INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

NOTE TO USERS

This reproduction is the best copy available.

UMI®

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Differential Regulation of Mammalian Phospholipase D isoforms and Their Functional Involvement in Receptor-Mediated Endocytosis and Oncogenic Invasion

by

Yingjie Shen

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

2001

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

UMI Number: 3024834

UMI®

UMI Microform 3024834
Copyright 2002 by Bell & Howell Information and Learning Company.
All rights reserved. This microform edition is protected against
unauthorized copying under Title 17, United States Code.

Bell & Howell Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

9/7/01
Date

Chair of Examining Committee
Dr. David Foster, Hunter College

9/17/01
Date

Executive Officer
Dr. ~~Richard L. Chappell~~

Dr. Thomas H. Haines, City College

Dr. Jesus Angulo, Hunter College

Dr. Thomas Schmidt-Glenewinkel, Hunter College

Dr. Dennis Sheilds, Albert Einstein College of Medicine of Yeshiva University

Supervising Committee

The City University of New York

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Abstract

Differential Regulation of Mammalian Phospholipase D isoforms and Their Functional Involvement in Receptor-Mediated Endocytosis and Oncogenic Invasion

by: Yingjie Shen

Advisor: Dr. David A. Foster

Phospholipase D (PLD) activity is elevated in response to a wide range of extracellular signals. By hydrolyzing the major membrane lipid phosphatidylcholine and generate potential second messenger phosphatidic acid, PLD mediates a variety of cellular physiology.

The research presented in this thesis focused on the enzymatic regulation and cellular functions of mammalian PLD1 and PLD2. These studies have revealed that epidermal growth factor (EGF) and tyrosine kinase v-Src can each elevate PLD activity in rodent fibroblasts, and that PLD2 and PLD1 are sequentially activated and differentially regulated in response to EGF stimulation. Data are also presented demonstrating that both PLD1 and PLD2 mediate EGF-induced receptor endocytosis and the mitogenic signals that are dependent on this process. And finally, it is shown that PLD2 is required for Src-induced cell protrusion and the related motility and oncogenic invasiveness.

These data suggest that PLD participates not only in mitogenic signal transduction, but in morphological transformation and metastasis of tumor cells as well, possibly through facilitating vesicle formation and cytoskeleton reorganization. The differential functions of PLD1 and PLD2 are also discussed.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Acknowledgments

The first words of gratitude go to my parents, who inspired my dream of being a scientist ever since I was a child, who have nurtured me with their love and care, and who have been my first and always teachers of science and life.

I thank my sister who sparked my interest in biology and led me into the field.

I thank my mentor Dr. David Foster for his advice, support, and the words he said to me on the first day I joined his lab: 'I don't want you to be a technician, I want you to be a scientist.' It was these words that enlivened me to overcome the fear of lacking lab experience and to always think not only over the projects but also beyond the projects.

I thank Dr. Thomas Schmidt-Glenewinkel for admitting me into this Ph.D. program several years ago and for his help on experiments.

I thank Dr. Thomas Haines not only for he urged me to read more literature, from which I've benefited ever since, but also because he encouraged me not to be afraid of ignorance but to always be ready to learn.

I thank Yang Zheng, Lizhong Xu and Roger Wooden, for their collaboration and help.

I am grateful to Ms. Romia Bull, for her extreme patience and for spending a tremendous amount of time helping me with English.

Finally, special thanks to Joan Zhang, Grace and Mike Hammond, and Jin Cai for their care and support..

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table of Contents

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

List of Tables

List of Schemes and Figures

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

List of Abbreviations

AP2	Clathrin Assembly / Adaptor Protein Complex 2
AP3	Clathrin Assembly Protein or Adaptor Protein 3
ARF	Adenosine-diphosphate Ribosylation Factor
BFA	Brefeldin A
1-BtOH	1-Butanol
t-BtOH	tertiary-Butanol
ChOH	Choline
CR	Conserved Region
CT, C-terminus	Carboxyl Terminus
DG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular-signal Regulated Kinase
GDP	Guanosine-diphosphate
GDS	Guanine-nucleotide Dissociation Stimulator

