyrosine residues serve as selective binding sites for proteins containing Src homology 2 (SH2) domain. SH2 domain is found in a remarkably diverse group of cytoplasmic signaling proteins. The SH2 domain of phosphoinositide-specific phospholipase C-gamma (PLC-γ) binds to phosphotyrosine 992 in the C-terminal domain of EGFR and mediate the association of cytoplasmic PLC-γ with activated EGFR (Kim, 1991; Fantl et al., 1992). The association of PLC-γ and EGFR has two functions: first, EGFR tyrosine kinase phosphorylates tyrosine residues (Tyr 783 and 1254) in PLC-γ and activates PLC-γ (Wahl, et al., 1990;

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Meisenhelder, et al. 1989; Margolis et al., 1989; Nishibe, et al., 1990; Kim, et al., 1991). Second, the association between EGFR and PLC-γ serves to localize the PLC-γ at the plasma membrane near its PIP_2 substrate. PLC-γ hydrolyzes PIP_2 to diacylglycerol (DG) and inositol-1,4,5-trisphosphate (IP_3). DG activates protein kinase C (PKC) and IP_3 induces Ca^{2+} releasing from intracellular stores (Berridge, 1987). Activated PKC phosphorylates other signaling proteins to induce cell division and proliferation. Besides PIP_2, other phosphatidylinositols (PI) can also be hydrolyzed to DG by PI-PLC. PI-derived DG is normally transient and rapidly converted to PA by DG kinase (Ford and Gross, 1990; Preiss et al., 1986; Lee, et al., 1991). Thus, PI-derived DG can not sustain a long activating on PKC. Mutants of the EGFR that block association with PLC-γ do not inhibit EGF induced mitogenesis (Mohammadi et al., 1992; Vega, et al., 1992; Seedorf, et al., 1992; Peters, et al., 1992). This suggests that PLC-γ generated DG does not seem to be essential for cell division and proliferation in response to EGF stimulation. There may exist some other

sources for generation of DG to activate PKC which may play an important role for cell division and proliferation in response to EGF stimulation.

Phosphatidylcholine (PC) is the major phospholipid in mammalian tissue. PC can also be directly hydrolyzed to DG by PC-phospholipase C (PC-PLC). Based on optimal pH for their activity, two PC-PLC isoforms have been detected and partially purified. One isoform exhibits alkaline pH optima, and the other isoform exhibits neutral pH optima. These two PC-PLCs do not

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hydrolyze PI (Billah and Anthes, 1990). PC-PLC-mediated generation of DG has been suggested to occur in a number of cells especially in response to certain cytokines. Tumor necrosis factor α, interferon α, interleukin 1, interleukin 3 and colony-stimulating factor 1 stimulate generation of DG from PC via a PC-PLC pathway in the absence of PI-PLC activation (Liscovitch, 1992). There are, however, few reports about the regulatory mechanism of PC-PLC activity.

PC is mostly regarded as a substrate for phospholipase D (PLD) which hydrolyzes PC to phosphatidic acid (PA) and choline. PLD generated PA can be hydrolyzed to DG by phosphatidic acid phosphatase (PAP). PLD generated PA has been reported to be an important second messenger for regulating the activity of some proteins involved in signaling transduction. PA binds to Ras GTPase activating protein (Ras-GAP) and inhibits its activity (Tsai, et al., 1989a). PA also stimulates a cytoplasmic Ras GTPase inhibitory protein (GIP) (Tsai, et al.,1989b). These two events result in elevated levels of the active, GTP-bound form of Ras, which are indeed known to occur in mitogen-stimulated cells (Tsai, et al., 1989a; 1989b; Golubic et al., 1991; Liscovitch, 1992). Also, PA, causes (a) an activation of cytosolic phospholipase A_2 ($cPLA_2$) and an increase in intracellular concentration of arachinonic acid (AA) (Murayam et al., 1987), (b) an inhibition of adenylate cyclase and a decrease in cyclic AMP (cAMP) (von Corven, et al., 1989), (c) a stimulation of phosphatidylinositol-4-phosphate kinase (Moritz, 1992), (d)

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Ca^{2+}-activated phosphorylation of specific proteins (Bocckino, 1991), and (e) a superoxide generation in neutrophils (Bauldry, 1991).

PLD activity is elevated in response to the activation of EGFR tyrosine kinase, oncogenic protein kinase v-Src and agonists stimulation. (Song, et al., 1994; 1991; Kaszkin, et al., 1992; Pessin, et al., 1990; Cook, et al., 1992; Fisher, et al., 1991). PLD activity is stimulated by ADP-Ribosylation Factor (ARF) (Brown, et al., 1993). ARF proteins constitute a subgroup in the Ras superfamily of monomeric GTP-binding proteins (Kahn et al., 1992). Recombinant myristoylated ARF_1 was found to be a better activator of PLD activity than was the nonmyristoylated form (Brown, et al., 1993). This result indicates that ARF may be an important component in the generation of lipid second messengers via PLD pathway. PIP_2 is required for optimal stimulation of PLD activity when substrate is supplied in the form of exogenous phospholipid vesicles (Brown, et al., 1993; Liscovitch et al., 1994). The role of PIP_2 is not due to some general effect on vesicle structure. It may serve as a binding site for ARF, PLD, or other potential cofactors that may participate in PLD signaling system (Brown, et al., 1993; Liscovitch, et al., 1994). Recently, PLD genes have been cloned from *Saccharomyces cerevisiae*, murine source, and HeLa cells (Rose, et al., 1995; Hammond, et al., 1995). The cloned PLD activity is also dependent upon ARF and PIP_2.

In v-src transformed Balb/c 3T3 cells, v-Src-induced PLD activation depends upon a GTPase cascade. Ral, a membrane-bound protein, constitutively associates with PLD through Ral's amino terminus. But Ral does

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not activate PLD alone (Jiang, et al., 1995b). Ral-GDS, the exchange factor responsible for the activation of Ral GTPases, serves as a conduit for bringing PLD into a complex with v-Src activated Ras (Jiang, et al., 1995a, 1995b).

PLD-generated PA can be further metabolized to form lyso phosphatidic acid (lyso PA) by type A_2 phospolipase, which removes a fatty acid from the *sn*-2 position of PA. Lyso PA is an important lipid second messenger and mitogen. Lyso PA activates p21Ras to trigger Ras signaling pathway (van Corven, et al., 1993). Lyso PA also activates PLC-γ to increases intracellular Ca^{2+}, and PKC activity (Jalink, et al., 1990).

Lyso PA, like PA, also activates $cPLA_2$ to increase intracellular concentration of arachidonic acid (Murayama et al., 1987). and inhibits adenylate cyclase to decrease cAMP. (von Corven, et al., 1989). However, Lyso PA fails to mimic PA in inhibiting GAP activity (Tsai, et al., 1989b).

PLD-generated PA can also be hydrolyzed to DG by PAP. The activation of EGF receptor tyrosine kinase and oncogenic tyrosine kinase v-Src have been shown to generate DG from PC via PLD/PAP signaling pathway (Susa, et al., 1989; Kaszkin, et al., 1992; Pessin, et al., 1990; Song, et al., 1991; 1994; Plevin, et al., 1991). Unlike PI-derived DG, this PC-derived DG is a poor substrate for DG kinase (Preiss, et al., 1986; Lee, et al., 1991; Ford and Gross, 1990). Thus PC-derived DG can sustain a long time to activate PKC. PC-derived DG is considered to play a major role for cell division and proliferation.

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PAP exists in two forms which differ in their enzymatic and physical properties, regulation, N-ethylmaleimide (NEM) sensitivity and subcellular distribution. The microsomal and cytosol fractions contain mainly the NEM-sensitive form (Mg^{2+}-dependent) of PAP. The NEM-sensitive form of PAP is not heat resistant, requires Mg^{2+} for its activity, and is inhibited by NEM. The plasma membrane and the mitochondrial fractions contain NEM-insensitive form (Mg^{2+}-independent) of PAP. The NEM-insensitive PAP is able to sustain high temperature, does not require Mg^{2+} for its activity and is not inhibited by NEM (Jamal, et al., 1991; Day, et al., 1992; Jamdar and Cao, 1994). It is postulated that NEM-insensitive PAP may be involved in PLD/PAP signal transduction pathway to generate the lipid second messenger (Truett, et al., 1992; Exon, 1990; Jamal, et al., 1991), whereas NEM-sensitive PAP may be involved in triacylglycerol formation by generating diacylglycerol via the glycerol-3-phosphate pathway (Brindley, 1987).

PAP activity can be regulated in different levels. In the long term, PAP activity is subjected to hormonal control at the level of enzyme synthesis. Glucocorticoids, glucagon (via cAMP), and growth hormone can increase the synthesis of PAP, whereas insulin suppresses the enzyme synthesis (Brindley, 1984; 1987). On the other hand, PAP activity is regulated in the short term by enzyme translocation. The NEM-sensitive PAP in the cytosol translocates to the endoplasmic reticulum to become functionally active in triacylglycerol synthesis. Oleate and acyl-CoA ester stimulate the membrane translocation of cytosolic PAP (Brindley, 1984; 1987; Martin, et al., 1987;

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Butterwith, et al., 1984). Okadaic acid and albumin displace the NEM-sensitive PAP activity from the membrane to the cytosol, and prevent the membrane translocation induced by oleate (Gomez-Munoz, et al., 1992; Tronchere, et al., 1994). The membrane translocation of NEM-sensitive PAP from the cytosol to the endoplasmic reticulum is also under hormonal control and is inhibited in the presence of cAMP or glucagon (Tronchere, 1994). However, there are few reports on the regulation of NEM-insensitive PAP activity. Linolenate, arachidonate and eicosapentaenoate are reported to produce small decreases in NEM-insensitive PAP activity (Gomez-Munoz, et al., 1992).

All above evidences indicate that PC can be metabolically converted into different lipid second messengers (PA, lyso-PA and DG) in response to different extracellular signals and under regulation of different enzymes in PC metabolic pathways. These lipid second messengers may activate their target signaling proteins and transduce the signals across the membrane into different signaling pathways in the cells. In PLD/PAP signaling pathway, PAP seems to play an important role in regulalting the relative abundance of lipid second messenger PA and DG. In this way, PAP may control the balance between PA and DG-activated signal transduction pathways in the cells. PLD has been reported to be activated in response to the stimulation of growth factors, some oncogenes and many agonists (Kaszkin, et al., 1992; Pessin, et al., 1989; 1990; Cook, et al., 1992; Fisher, et al., 1991; Song et al., 1991;

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1994; Foster, 1993). However, the activation of PAP by growth factors, oncogenes and agonists is still unknown.

As has been discussed, PIP_2 and PC are two major phospholipid sources for DG production (Fig. 1.). DG can be derived from PIP_2 via PLC-γ signaling pathway. DG can also be derived from PC via PC-PLC or PCD/PAP signaling pathways. My research project is to define phospholipid signaling pathways which generate lipid second messenger DG in response to stimulation of oncogenic protein tyrosine kinase v-Fps and EGF. The work is separated into two parts. The first part focused on the effect of v-Fps on lipid second messenger generation. (1) Since PKC is involved in v-Fps induced signal transduction (Spangler, et al., 1989; Alexandropoalos et al., 1992; 1993), we examined the effect of v-Fps activity on DG levels. (2) PC and PI are two major phospholipid precursors of DG. Protein tyrosine kinase receptors and oncogenic protein tyrosine kinase v-Src induce an increasd production of DG from PC via the PLD/PAP signaling pathway (Pessin et al., 1990; Plevin, et al., 1991; Kaszkin, et al., 1992; Cook and Wakelam, 1992; Fisher, et al., 1991; Song, et al., 1994). If v-Fps induces an increase in DG production, it is of interest to identify the phospholipid source of the DG and define the phospholipid signaling pathway which is activated by v-Fps.

The second part of this study focused on the regulation of PAP activity in response to EGF stimulation. (1) EGF can induce a two phase induction of DG. The first transient phase DG is derived from PI. It is followed by a sustained increase of second phase DG which is derived from PC via

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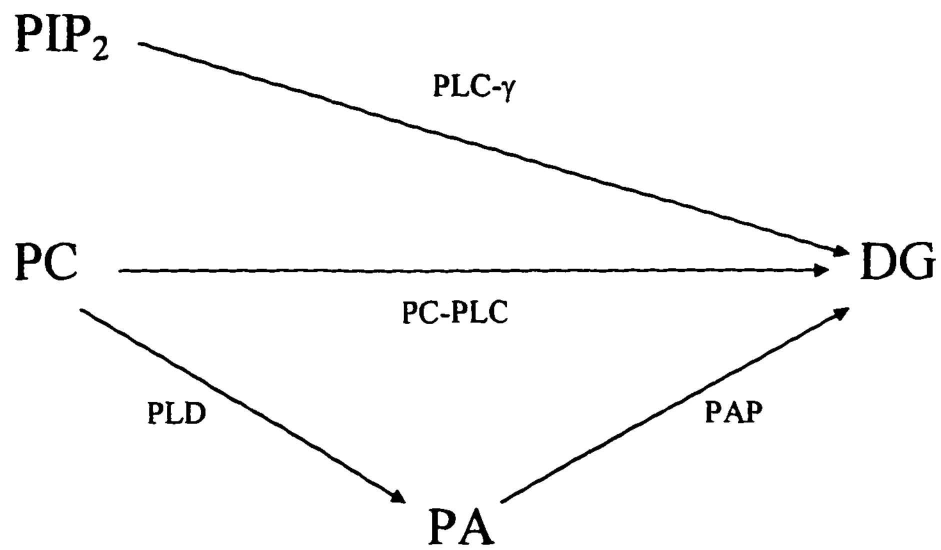

Fig. 1. The phospholipid source for DG.
DG can be generated from PIP_2 via PLC-γ signaling pathway. DG can also be generated from PC via PC-PLC or PLD/PAP signaling pathways.

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PLD/PAP signal pathway (Song, et al., 1994). Though PLD is activated under EGF stimulation, it has not yet been demonstrated whether PAP is activated by EGF. We examined the PAP activity in response to the stimulation of EGF. (2) Many reports have provided indirect evidence to indicate that PAP is a regulatory site for DG generation (Martinson, et al. 1990; Gomez-Munoz, et al., 1992). We wish to define the regulatory mechanism of PAP by EGF.

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SECTION II

Thesis Progress

Part I:

PC-specific PLD and PAP Activity is Elevated in v-Fps-transformed Cells

RESULTS

Activation of tyrosine kinase v-Fps leads to increased levels of DG.

Previous studies demonstrated that oncogenic protein tyrosine kinase v-Fps activates PKC in both avian and murine fibroblasts (Spangler, et al., 1989; Alexandropoulos, et al., 1991; 1992). Since PKC is activated by DG (Nishizuka, 1986), increases in DG levels in response to the activation of v-Fps were examined by using 3Y1 rat fibroblasts transformed with the tsNY225 temperature-sensitive strain of Fujinami sarcoma virus (Alexandropoulos, et al., 1992). The tsNY225 cells and parental 3Y1 cells were metabolically prelabled with [^{3}H]-myristate. Quiescent tsNY225 cells maintained at the non-permissive temperature for v-Fps (39.5°C) were shifted to the permissive temperature (34°C) to activate the protein-tyrosine kinase activity of v-Fps, and levels of [^{3}H]-labeled DG were examined. As shown in Fig. 2A, activation of v-Fps can induce a two fold increase in [^{3}H]-myristate labeled DG

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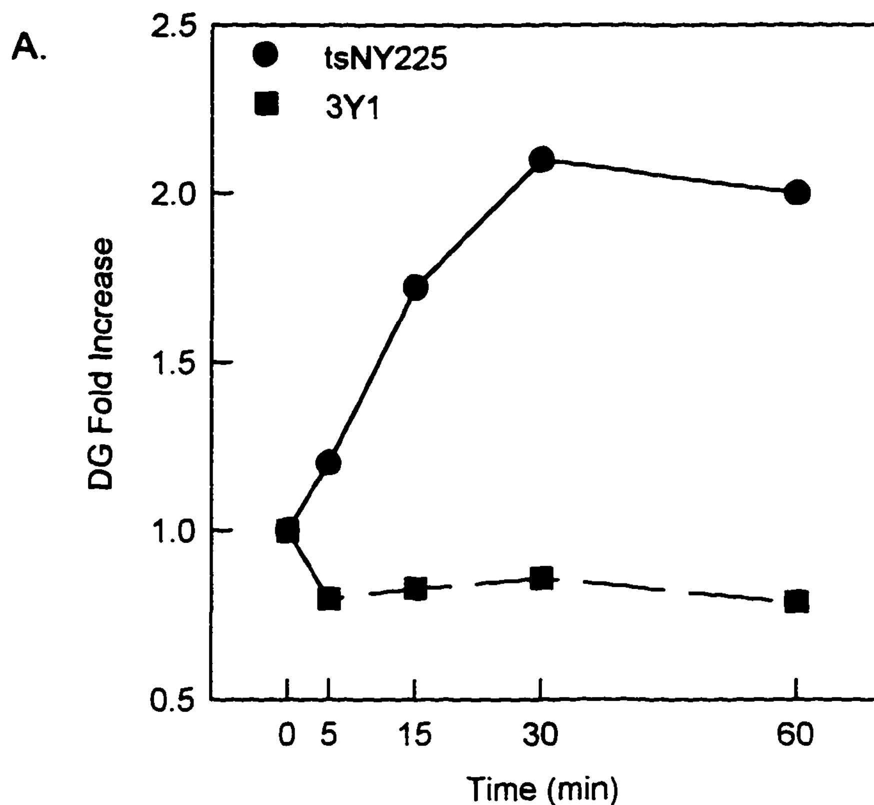

Fig. 2. v-Fps-induced DG.
A, tsNY225 and 3Y1 cells were prelabeled for 24 hr with [^{3}H]-myristate. Cells were shifted from the non-permissive (39.5°C) to the permissive (34.0°C) temperature for v-Fps and levels of DG were determined at the times shown. Data are presented as the fold increase in DG. The cpm values for DG at 39.5°C were 5929 +/- 179 and 4933 +/- 95 for tsNY225 and 3Y1 cells respectively. B, DG levels were determined in tsNY225 cells prelabeled with either [^{3}H]-myristate or [^{3}H]-arachidonate for 24 hr. Cells were then shifted from the non-permissive to the permissive temperature for v-Fps and DG levels were determined as in A. The cpm values for DG at 39.5°C for [^{3}H]-arachidonate-prelabeled cells was 5249 +/- 188. The data presented in A and B represent mean values for duplicates from representative experiments that were repeated at least three times.

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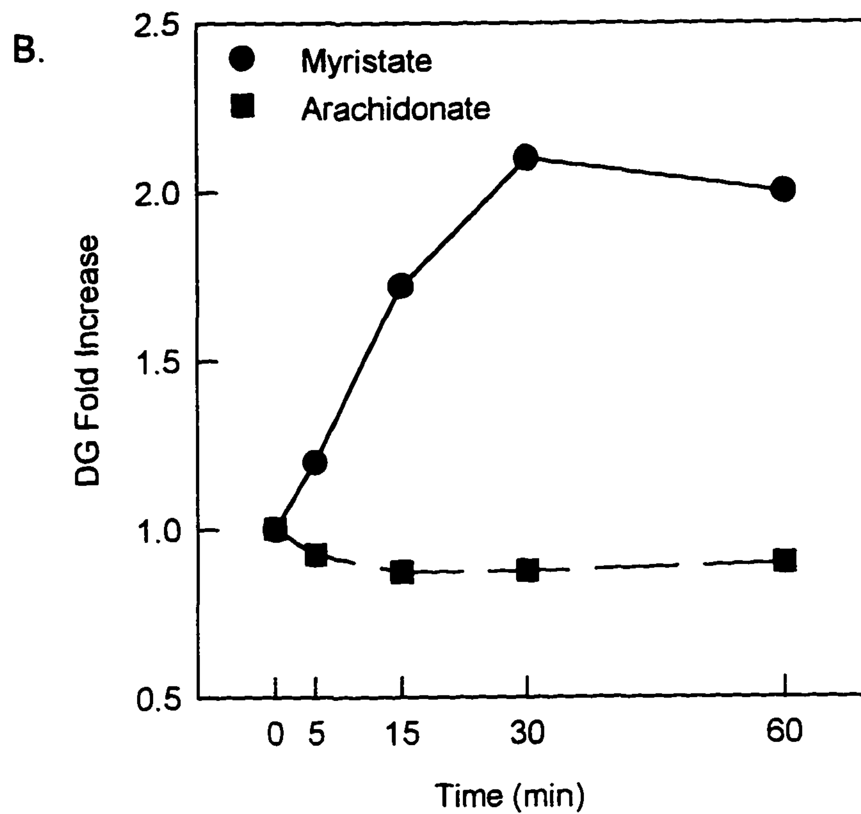

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production, and the increased DG could be detected within 5 min after temperature shift. This treatment did not increase [^{3}H]-myristate labeled DG levels in the parental 3Y1 cell line (Fig. 2A). We also examined DG production in cells prelabeled with [^{3}H]-arachidonate. As shown in Fig. 2B, temperature shift to activate v-Fps can not induce an increase in [^{3}H]-arachidonate labeled DG production. The result suggest that v-Fps induced DG production has its own fatty acid profiles. The v-Fps induced DG production tend to have short saturated aliphatic groups instead of long unsaturated aliphatic groups.

Identifying the source of v-Fps-induced DG production by differentially labeling of phospholipids.

As in DG species, phospholipids have characteristic fatty acid compositions which permit differential labeling with different radioactively labeled fatty acids (Augert, et al., 1990; Cabot, et al., 1988; Huang and Cabot, 1990; Martin and Michaelis, 1988; Swendsen, et al., 1987; Takayama, et al., 1987; Welsh, et al., 1990). PI is preferentially labeled with arachidonate which is esterified to the *sn*-2 position of the glycerol backbone in PI. In contrast, PC is mostly prelabeled with myristate. Since PI and PC are two major source for DG production, we made use of the differential labeling of PI and PC to identify the source of DG produced in response to the activation of v-Fps. The differential fatty acid labeling of PI and PC in 3Y1 cells and tsNY225 cells is shown in table 1. [^{3}H]-myristate is incorporated almost exclusively into PC in

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	Percent of label incorporated into:			
	tsNY225 cells		3Y1 cells	
	PC	PI	PC	PI
[^{3}H]-myristate	55.1 (+/- 2.3)	2.9 (+/- 0.2)	31.8 (+/- 2.0)	1.9 (+/- 0.1)
[^{3}H]-arachidonate	31.0 (+/- 1.1)	21.9 (+/- 0.1)	18.7 (+/- 1.4)	10.4 (+/- 0.4)

Table 1. Differential Labeling of Phospholipids.
tsNY225 and 3Y1 cells were prelabeled with the indicated phospholipid precursor ([^{3}H]-arachidonate or [^{3}H]-myristate). Cell were harvested 30 min after temperature shift. The percentage of label incorporated into PC and PI was calculated by determining the radioactivity incorporated into either PC and PI and dividing this value by the total cpm present in the $CHCl_3$:MeOH lipid extract as described in Experimental Procedures. Total cpm were determined prior to TLC and the cpm for PC and PI were determined after TLC. Therefore, total cpm contains some background cpm and thus, the absolute percentages are likely to be higher than the values shown. Data are from a representative experiment performed in duplicate that was repeated three times.

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both the tsNY225 and 3Y1 cells (Table 1). This is consistent with what has been reported previously in virtually all cell lines that has been examined (Song, et al., 1991; Song, et al., 1994; Huang & Cabot, 1990). [^{3}H]-arachidonate is incorporated almost equally into both PI and PC. In cell membrane, PC is approximately 35% of the total phospholipid, but PI is only approximately 3.5% of the total phospholipid, much less than PC (Dugan, et al., 1986). The equivalent incorporation of arachidonate into PC and PI indicates that arachidonate is incorporated with high efficiency into PI. Since the v-Fps-induced increase in DG was only observed in [^{3}H]-myristate prelabeled tsNY225, which is incorporated almost exclusively into PC, the data suggest that PC is the source of v-Fps-induced DG production. The lack of an increase in DG in [^{3}H]-arachidonate labeled tsNY225 suggests that PI and arachidonate incorporated PC are not the source for v-Fps-induced DG.

Propranolol blocks v-Fps-induced DG production in tsNY225-transformed cells.

DG can be generated from PC via either PC-PLC or PLD/PAP signal pathway. In PLD/PAP signal pathway, the primary metabolite of PLD activity is PA. PA must be hydrolyzed by PAP to generate DG. PAP activity has been shown to be inhibited by propranolol (Song, et al., 1991; Billah, et al., 1990) but PC-PLC has not been reported to be affected by propranolol. Propranolol, therefore, can help us to distinguish the PLD/PAP signal pathway from PC-PLC signal pathway. If the DG generated in response to v-Fps is via PAP,

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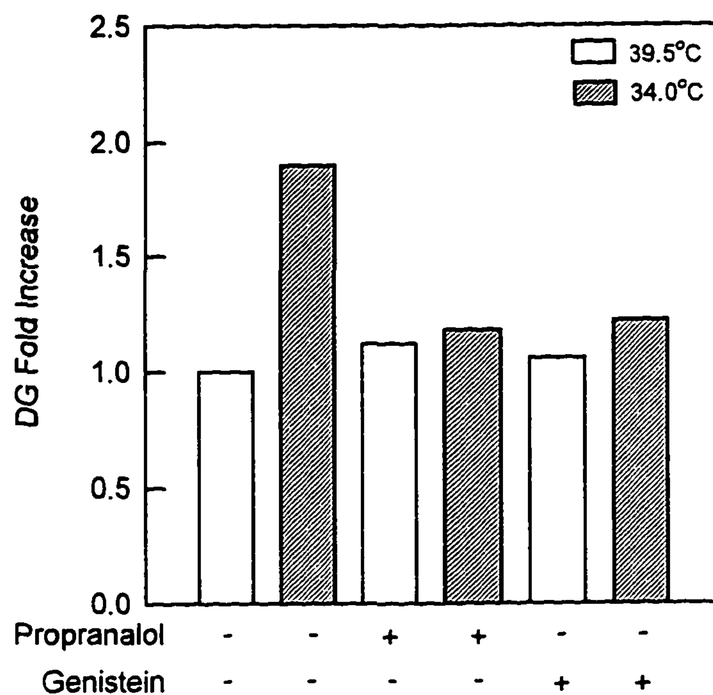

Fig. 3. The effect of propranolol and genistein on v-Fps- induced DG.
The experiments were carried out in the presence and absence of either propranolol (300μM) or genistein (150μM). Propranolol was added 5 min prior to temperature shift and genistein was added 4 hr prior to temperature shift. Cells were harvested 30 min after temperature shift. The cpm value for DG at 39.5°C was 5260 +/- 179. The data are the averages of duplicate cultures from a representative experiment using [^{3}H]-myristate as a label.

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propranolol should inhibit v-Fps induced DG production. As shown in Fig. 3, v-Fps-induced DG production was blocked by propranolol. This suggests that the DG generated in response to v-Fps was derived from PA and implicates a PLD activity that generates PA. The increased DG was also blocked by the tyrosine kinase inhibitor genistein- further suggesting that the increase in DG observed was in response to increased v-Fps kinase activity.

PA levels are elevated in response to v-Fps.

Since v-Fps-induced DG was sensitive to the PAP inhibitor propranolol, we examined PA levels in tsNY225 cells at either temperatrure relative to the parental 3Y1 cells. There was significantly higher level of PA at the permissive temperature for v-Fps relative to the non-permissive temperature for v-Fps (Table 2); however, there were also higher levels of PA at the non-permissive temperature in tsNY225 cells than there were in the parental 3Y1 cells. The higher levels of PA, like that observed for the increased DG, was only observed when the cells were prelabeled with [^{3}H]-myristate. No difference in PA levels was observed either between the permissive temperature for v-Fps or between the tsNY225 and parental 3Y1 cells when [^{3}H]-arachidonate was used to prelabel the cell (Table 2). Thus, consistent with the observation that propranolol blocks v-Fps-induced DG production, v-Fps increases PA levels.

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	$[^3H]$-PA (% total cpm)			
	$[^3H]$-myristate		$[^3H]$-arachidonate	
	tsNY225 cells	3Y1 cells	tsNY225 cells	3Y1 cells
39.5°C	0.49 +/- 0.03	0.34 +/- 0.01	0.27 +/- 0.01	0.29 +/- 0.01
34.0°C	0.64 +/- 0.02	0.32 +/- 0.01	0.29 +/- 0.03	0.26 +/- 0.02

Table 2. PA levels in tsNY225 and 3Y1 cells.
tsNY225 and 3Y1 cells were prelabeled with either $[^3H]$-myristate or $[^3H]$-arachidonate. The cells were harvested before and 30 min after shifting from the non-permissive to the permissive temperature for v-Fps. The percentage of label incorporated into PA was calculated by determining the radioactivity incorporated into PA and dividing this value by the total cpm present in the $CHCl_3$:MeOH lipid extract as in Table 1. Data are from a representative experiment performed in duplicate that was repeated three times.

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PLD activity is elevated in response to v-Fps.

The increased levels of PA in response to v-Fps and the sensitivity of v-Fps-induced DG to propranolol suggested that the source of the DG was PA, the primary metabolite of PLD activity. We therefore examined whether activating the kinase activity of v-Fps results in an increase in PLD activity. PLD exhibit a unique transphosphatidylation activity (Huang and Cabot, 1990; Kobayashi and Kanfer, 1987; Randall, et al., 1990) In this reaction, the phosphatidy group of phospholipids is transferred to a primary alcohol, ethanol or butanol, to form phosphatidylethanol or phosphatidylbutanol. The transphosphatidylation reaction has been used as an indicator of PLD activity (Song, et al., 1991;Song and Foster, 1993; Cook and Wakelam, 1992; Song, et al., 1994; Huang and Cabot, 1990; Billah, et al., 1990). This reaction has also been used to distinguish between PC-PLC and PLD signaling pathways (Huang and Cabot, 1990). Upon shift from the non-permissive to the permissive temperature for v-Fps, we detected increased transphosphatidylation (Table 3). As demonstrated for v-Fps-induced increase in PA, the effect was limited to cells prelabeled with [^{3}H]-myristate. As observed for PA levels in the tsNY225 and 3Y1 cells, there was elevated PLD activity in the tsNY225 cells relative to the parental 3Y1 cells at the non-permissive temperature for v-Fps.

Though the transphosphatidylation of PLD has been shown to be activated by EGF, v-Src and many agonists in different cell lines (Song, et al., 1991; 1994; Kaszkin et al., 1992; Pessin, et al., 1990; Cook et al., 1992

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	$[^3H]$-PBt (% total cpm)			
	$[^3H]$-myristate		$[^3H]$-arachidonate	
	tsNY225 cells	3Y1 cells	tsNY225 cells	3Y1 cells
39.5°C	0.10 +/- 0.02	0.07 +/- 0.01	0.11 +/- 0.03	0.11 +/- 0.01
34.0°C	0.14 +/- 0.01	0.07 +/- 0.03	0.12 +/- 0.01	0.11 +/- 0.02

Table 3. PLD activity in tsNY225 and 3Y1 cells.
tsNY225 and 3Y1cells were prelabeled with either $[^3H]$-myritate or $[^3H]$-arachidonate. The cells were harvested before and 30 min after shifting from the non-permissive to the permissive temperature for v-Fps. The cells were incubated in the presence of 0.7% butanol for 30 min prior to harvest. The transphosphatidylation products were separated by TLC and quantified by measuring the radioactivity incorporated into PBt. The data are expressed as the percentage of the total radioactivity incorporated into PBt. The data are mean values from a representative experiment performed in duplicate that was repeated at least three times.

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Fisher, et al., 1991), tsNY225 cells has higher transphosphatidylation activity than the parental 3Y1 cells and activation of v-Fps did not induce an increase in transphosphatidylation activity which is comparable to the v-Fps-induced increase in DG production. The most likely explanation for this phenomenon is that the temperature-sensitive defect in the v-fps gene in the tsNY225 cells has a "leaky" phenotype. While the tsNY225 cell line has a very tight temperature-sensitive phenotype for transformation at the permissive and non-permissive temperatures, it is possible that there is a threshold kinase activity for transformation that is not reached at the non-permissive temperature. The different morphology of the tsNY225 cells at non-permissive and permissive is shown in Fig. 4A. At permissive temperature (34°C), the tsNY225 cells take on a rounded refractile morphology characteristic of transformed cells; whereas at 39.5°C, the cells retain a flat non-transformed morphology that is indistinguishable from the parental 3Y1 cells. We also examined phosphotyrosine levels in 3Y1 and tsNY225 cells that had been maintained in 39.5°C and in cells that had been shifted to 34°C for 30 min. As shown in Fig. 4B, increased levels of phosphotyrosine were observed in the tsNY225 cells upon shift from the non-permissive to the permissive temperature for v-Fps. However, as expected, there were substantially higher phosphotyrosine levels in the tsNY225 cells maintained at the non-permissive temperature for v-Fps relative to the parental 3Y1 cells. Thus, the increased PLD activity and PA levels observed at the non-permissive temperature for v-Fps can be explained by an elevated kinase activity in these cells. This

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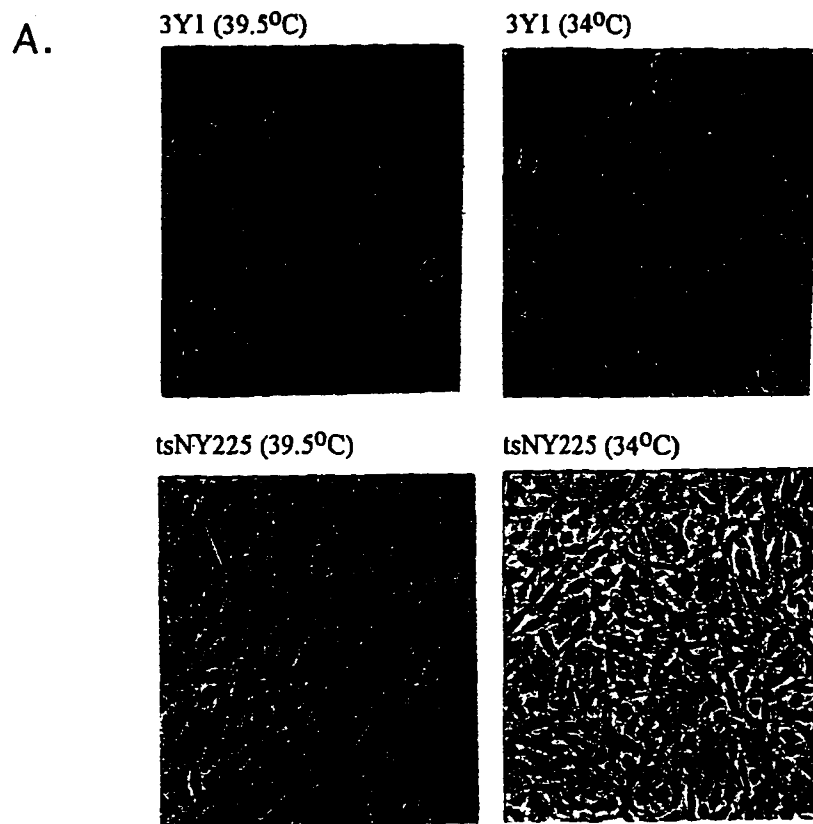

Fig. 4. tsNY225 cells have a leaky phenotype for protein-tyrosine kinase activity, but not for transformation.

A, Morphology of tsNY225 and 3Y1 cells maintained at 39.5°C or 34°C. B, Phosphotyrosine levels were determined by "Western" analysis using an antiphosphotyrosine antibody in tsNY225 and 3Y1 cells maintained at 39.5°C and in cells shifted to 34°C for 30 min.

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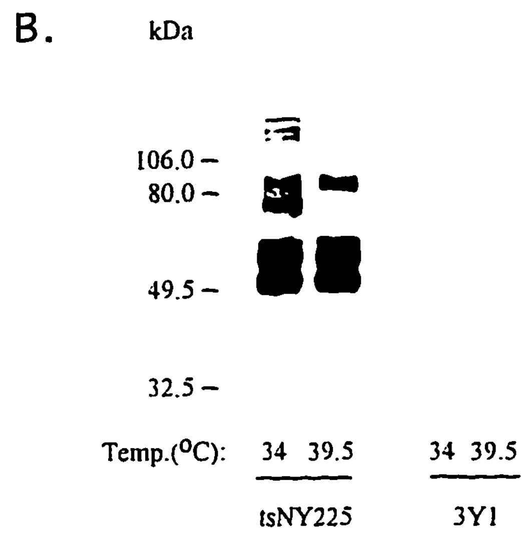

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observation suggests that the v-Fps-induced PLD activity is not a direct effect of transformation since an elevated PLD activity can be observed at the non-permissive temperature for v-Fps but the cells are not transformed at this temperature.

v-Fps activates PAP activity in tsNY225 cells.

Though the PLD activity and PA levels were both elevated in the tsNY225 cells even at the non-permissive temperature for v-Fps, cells are not transformed at non-permissive temperature. Increasing the kinase activity of v-Fps results in a propranalol-sensitive increase in DG levels without a comparable increase in PLD activity. It suggests that PAP activity may be elevated in response to increased v-Fps kinase activity. We therefore examined PAP activity in tsNY225 cells to determine whether the increased DG observed in response to temperature shift could be explained by an increase in PAP activity. There are currently no direct assays for PAP activity in intact cells. However, PAP activity can be measured *in vitro* by adding cell extracts or lysates to liposomes containing radiolabeled PA and examining the conversion of PA to DG. An *in vitro* PAP assay was developed using strategies employed by Brindley and co-workers (Jamal et al., 1991; Martin et al., 1993). tsNY225 cells maintained at the non-permissive temperature for v-Fps and shifted to the permissive temperature for v-Fps for 20 min were used to prepare for the cell homoginates. The cell homoginate PAP activity to convert PA to DG production was measured by using exogenous liposome

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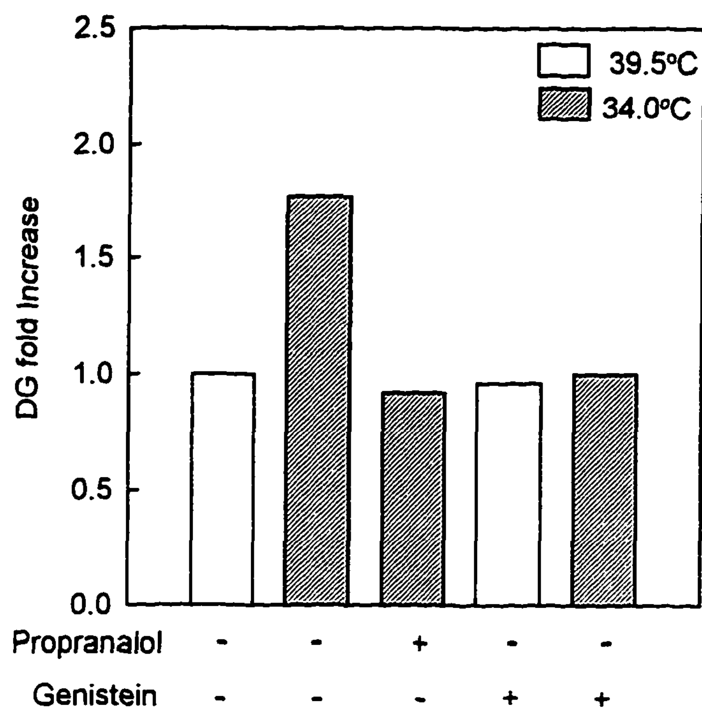

Fig. 5. PAP activity is increased in tsNY225 cells in response to temperature shift.

Cell extracts were prepared from tsNY225 cells and added to liposomes containing PA, PC and PIP_2. PAP activity was determined by measuring the DG generated in liposomes as described in Experimantal Procedures. PAP activity was determined in tsNY225 cells maintained at the non-permissive temperature for v-Fps and shifted to the permissive temperature for 20 min. The effect of v-Fps on PAP activity was also determined in the presence of propranalol (300 μM, 5 min) and genistein (150 μM, 4 hr). Data are mean values from a representative experiment performed in duplicate and repeated at least three times. The cpm value of DG with cell extract from tsNY225 in 39.5°C is 1628 +/- 115.

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containing [^{14}C]-PA. As shown in Fig. 5, shifting from the non-permissive to the permissive temperature for v-Fps led to an increase in PAP activity as measured by DG production that was comparable to the increase in DG seen in response to v-Fps *in vivo*. Since the PLD is already active at the non-permissive temperature for v-Fps, the increase in DG observed in response to v-Fps is like due to an activation of PAP activity.

v-Fps activates NEM-insensitive PAP activity in tsNY225 cells.

Two forms of PAP can be assayed specially by selective inhibition of NEM-sensitive PAP activity with NEM (Jamal, et al., 1991). NEM-sensitive PAP is stimulated by Mg^{2+}. It translocates from the cytosol to the endoplasmic reticulum in response to an accumulation of oleate and acyl-CoA ester (Brindley, 1987; Freeman and Mangiapane, 1989). NEM-insensitive PAP activity is Mg^{2+}-independent. It is located in the plasma membrane , an ideal location that can play a role in PLD signal transduction pathway (Jamal, et al., 1991). There are two forms of PAP activity in tsNY225 cells. We found that 80% of total PAP activity in membrane fraction is NEM-insensitive and Mg^{2+}-independent. PAP activity in the cytosol fraction is completely NEM-sensitive and Mg^{2+}-dependent. We, therefore, determined the NEM-insensitive PAP activity in tsNY225 cells in non-permissive temperature and shifting to permissive temperature 20 min to see whether the PAP activity is elevated in response to the activation of v-Fps. The membrane fractions from tsNY225 cells maintained at the non-permissive temperature for v-Fps and shifted to

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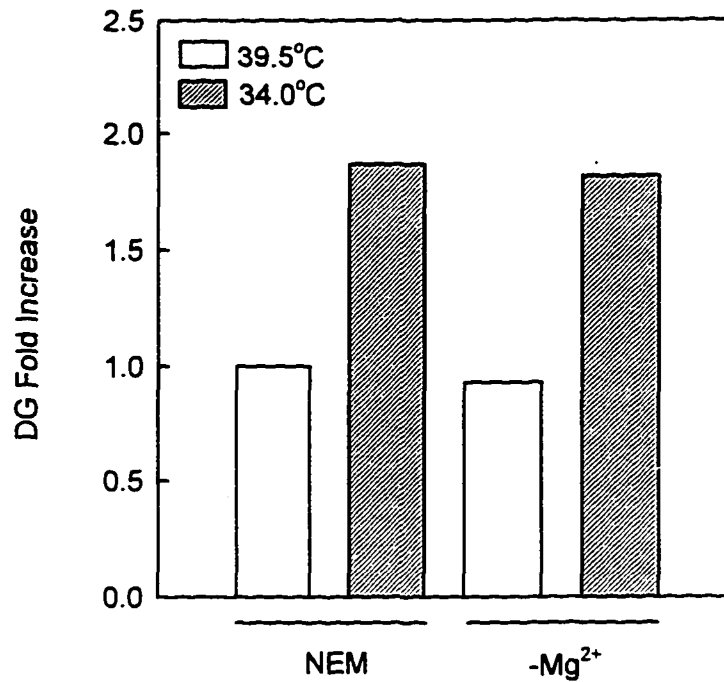

Fig. 6. NEM-insensitive PAP activity is increased in tsNY225 cells in response to temperature shift.

Cell membrane fractions were prepared from tsNY225 cells maintained at the non-permissive temperature and shifted to the permissive temperature for 20 min. The membrane fractions were treated with 2 mM NEM for 5 min in 30°C to eliminate the NEM-sensitive PAP activity. The NEM-insensitive PAP activity was determined as in Fig. 4. The effect of Mg^{2+} on NEM-insensitive PAP activity was determined in the absence of Mg^{2+}. Data are mean value from a representative experiment performed in duplicate and repeated at least three times.

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permissive temperature for v-Fps for 20 min were treated with NEM to eliminate the NEM-sensitive PAP activity. In the NEM-treated membrane fractions, the NEM-insensitive PAP activity to convert PA to DG were measured as described in Fig. 5. As shown in Fig. 6., the temperature shift to activate tyrosine kinase v-Fps resulted in an increase in NEM-insensitive PAP activity as measured by DG production that was comparable to the increase in DG production *in vivo* in response to the activation of v-Fps. The increased PAP activity is independent on Mg^{2+}. Therefore, v-Fps activates NEM-insensitive PAP activity which conjugates with PLD to regulate the DG production in tsNY225 cells.

DISCUSSION

PLD has recently been implicated in the transduction of intracellular signals initiated by protein-tyrosine kinase (Song, et al., 1991; 1993; 1994; Plevin et al., 1991; Fisher, et al., 1991; Cook and Wakelem, 1992; Kaszkin et al., 1992). We report here that PLD is constitutivly activated in tsNY225 cells transformed by the oncogenic protein-tyrosine kinase v-Fps. The primary metabolite of PLD is PA, which can be metabolized to DG by PAP. Increasing the kinase activity of v-Fps in tsNY225 cells leads to a propranalol-inhibiting DG increase. The data suggests that v-Fps may activate PAP activity to generate an increase in DG production from PLD-generated PA. Consistent with this data, PAP activity is elevated in cell extracts from tsNY225 cells that had been transferred from the non-permissive to the permissive temperature.

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v-Fps-activated PAP is NEM-insensitive and located in the membrane. The data confirm the hypothesis that NEM-insensitive PAP in the membrane in an important site to regulate the DG production via PLD/PAP signaling pathway. Thus, the induction of DG by v-Fps involves the concerted activation of two phospholipid metabolizing enzymes, PLD and PAP. Metabolism of PC by PLD/PAP signaling pathway may be important for the mitogenic transforming properties of protein tyrosine kinase.

The v-Fps-induced DG production was only observed when the cell were prelabeled with [^{3}H]-myristate which is incorporated almost exclusively into PC in tsNY225 cells. No increase in DG was observed when cells were prelabeled with [^{3}H]-arachidonate, which is incorporated with high efficiency into PI. Thus, the v-Fps induced DG is most likely not come from PLC-γ mediated hydrolysis of PI-4,5-bisphosphate. The observation that [^{3}H]-arachidonate is also incorporated into PC and that no arachidonate labeled DG is generated suggests that v-Fps-activated PLD is specific for a species of PC lacking arachidonic acid. Different aliphatic DG species are differentially metabolized by DG kinase to PA (Ford and Gross, 1991). DG produced from PC contains short saturated fatty acid and is a poor substrate for DG kinase, but DG produced from PI has arachidonate and is quickly converted to PA by DG kinase (Lee, et al., 1991). Thus, DG produced from PC via PLD/PAP signaling pathway could sustain the activation of PKC and give a more prolonged effect on cell growth than DG from PI. PLD/PAP signaling pathway may play a major role in cell growth and transformation in

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response to the activation of tyrosine kinase receptors and tyrosine kinase oncogenes. On the other hand, it has been suggested that different PKC isoforms could be differentially activated by different DG species (Leach, et al., 1991; Kolesnick and Paley, 1987). Therefore, the different aliphatic composition of DG and its phospholipid precursors may be used by cells to achieve signaling specificity in response to the activation of tyrosine kinase receptors and tyrosine kinase oncogenes.

Martin et al. (1993) recently reported that PLD and PAP are coordinately regulated in rat fibroblasts transformed by v-Fps and v-HaRas. Levels of PAP was reported to be lower in the transformed cells than in their non-transformed cells. This result might reflect the long term regulation of PAP activity at the level of enzyme synthesis in oncogene transformed cell lines. Our data indicate that PAP activity is elevated in response to an increase in the kinase activity of v-Fps. This is an initial response to the activated v-Fps kinase activity but not a long term response to the transformed phenotype. The cytosolic PAP membrane translocation has been described as a short term regulation of PAP activity in response to oleate stimulation (Brindley, 1984). This short term regulation of PAP activity mostly occurs in the endoplasmic reticulum and is involved in regulating triacylglycerol synthesis (Brindley, 1984; 1987). We found that v-Fps-induced increase in PAP activity is NEM-insensitive. It suggests that cytoplasmic NEM-sensitive PAP does not translocate to the plasma membrane in response to activation of v-Fps. In our experiment, we did not find membrane

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translocation of cytoplasmic PAP (data not shown). Our data suggest a novel short term regulating mechanism of PAP activity other than its membrane translocation. This novel mechanism might be important in regulating DG production in PLD/PAP signaling pathway. Regardless, both our data and Martin's results indicate that PAP can be regulated at the levels of both protein expression and enzyme activity. This PAP is correlated with the control of cell proliferation.

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Part II:

Regulation of PAP by EGF: Reduced Association with EGFR Followed by Increased Association with PKC ε

In tsNY225 temperature sensitive cell lines, it is difficult to maintain accurate temperature. The temperature sensitive mutant is apparently leaky for PLD activation. Thus, tsNY225 cell line is not an idea system to study the regulatory mechanism of PLD/PAP signaling pathway in detail. In A431 human epidermoid carcinoma-derived cell line, which overexpresses EGF receptors, EGF also induces a sustained increase in DG production via the PLD/PAP signal transduction pathway. PLD is activated by EGF stimulation (Song, 1994). It is not clear whether PAP is activated by EGF. To further establish PAP as a regulatory site for DG production, we investigated the effect of EGF on PAP activity in A431 cells. It is postulated that the v-Fps-encoded protein tyrosine kinase may inappropriately phosphorylate signaling proteins that are involved in receptor tyrosine kinase induced signal transduction pathways, and stimulate the cell to proceed in unrestrained proliferation (Hunter and Cooper, 1995; Foster, 1993). Thus, the study of receptor tyrosine kinase on PAP activity may shed light on the effect of v-Fps on PAP activity.

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RESULTS

PAP activity in A431 cells.

In A431 cells, EGF induces a PC-specific PLD activity with kinetics that parallel EGF-induced DG derived from PC. This implies that the PA generated by the action of PLD is converted to DG by PAP. Consistent with this result, propranalol, which inhibits the conversion of PA to DG, inhibits EGF-induced DG production (Song et al., 1994). We therefore wanted to determine whether PAP activity is also regulated by EGF in A431 cells. In A431 cells, PAP activity could be detected in both the cytosol and membrane fractions (Table 4). Unlike two forms of PAP in tsNY225 cells, we found that virtually all of the PAP activity in A431 cells, both the membrane and cytosol, was sensitive to NEM (Table 4). In A431 cells, PAP activity from both the membrane and cytosol was dependent upon Mg^{2+}. These properties are characteristic of the PAP previously designated PAP1 (Jamal et al., 1991). Thus, unlike tsNY225 cells, we were unable to detect significant biochemical differences between the membrane and cytosolic PAP activities in A431 cells. As expected, the PAP activity was sensitive to the amphiphilic cations, propranolol, chlorpromazine and sphingosine, which have been shown previously to inhibit PAP activity (Koul and Houser, 1987; Jamal et al., 1991).

It has been reported that liposome composition can also affect *in vitro* PAP activity (Jamal et al., 1991). We therefore examined the effect of the phospholipid composition of the liposomes used in the PAP assay. In addition to PA, PIP_2 was somewhat stimulatory. The addition of

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	PAP activity $[^{14}C]$-DG (cpm/min/mg protein x 10^{-3})			
	Membrane		Cytosol	
Liposomes only	0.05 +/- 0.01		0.03 +/- 0.01	
Complete	2.78 +/- 0.09	(1.00)	0.84 +/- 0.12	(1.00)
- Mg^{2+}	0.10 +/- 0.0	(0.04)	0.13 +/- 0.09	(0.15)
-Ca^{2+}	2.74 +/- 0.13	(0.99)	0.96 +/- 0.07	(1.14)
+ NEM	0.05 +/- 0.01	(0.02)	0.03 +/- 0.01	(0.04)
heat	0.23 +/- 0.08	(0.08)	0.14 +/- 0.04	(0.16)
+ propranolol	0.51 +/- 0.23	(0.18)	n.d.	
+ sphingosine	0.05 +/- 0.01	(0.02)	n.d.	
+ chlorpromazine	0.11 +/- 0.02	(0.04)	n.d.	

Table 4. PAP activity in A431 cells.
PAP activity in membrane and cytosolic fractions from A431 cells was determined as described in Experimental Procedures. The complete assay was performed by using liposomes containing only PA, PC and PIP_2 as shown in Table 5. The effect of Mg^{2+}, or Ca^{2+}, NEM (2 mM), and heat denaturation (55°C, 20 min) is shown. The effect of the amphiphilic cations propranolol (2 mM), sphingosine (0.1 mM), and chlorpromazine (0.3 mM) on membrane PAP activity was determined by inclusion of three compounds in the liposome preparations. The relative effects are shown in parentheses after normalizing to the PAP activity found in the complete assay mix. The data represent the average of duplicates (+/- range) from a representative experiment repeated three times. n.d., not determined

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	PAP Activity [^{14}C]-DG (cpm/min/mg protein x10^{-3})	
Liposome Composition	-EGF	+EGF
PA only	1.07 +/- 0.17 (1.0)	1.59 +/- 0.10 (1.5)
PA + PC (1.0 : 2.0)	1.18 +/- 0.10 (1.1)	1.72 +/- 0.12 (1.6)
PA + PC + PIP2 (1.0 :2.0 :1.4)	3.40 +/- 0.15 (3.2)	5.62 +/- 0.24 (5.3)
PA + PC + PIP2 + DG (1.0 : 2.0 : 1.4 : 0.2)	2.13 +/- 0.14 (2.0)	5.76 +/- 0.33 (5.4)
PA + PC + PIP2 + PS (1.0 : 2.0 : 1.4 : 6.0)	4.13 +/- 0.30 (3.9)	6.15 +/- 0.46 (5.8)
PA + PC + PIP2 +PS +DG (1.0 : 2.0 : 1.4 : 6.0 : 0.2)	5.28 +/-0.36 (4.9)	6.57 +/- 0.40 (6.1)

Table 5. Effect of Liposome Composition and EGF on PAP Activity from A431 Cells.

The effect of phospholipid composition and EGF (100nM) on PAP activity was investigated as shown with the relative concentration of the phospholipids used in generating the liposomes given in parentheses. The relative effects on PAP activity are shown in parentheses after normalizing to the PAP activity found using liposomes containing PA only in the absence of EGF. The PAP assay was performed as in table 4. By using cell lysate. The data represent the average of duplicates (+/- range) from a representative experiment that was repeated twice.

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phosphatidylserine (PS) and DG was also stimulatory for PAP activity (Table 5).

PKC is involved in EGF-induced PAP activity.

It has been known that lipids can function as activators of signal transduction proteins. PS and DG are PKC activator (Nishizuka, 1992). PIP_2 interact with some unknown factor to activate PLD activity (Brown et al., 1993; Liscovitch, et al., 1994). EGF treatment of A431 cells can induce an increase of PAP activity in cell lysate. The EGF stimulated PAP activity varied depending on the different liposome compositions (Table 5). Since PS and DG can increase PAP activity, it suggests that PKC is the most possible candidate that regulates PAP activity. We therefore examined whether EGF can still induce an increase of PAP activity after PKC activity has been blocked in A431 cells. Chelerythrine chloride inhibits the catalytic domain of PKC, and shows competitive kinetics with the PKC substrate (histone IIIS) for phosphorylation (Herbert, 1990). A431 cells were pretreated with chelerythrine chloride, and stimulated with EGF. PAP activity in cell lysate was measured as described in table 5. Chelerythrine chloride pretreatment blocked EGF induced increase in PAP activity in cell lysate (Fig. 7). In order to confirm that the inhibition of PAP activity is not due to a direct inhibiting effect on PAP by chelerythrine chloride itself, we measured the cell lysate PAP activity with liposome which contains chelerythrine chloride. Chelerythrine chloride itself has no directly inhibition effect on PAP activity (data not shown).

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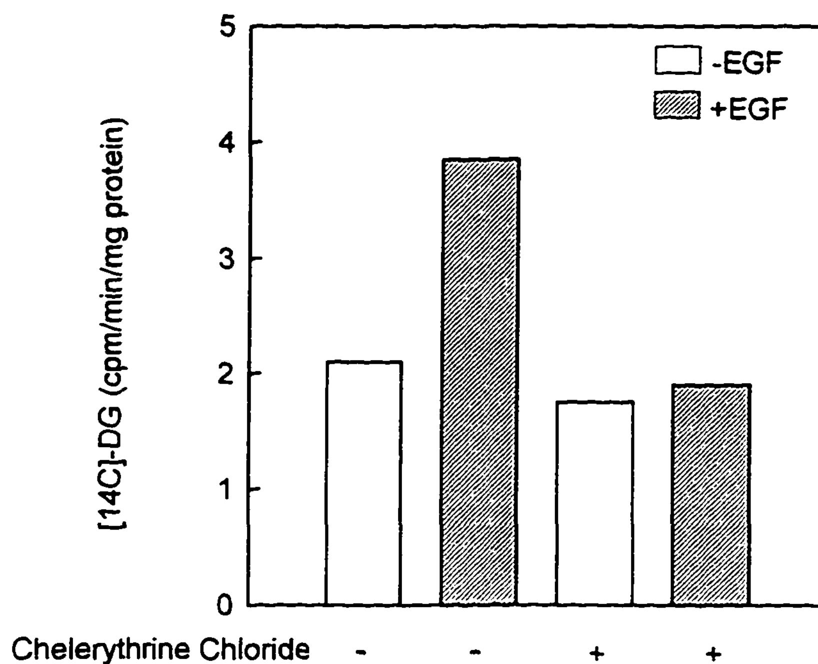

Fig. 7. The effect of chelerythrine chloride on EGF-induced PAP activity. PAP activity in complete cell lysate was determined with liposome containing PA, PC, PIP_2 and DG as described in table 5. Chelerythrine chloride (10 μM) was added into cell medium 12 hr prior to EGF stimulation. The data are the average of duplicates from a representative experiment that was repeated three times.

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PAP becomes associated with PKC ε in response to EGF.

Since inhibition of PKC by chelerythrin chloride blocks EGF-induced increase in PAP activity, it suggests that PKC is involved in regulation of PAP activity. Besides protein phosphorylation by PKC, a number of proteins that interact with PKC isoforms have been described (Chapline et al., 1996). We therefore investigated whether PAP activity could be detected in association with any of the PKC isoforms present in A431 cells. The predominant PKC isoforms present in A431 cells are the α, ε, and ζ isoforms (our unpublished results). We developed an *in vitro* assay to measure the PAP activity in PKC immunoprecipitates from membrane lysates of A431 cells that had been either treated or untreated with EGF. The PKC antibody immunoprecipitates were recovered and mixed with liposomes containing PC, PA, PIP_2, PS and DG (unless otherwise indicated). The PAP activity in PKC isoform immunoprecipitates were determined by measuring the conversion of PA to DG. As shown in Fig. 8A, a very strong EGF-dependent PAP activity was detected in PKC ε, but not PKC α or ζ immunoprecipitates. In order to confirm that PAP activity coimmunoprecipitated with PKC ε antibody is specific for PKC ε, we preincubated PKC ε antibody with a PKC ε peptide against which the antibody was raised. As shown in Fig. 8B, the preincubation prevented precipitation of PAP with the PKC ε antibody. The association of PAP activity with PKC ε occurred at 1 min after EGF treatment, and sustained for at least 30 min (Fig. 8C). The kinetics of association of PAP with PKC ε was parallel

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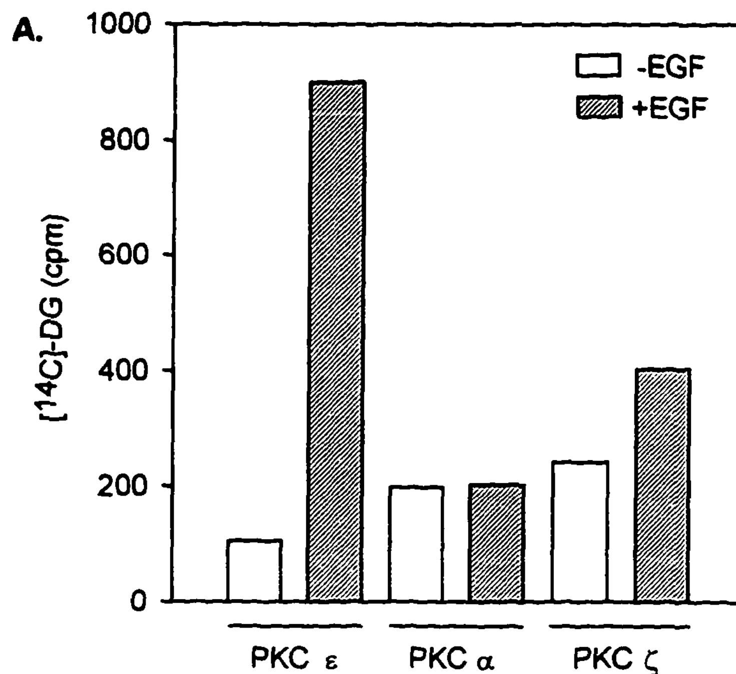

Fig. 8. PAP activity can be co-immunoprecipitated with PKC ε after EGF treatment.

(A). The membrane fraction of A431 cells that were either untreated or treated with EGF (100 nM, 5min) were harvested and then lysed with 1% triton X-100. The lysate was then incubated with antibodies specific for the α, ε, and ζ isoforms of PKC (Transduction Laboratories) (12 hr, 4°C). The Antigen-antibody complexes were recovered with protein A agarose for PKC α and ε, and protein G agarose for PKC ζ. The recovered antigen-antibody complexes were added directly to the complete liposome mixture as described in table 5 and PAP activity in immunoprecipitates was determined as described in Experimental Procedures. (B). To establish that the effect was specific for PKC ε, a PKC ε-specific peptide against which the antibody was raised was included in the immunoprecipitation and the ability to immunoprecipitate PAP activity was determined. (C). The time course for association of PAP activity with PKC ε after EGF treatment was determined as shown. The data are presented from a representative experiment that was repeated 3 times.

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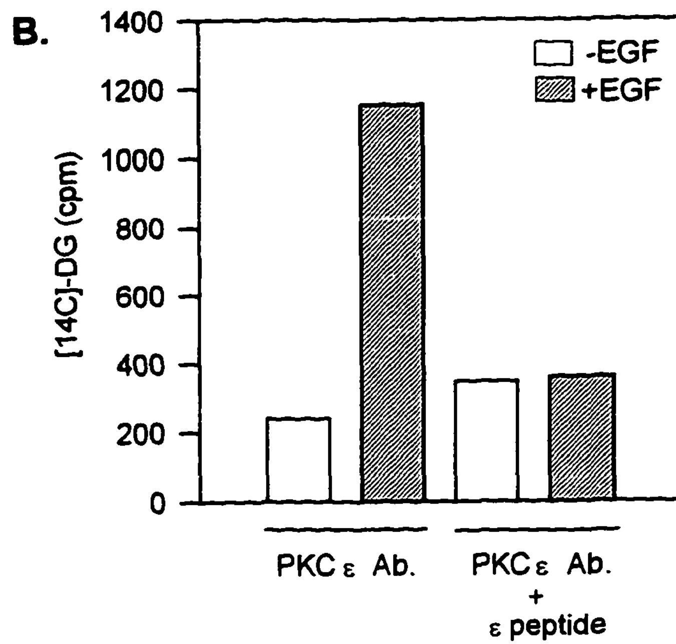

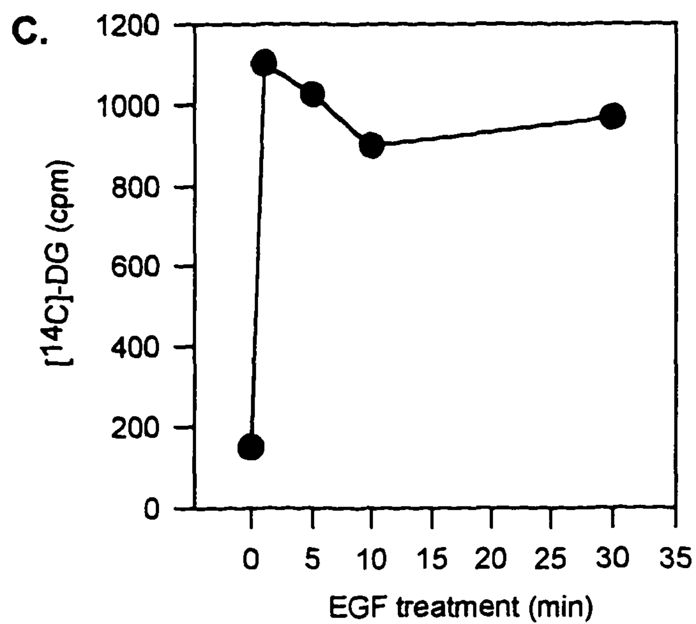

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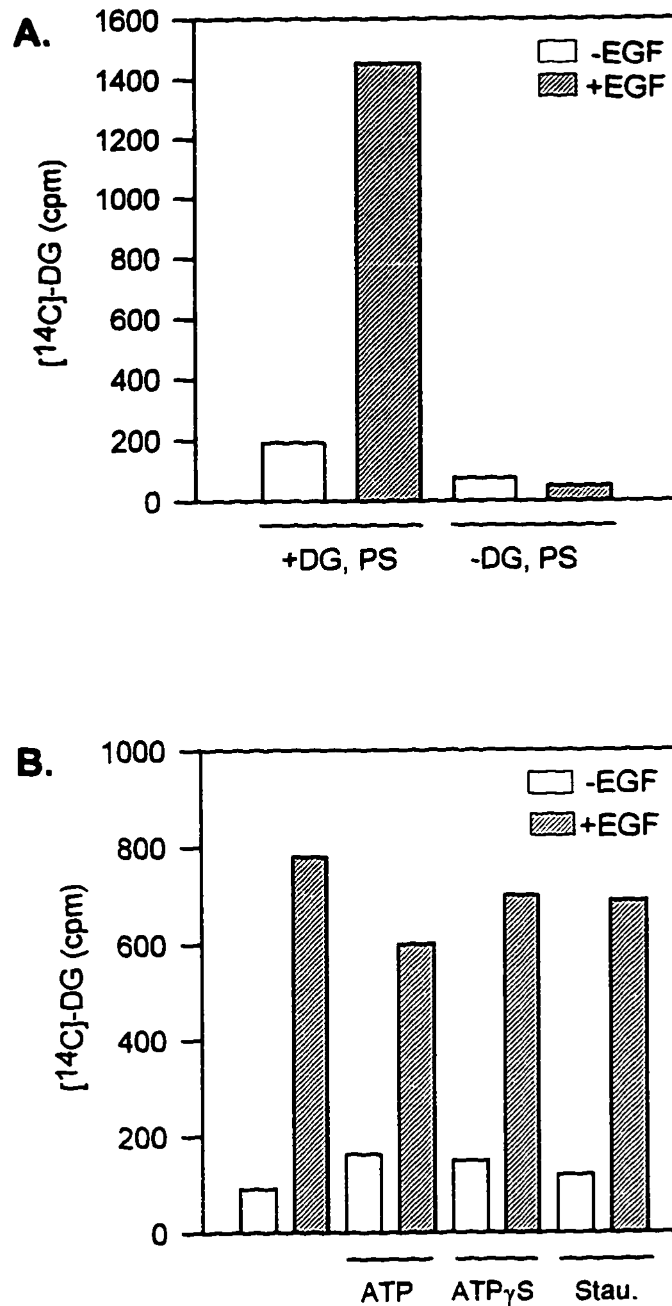

Fig. 9. PKC ε-associated PAP activity is dependent upon DG and PS, but independent of ATP.

The PAP activity associated with PKC ε was examined using liposomes containing and lacking the PKC co-factors DG and PS as shown (**A**). The effect of ATP (0.1 μM), ATPγS (0.1μM), and staurosporine (70 nM) on the PKC ε-associated PAP activity is also presented (**B**).

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to the kinetic of EGF-induced DG production in A431 cells (Song, et al., 1994).

The *in vitro* PAP activity from A431 cells was significantly enhanced when PS and DG were included in the liposomes used in the PAP assay (Table 5). We therefore examined the effect of DG and PS in the liposomes when the PKC ε-associated PAP activity was determined. In the absence of DG and PS, we were unable to detect any PAP activity in the PKC ε immunoprecipitates in response to EGF (Fig. 9A). The DG and PS requirement suggests that PKC ε activation is important in the *in vitro* liposome assay. However, as shown in Fig. 9B, there was no effect of ATP on the PAP activity associated with PKC ε, suggesting that the PKC ε kinase activity is not required. Consistent with this result, staurosporine, which inhibits PKC kinase activity by competing for ATP binding, also had no effect on the *in vitro* PAP activity in PKC ε immunoprecipitates (Fig. 9B). These surprising results suggest that while the PKC co-factors DG and PS are required to observe the PKC ε-associated PAP activity *in vitro*, the kinase activity of PKC ε is apparently not required for the *in vitro* PAP activity.

PAP activity is associated with the EGF receptor in A431 cells.

Since the EGF receptor has previously been demonstrated to form complexes with signaling molecules involved in lipid second messenger generation (Foster, 1993; Cantley, et al.,1991; Ullrich and Schlessinger, 1990), we tested PAP activity in EGF receptor immunoprecipitates.

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Membranes lysate were prepared from EGF-treated and untreated A431 cells and incubated with antibodies raised against the EGF receptor. The immunoprecipitates were recovered and PAP activity was determined. As shown in Fig. 10A, EGF treatment induces a decrease of PAP activity in the EGFR immunoprecipitates. In response to EGF stimulation, the loss of PAP activity in EGFR immunoprecipitates was both time (Fig. 10A) and dose dependent (Fig. 10B). The loss of PAP activity in EGFR immunoprecipitates was detectable within 1 min and at approximately 1nM EGF. Since the EGF receptor antibodies are raised against the EGF binding site, we had to further confirm that the reduced PAP activity in EGF receptor antibody immunoprecipitates was not due to incapability of the EGF receptor antibodies to precipitate dimerized EGF receptors. Membrane lysates from EGF treated and untreated A431 cells were incubated with EGF receptor antibodies. The quantity of immunoprecipitated EGF receptor was determined with EGF receptor antibodies in western blot. As shown in Fig. 10C, there were no differences in the amount of EGF receptor precipitated from the EGF-treated and untreated cells. The data suggested that either the PAP activity was reduced or the PAP protein was released from the receptor in response to EGF. Since we could detect overall increases in total PAP activity in response to EGF, we considered the first possibility unlikely. Thus, the data in Fig. 10. suggest that the PAP associated with the EGF receptor is released in response to EGF treatment. Since the kinetics of EGF-induced PAP dissociation from EGF receptor is parallel to the kineties of EGF-induced PAP

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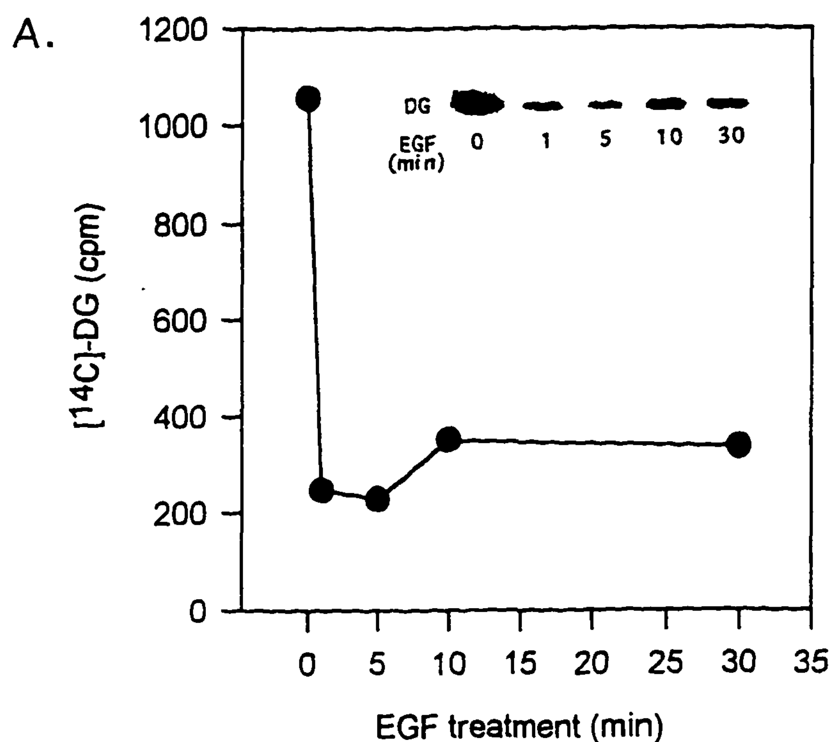

Fig. 10. PAP activity in EGF receptor immunoprecipitates.
(A). The membrane fraction from A431 cells that were either untreated or treated with EGF (100 nM) for the indicated times were harvested and then lysed with 1% Triton X-100. The lysate was then incubated with antibodies specific for the EGF receptor (Oncogene Science) (12 hr, 4°C). Antigen-antibody complexes were recovered with protein G agarose and added directly to the complete liposome mixture described in Table 5 and PAP activity in immunoprecipitates was determined as described in Experimental Procedures. (B). The dose response for the effect seen in (A) was determined as shown. (C). The amount of EGF receptor immunoprecipitated in EGF-treated and untreated A431 cells was determined by western blot analysis. The data is presented from a representative experiment that was repeated 3 times.

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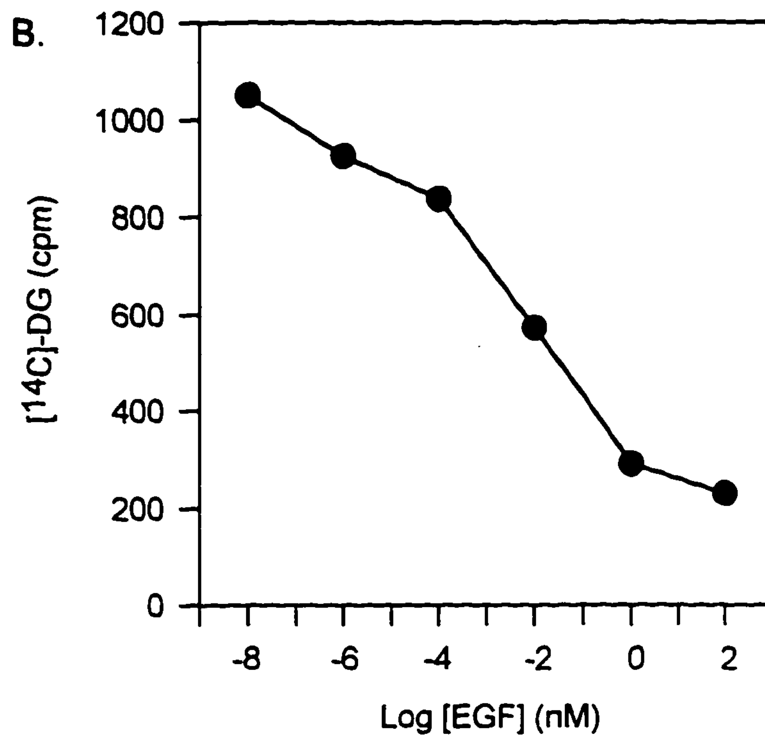

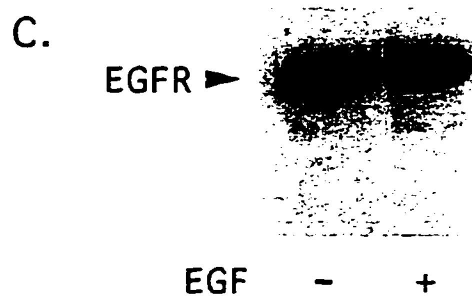

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association with PKCε, it suggests that EGF induces PAP to dissociate from EGFR and form a complex with PKCε.

AG1478 induces a decrease in PAP activity in EGFR immunoprecipitates

In order to further establish that the PAP, which dissociates from EGFR and associates with PKC ε, does regulate EGF-induced DG production in PLD/PAP signaling pathway,we tried to find some inhibitors which can either inhibit PAP dissociation from EGFR or block PAP association with PKC ε. AG1478 is a specific inhibitor of EGFR tyrosine kinase. In A431 cells, AG1478 pretreatment blocks EGF-stimulated receptor tyrosine kinase for autophosporylation (Fry, et al., 1994; Levitzki and Gazit, 1995). We examined the effect of AG1478 on EGFR-associated PAP activity in vivo. The membrane lysate were prepared from A431 cells pretreated with AG1478 before EGF stimulation, and incubated with EGF receptor antibodies. The immunoprecipitates were recovered and mixed with liposomes to measure PAP activity as described in Fig. 10. AG1478 pretreatment dramatically decreased the PAP activity in the EGFR immunoprecipitates from both EGF treated and untreated A431 cells (Fig. 11). In order to confirm that the decreased PAP activity in the immunoprecipitates from AG1478 pretreated cells is not due to any inhibitory effect of AG1478 itself, we directly examined the inhibitory effect of AG1478 on PAP activity in the immunoprecipitates with liposomes containing AG1478. We found that AG1478 itself has no direct inhibitory effect on PAP activity in the immunoprecipitates (data not shown). Therefore, the AG1478-induced decrease of PAP activity in EGFR immunoprecipitates is not due to inhibitory effect of AG1478 on PAP activity. AG1478 may induce PAP to dissociate from EGFR to decrease PAP activity in EGFR.

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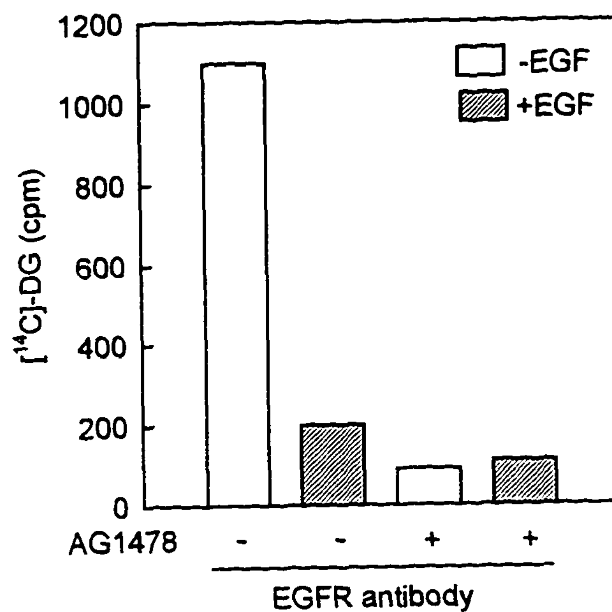

Fig. 11. PAP activity in EGFR immunoprecipitates from AG1478 pretreated cells.
AG1478 was added in cell medium 2 hr prior to EGF stimulation. PAP activity in EGFR immunoprecipitates was determined as in Fig. 10. The data is presented from a representative experiment that was repeated at least three times.

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AG1478 induces PAP translocation from the membrane to the cytosol.

In order to further establish that AG1478 induces PAP to dissociate from membrane EGFR, we determined PAP activity in the membrane and cytosol fractions from AG1478 treated and untreated A431 cells. The membrane and lytosol fractions were prepared and mixed with liposome to measure PAP activity as described in exprimentanl procedures. We found that AG1478 treatment causes a decrease of PAP activity in the membrane fractions and a corresponding increase of PAP activity in the cytosol fractions (Fig. 12). The data suggest that AG1478 causes PAP to translocate from the membrane EGFR to the cytosol.

AG1478 blocks EGF-induced PAP association with PKC ε.

EGF induces PAP to dissociate from EGFR and associate with PKC ε. Since AG1478 cause PAP to translocate from membrane EGFR to the cytosol, we examined whether AG1478 has any effect on EGF-induced PAP association with PKC ε. The membrane lysate were prepared from A431 cells pretreated with AG1478, and incubated with PKC ε antibodies. The PAP activity in PKC ε immunoprecipitates was determined as described in Fig. 8. Upon AG1478 pretreatment, AG1478 blocked the EGF-induced increase of PAP activity in PKC ε immunoprecipitates (Fig. 13). By using liposome containing AG1478, we confirmed that the loss of PAP activity in PKC ε immunoprecipitates was not due to the direct inhibitory effect of AG1478 on PAP activity (data not shown). The data suggest that AG1478 may block the PAP association with PKC ε. Combining the effect of AG1478 on PAP activity in PKC ε and EGFR immunoprecipitates with its effect on PAP activity in the membrane and cytosol fractions, we proposed a model that AG1478 induces

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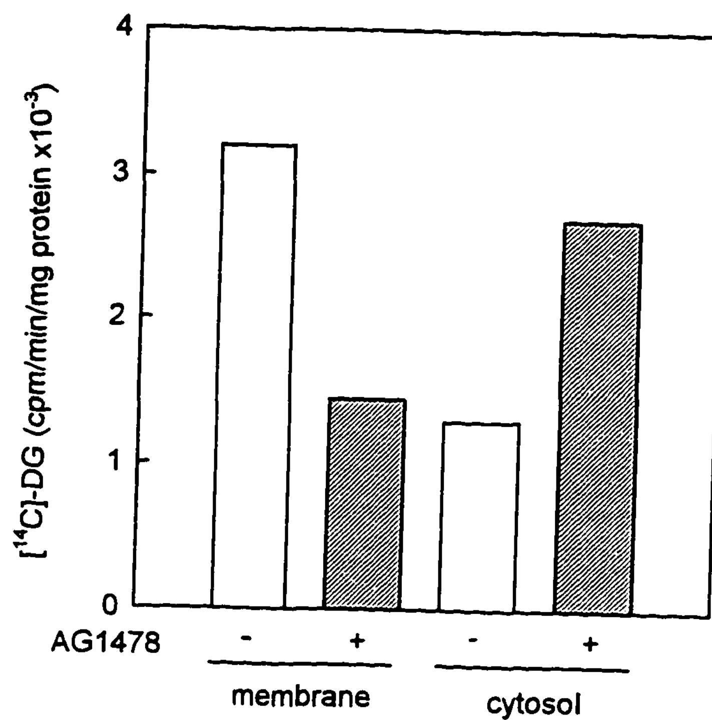

Fig. 12. The effect of AG1478 on PAP activity in membrane and cytosol fractions.

Cells were treated with AG1478 as described in Fig. 10. PAP activity in membrane and cytosolic fractions was determined as described in Experimental Procedures. The data are the average of duplicates from a representative experiment that was repeated twice.

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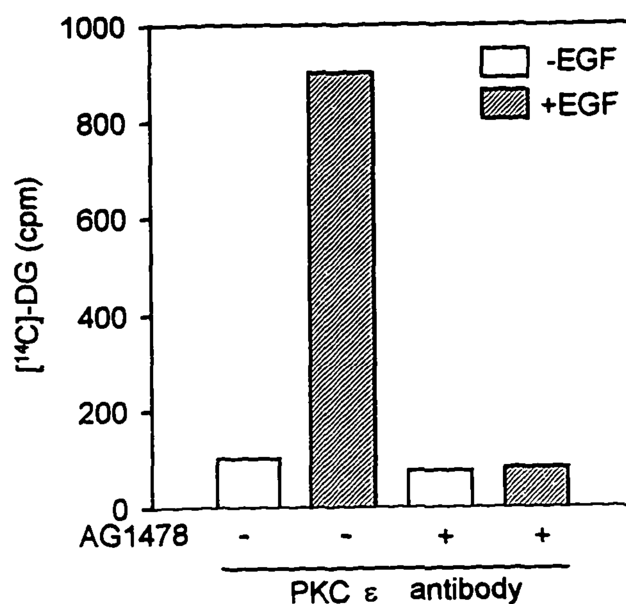

Fig. 13. PAP activity in PKC ε immunoprecipitates from AG1478 pretreated cells.

AG1478 was added in cell medium 2 hr prior to EGF stimulation. PAP activity in PKC ε immunoprecipitates was determined as in Fig. 8. The data is presented from a representative experiment that was repeated at least three times.

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PAP to translocate from membrane EGFR to the cytosol and blocks PAP association with PKCε.

AG1478 inhibits EGF-induced DG production.

Since AG1478 pretreatment decreases the PAP activity in PKC ε immunoprecipitates. We made use of this effect to examine whether EGF-induced PAP association with PKC ε is critical in regulating EGF-induced DG production in PLD/PAP signaling pathway. If PKC ε-associated PAP really regulates the DG production from PA, AG1478 pretreatment to block EGF-induced PAP association with PKC ε should inhibit EGF-induced DG production. We pretreated A431 cells with AG1478 and measured DG levels in response to EGF stimulation as described previously (Song et al., 1994). As shown in Fig. 14, AG1478 pretreatment inhibited EGF-induced increase in DG production. The EGF-induced membrane translocation of cytosolic PKC was also blocked by AG1478 pretreatment (data not shown). The data suggest that it is the PKC ε-associated PAP activity that regulates the EGF-induced DG production in PLD/PAP signaling pathway.

DISCUSSION

Upon EGF treatment, PLCγ is activated which generates DG (Margolis, et al., 1989). This would lead to the observed increase in membrane localization of PKC ε in response to EGF. Our data suggest a novel PKC-involved signal transduction pathway in which EGF induces PAP to dissociate from dimerized EGF receptor and to associate with membrane bound PKC ε through a mechanism that remains to be determined. This model is shown schematically in Fig. 15. The kinetics of the EGF-induced PAP dissociation from EGF receptor and association with PKC ε parallels the kinetics of

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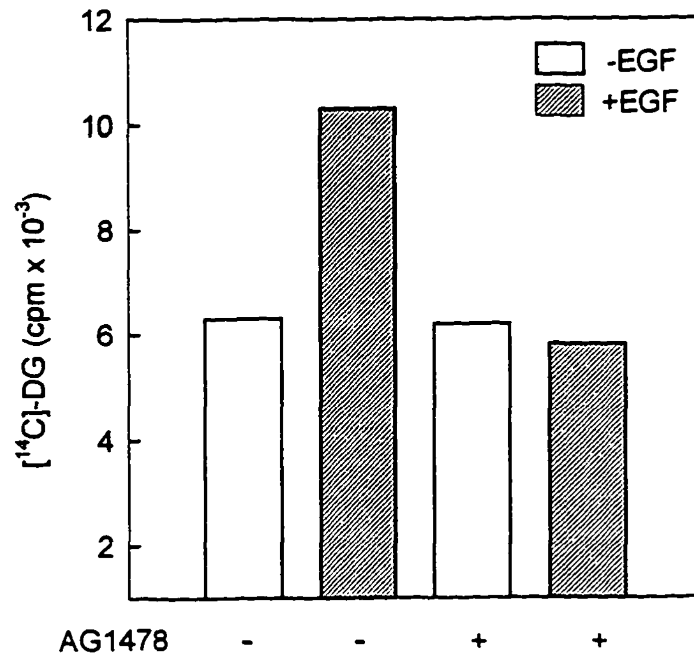

Fig. 14. The effect of AG1478 on EGF-induced DG production.
A431 cells were prelabeled with [^{3}H]-myristate. Cells were pretreated with AG1478 as described in Fig. 10 and levels of DG production were determined as described in Experimental procedures. The data are average values for duplicates from representative experiments.

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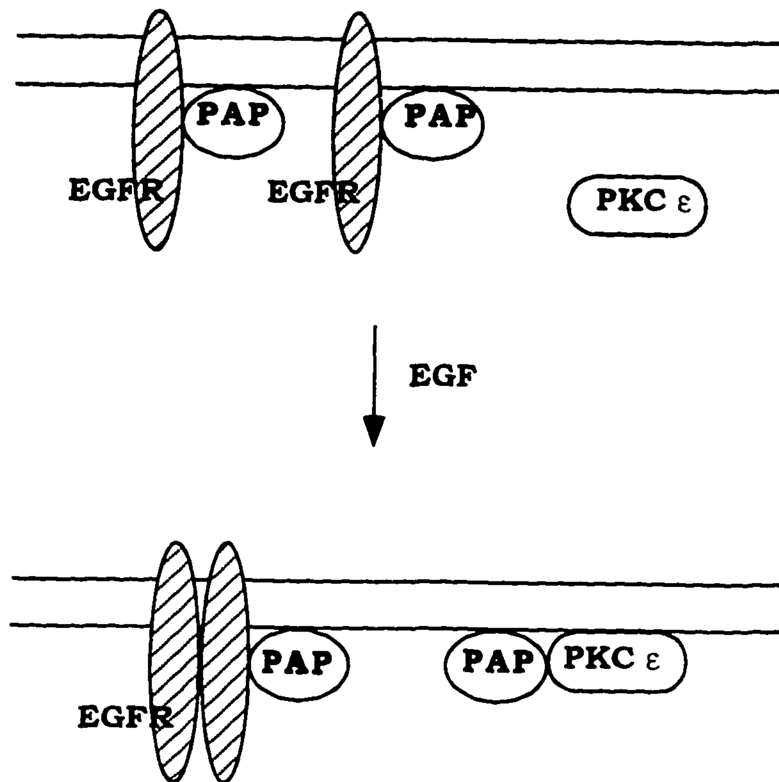

Fig. 15. A model for EGF regulation of PAP activity.
Upon stimulation by EGF, the EGF receptor (EGFR) dimerizes which changes the affinity of EGFR for PAP. PKC ε which becomes membrane associated in response to EGF is then able to associate with PAP. It is not yet clear whether the proposed interaction are direct or indirect. The role of PKC ε may be to facilitate access of PAP to its substrate PA.

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EGF-induced PLD activity. It also correlates well with EGF-induced increase in DG production from the PLD-derived PA that has been reported previously (Song et al., 1994). AG1478 inhibits EGF-induced DG production via inducing PAP to dissociate from membrane EGFR and translocate to the cytosol, and blocking PAP association with PKC ε. The data indicate that PAP is an important regulating site for EGF-induced DG production in PLD/PAP signaling pathway.

Upon EGF binding, EGFR dimenizes and activates its tyrosine kinase for autophosphorylation. EGF also induces PAP to dissociate from EGFR. It is of interest to determine whether EGF activated receptor tyrosine kinase is critical for EGF-induced PAP dissociation from EGFR. In A431 cells, AG1478 blocks EGF-stimulated receptor tyrosine kinase for autophosphorylation (Fry, et al., 1994; Levitzki and Gazit, 1995). We found that AG1478 decreases the PAP activity in EGFR immunoprecipitates from EGF treated or untreated A431 cells. It seems that AG1478-induced inhibiting of EGFR tyrosine kinase and decreasing of PAP activity in EGFR immunoprecipitates are contrary to EGF-induced activating of EGFR tyrosine kinase and decreasing of PAP activity in EGFR immunopecipitates. AG1478 is a quinazoline (Levitzki and Gazit, 1995). We consider that, besides its inhibiting effect on receptor tyrosine kinase, AG1478 may function to directly interact with EGFR or PAP to block the formation of EGFR and PAP complex in the membrane. Thus, AG1478 limits us to clearly define whether EGFR tyrosine kinase activity is required for PAP dissociation from EGFR. We may have to overexpress tyrosine kinase deficient EGFR to characterize the tyrosine kinase requirement for EGF-induced PAP dissociation.

NEM is a thiol reagents. It reacts with the side chain of Cysteine residue. Since NEM-sensitive PAP in A431 cells regulates DG production, it

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suggests that Cysteine residues of PAP is essential for the biological function of PAP. However, NEM treatment has no effect on PAP function to regulate DG production in tsNY225 cells. The possible reason for this paradox is that the functional Cysteine residues of PAP in tsNY225 cells may be covered by some proteins in a signaling complex where PAP functions as a component to generate DG. The covered side chain of Cysteine residues are protected from chemical modification by NEM. Thus, the unmodified Cysteine residues of PAP in tsNY225 cells can still play their biological function in the presence of NEM. It is not a good criterion to distinguish two forms of PAP by NEM. We have to purify PAP to further characterize whether the PAP in tsNY225 and A431 cells is the same form or two different forms.

The requirement for PS and DG in order to see the PKC ε-associated PAP activity suggests a requirement for PKC activity. However, since neither ATP, ATPγS, nor the ATP analog staurosporine has any direct effect on the PKC ε-associated PAP activity, It suggests that PKC may have kinase-independent roles in the regulation of intracellular signals. In this regard, it is of interest that PKC has been shown to stimulate PLD activity via an ATP-independent mechanism (Conricode, et al., 1992: Singer, et al., 1996). It is possible that PS and DG are functioning to localize PKC ε and the associated PAP to the liposomes where the PA substrate is present. PKC ε may serve to either allosterically modify PAP or only localize PAP to its PA substrate on membrane. PKC ε may also localize PAP in a molecular complex where PAP functions as a component of a signaling complex that generates DG. PAP-generated DG further activates other PKC isoforms. The activated PKCs phosphorylate their substrates and stimulate cell growth and differentiation.
PS and DG also have some stimulatory effect on PAP activity from EGF-untreated cell lysate. We have to determine whether this effect is due to the

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activation of PKC by PS and DG or the direct stimulatory effect of PS and DG on PAP activity. PKC antibodies can be used to immunoprecipitate PKC and clear PKC from the cell lysate. If PS and DG have no stimulatory effect on PAP activity in PKC-precleared cell lysate, it suggests that PS and DG may activate PKC to increase PAP activity in the cell lysate. If PAP activity in PKC-precleared cell lysate can be activated by PS and DG, it suggests that PS and DG may have some direct stimulatory effect on PAP activity. Then, EGFR immunoprecipitates may be used to test the direct effect of PS and DG on PAP activity in the presence and absence of PS and DG in the liposome.

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SECTION III

Summary

Activation of v-Fps tyrosine kinase induces a sustained two fold increase in [^{3}H]-myristate labeled DG production, but not [^{3}H] arachidonate labeled DG production. PC and PI are two major phospholipid sources for lipid second messenger DG. [^{3}H]-myristate is incorporated almost exclusively into PC and [^{3}H]-arachidonate is incorporated almost equally into PC and PI. The differential fatty acid labeling of PC and PI helped us to identify PC as the source of v-Fps-induced DG production. PC can either be hydrolyzed by PC-PLC to generate DG directly or be hydrolyzed by PLD to generate PA which is then converted to DG. PC-PLC and PLD/PAP signaling pathways can be distinguished by two methods. (1) Propranalol inhibits PAP activity, thus blocks DG generation from PLD/PAP signaling pathway, but not DG generation from PC-PLC signaling pathway (Song, et al., 1991; Billah, et al., 1989). (2) Transphosphatidylation is used to distinguish PLD from PC-PLC (Huang and Cabot, 1990). We found that propranolol blocks v-Fps-induced DG production in tsNY225 cells; and transphosphatidylation indicated that there is elevated PLD activity in tsNY225 cells relative to parental 3Y1 cells at the permissive and non-permissive temperature for v-Fps. Consistent with increased PLD activity, there are higher levels of PA in tsNY225 cells than there are in 3Y1 cells at the permissive and non-permissive temperature. These data suggest a constitutive activation of PLD in tsNY225 cells. Thus, the PLD activity in tsNY225 cells does not parallel the v-Fps-induced two fold increase in DG production. However, since propranolol blocks v-Fps-induced DG production , PAP may be activated in response to the kinase activity of v-Fps and convert the PLD-generated PA to DG. We therefore examined PAP

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activity in response to activation of tyrosine kinase v-Fps. PAP activity is increased approximately two fold in response to v-Fps activation, which is comparable to the increase in v-Fps-induced DG production. These data indicate that v-Fps activates both PLD and PAP to generate an increase in DG production.

In tsNY225 temperature-sensitive cell lines, it is difficult to maintain accurate temperature and the temperature sensitive mutant is apparently leaky for PLD activation. Thus, tsNY225 cell line is not an ideal system to study the regulatory mechanism of PLD/PAP signaling pathway in detail. In A431 cells, EGF also induces a sustained increase in DG production via the PLD/PAP signal transduction pathway. PLD is activated by EGF stimulation (Song, 1994). It is not known whether PAP is activated by EGF. We found that upon EGF stimulation, PAP activity in A431 cell lysates is elevated. PKC inhibitors block the EGF-induced increase in PAP activity. These data suggest that PKC is involved in regulating PAP activity. We investigated whether PAP activity could be detected in immunoprecipitates of different isoforms of PKC. Upon EGF stimulation, there is a sustained increase in PAP activity in PKC ε immunoprecipitate, but not in PKC α, or PKC ζ immunoprecipitates. Since ATP, ATPγS and staurosporine have no effect on the PAP activity in the PKC ε immunoprecipitate, PKC ε kinase activity is not required for the EGF-induced PAP activity in the immunoprecipitate. In contrast to the increase in PAP activity in PKC ε immunoprecipitates, EGF induces a corresponding decrease in PAP activity in EGFR antibody immunoprecipitates. The kinetics of increased PAP activity in PKC ε antibody immunoprecipitate and corresponding decreased PAP in EGFR antibody immunoprecipitate is comparable to the kinetics of EGF-induced DG production. These data suggest that the EGF-induced PAP activity may

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contribute to regulate EGF-induced DG production. AG1478 induces a dramatic decrease in PAP activity in both EGFR and PKC ε immunoprecipitates. Concomitant with the loss in PAP activity in the immunoprecipitates, AG1478 correspondingly induces a decrease in membrane PAP activity and an increase in cytosolic PAP activity. These data suggest that AG1478 induces PAP translocation from membrane EGFR to the cytosol and blocks PAP association with membrane PKC ε. Consistent with a decrease in PAP activity in membrane EGFR and PKC ε, AG1478 blocks EGF-induced increase in DG production and EGF-induced PKC membrane translocation. The data implicate that the PAP activity, which dissociates from EGFR and associates with PKC ε in response to EGF stimulation, play an important role in regulating DG production in PLD/PAP signaling pathway. We propose a novel mechanism for EGF-regulated PAP activity in the PLD/PAP signaling pathway in which EGF induces the dissociation of PAP from EGFR and association with PKC ε. PKC ε localizes PAP to its PA substrate.

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SECTION IV

EXPERIMENTAL PROCEDURES

Cells and Cell Culture Conditions.

A431 cells and 3Y1 rat fibroblasts obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum (GIBCO). tsNY225-transformed 3Y1 expressing a temperature-sensitive v-Fps was obtained from Teruko Hanafusa and maintained as 3Y1 rat fibrablasts.

Materials.

[^{3}H]-Myristate (NET-830), [^{3}H]-arachidonate (NET-298Z) and [^{14}C]-dipalmitol PA (NEC-799) were obtained from New England Nuclear. PI, PC, PA, and DG standards were obtained from Sigma. PBt and PEt were obtained from Avanti Polar Lipids. Thin layer chromatography (TLC) plates (Silica gel 60A) were from Scientific Products. EGF was obtained from Calbiochem. Propranolol, chlorpromazine, sphingosine, PS and PIP_2 were obtained from Sigma. Antibodies raised against the EGF receptor was obtained from Oncogene Science and antibodies raised against PKC isoforms were obtained from Transduction Laboratories.

Extraction of phospholipase products and characterization of phospholipid metabolites by TLC.

Confluent 60 mm culture dishes were prelabeled for 24h in 5 ml of media containing 0.5% newborn calf serum. Isotopes were added to the culture media as follows: [^{3}H]-myristate, 5 μCi (40Ci/mmol); [^{3}H]-arachidonate,

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2 μCi (240Ci/mmol). Cells were washed twice with isotonic tris-saline buffer and rapidly treated with 0.6 ml of MeOH:6N HCl (50:2; v/v). Lipids were extracted by the addition of 0.6 ml of $CHCl_3$. Phase separation was obtained by adding 200 μl of 1M NaCl. The organic phase was reextracted with 0.6 ml of 0.35M NaCl and 0.2 ml of MeOH:6N HCl (50:1; v/v), dried under a stream of nitrogen and redissolved in $CHCl_3$:MeOH (90:10); v/v). Phospholipid metabolites were characterized by TLC as described previously (Song, et al., 1991). The following solvent systems were used : For DG, hexane:diethylether:MeOH:HAc (90:20:3:2; v/v); For PC and PI, $CHCl_3$:MeOH:HAc:H_2O (50:25:8:4; v/v); for PBt, PEt and PA, the upper phase of ethylacetate:trimethylpentane:HAc:H_2O (90:50:20:100; v/v). Lipid standards were visualized by treating TLC plates with iodine vapor. To quantitate metabolically labeled compounds, appropriate regions of TLC plates were scraped and counted in a scintillation counter.

In vitro PAP activity.

PAP activity was determined using procedures developed by Brindley and co-workers (Jamal et al.,1991; Martin et al.,1993) as follows: Cells were grown to confluence in 150 mm culture dishes and then placed in serum-free media overnight as described previously (Song et al., 1994). The cells were then washed in cold isotonic buffer, scraped from the plates, and suspended in 2 ml of hypotonic buffer A (20 mM Tris-HCl, pH 7.3; 5 mM Na_2HPO_4; 1 μM $ZnCl_2$; 0.2 mM PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotinin; 400 μM Na_4VaO_4; 1 mM dithiothreotol). The suspended cells were then broken by Dounce homogination (15 strokes with type B pestle). Disrupted cells were centrifuged at 500 x g for 10 min to clear nuclei and unbroken cells. The post-nuclear homogenate was used as the whole cell lysate. The post-nuclear

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homogenate can be separated into membrane and cytosolic fractions by centrifugation at 100,000 x g for 60 min. The supernantent is saved as the cytosolic fraction. The membrane fraction is recovered from the pellet by resuspending in buffer A. Protein levels were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Liposomes were made by mixing lipids in chloroform, drying under a stream of nitrogen resuspending in assay buffer (20 mM Tris-HCl (pH = 7.3), 5mM Na_2HPO_4, 140 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$) with vortexing and then sonicating for 3 min as described previously (Jiang et al., 1995). Unless otherwise indicated, the liposome suspension was 5 μg/ml dipalmitoyl-PA, 10 μg/ml PC (from egg yolk), 10 μg/ml PIP_2, 30 μg/ml PS, 3 μ g/ml DG and 20,000 cpm [^{14}C]-dipalmitoyl-PA (100 Ci/mM). The sonicated solution was kept at room temperature for 2 hours to equilibrate.

The PAP assay was initiated by the addition of 10 μl homogenate (20 μ g protein) to prewarmed (37°C) liposome suspension. Where appropriate, fatty acid-free bovine serum albumin was added to maintain constant protein concentration (1mg/ml) in the reaction mixture. The final volume of the reaction mixture was 140 μl. The mixture was incubated for 15 min at 37°C unless otherwise indicated. The reaction consumed less than 20% of the [^{14}C]-PA. The reaction was terminated by the addition of 800 μl chloroform/methanol (6:2; v/v). The mixture was vortexed and the organic phase was recovered and dried under a stream of nitrogen. DG and PA was resolved simultaneously by using DG and PA solvent systems sequentially: First, the TLC plate was run half way up in PA solvent, then dried the plate. Second, the plate was run all way up in DG solvent.

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Co-immunoprecipitation of PAP.

A431 cell membrane fractions were resuspended with buffer A containing 1% triton X-100 and 140 mM NaCl. Insoluble material was cleared by centrifugation (12,000 x g, 30 min). The 1 ml of supernatant (I mg protein) was then incubated with the indicated antibody (2 μg) overnight at 4°C with shaking. The antigen/antibody complexes were then recovered with 20 μl protein A sepharose suspension (Santa Cruz Biotechnology) (2 hr, 4°C with shaking) by microcentrifugation and washing four times in buffer A containing 1% triton X-100 and 140 mM NaCl. Immunoprecipitates were then added to the liposomes and PAP activity was determined as described previously.

Western analysis of tyrosine-phosphorylated proteins and EGFR.

Extraction of proteins from cultured cells was previously described (Spangler, et al., 1989). Transfer to nitrocellulose, blocking and washing of filter was performed as decricbed previously (Joseph, et al., 1992). Nitrocellulose filters were then incubated with an antibody raised against phosphotyrosine or EGFR (Transduction Laboratories). Detection of the antibody was accomplished using the ECL system (Amersham) according to the vendors instructions.

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SECTION V

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