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

GTP	Guanosine-triphosphate
GTPase, G protein	GTP-binding Protein
GAP	GTPase Activating Proteins
GEF	Guanine-nucleotide Exchange Factor
kDa	kiloDalton
Lyso-PA, LPA	Lyso-Phosphatidic Acid
MAP	Microtubule Associated Protein
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
NGF	Nerve Growth Factor
N-terminus	Amino Terminus
PA	Phosphatidic Acid
PAP	Phosphatidic Acid Phosphatase
PBS	Phosphate Buffered Saline
PBt	Phosphatidylbutanol
PC	Phosphatidylcholine
PDGF	Platelet-Derived Growth Factor
PH Domain	Pleckstrin Homology Domain
PI4-P5Kα	phosphatidylinositol 4-phosphate 5 kinase alpha
PIP_2	Phosphatidylinositol-4,5-bisphosphate

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PIP_3	**Phosphatidylinositol-3,4,5-trisphosphate**
PKC	**Protein Kinase C**
PLA_2	**Phospholipase A_2**
PLD	**Phospholipase D**
PC-PLD	**Phosphatidylcholine-Specific Phospholipase D**
PX Domain	**Phox Domain**
SDS-PAGE	**Sodium-Dodecyl-Sulfate Polyacrylamide-Gel-Electrophoresis**
TGF	**Transforming Growth Factor**

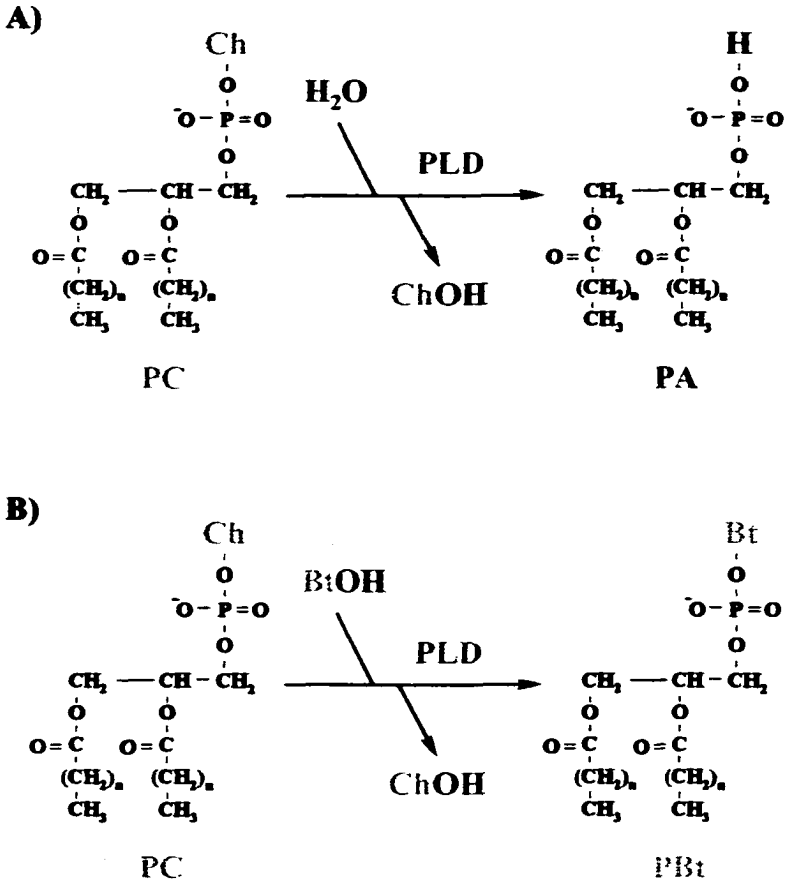
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 1 Introduction

1.1 Enzymology of Phospholipase D.

Phospholipase D (PLD) (EC 3.1.4.4) is an enzyme that catalyzes the hydrolysis of glycerophospholipids into phosphatidic acid (PA) and a water-soluble headgroup (1) (Scheme 1.1). PLD activities characterized from different sources exhibit substrate specificities for various phospholipids, such as phosphatidylcholine (PC), phosphoinositides, phosphatidylethanolamine, phosphatidylserine, lyso-phosphatidylcholine or lyso-phosphatidylserine (2). The PC-specific PLD (PC-PLD) hydrolyses PC to phosphatidic acid (PA) and choline (ChOH), and can also catalyze a transphosphatidylation reaction whereby the enzyme preferentially utilizes a short-chain primary alcohol over water to substitute the polar headgroup and generate phosphatidylalcohol instead of PA (3) (Scheme 1.1). The resultant phosphatidylalcohols are produced only by PLDs, and are not normally found in biological membranes. Because of their unique origin, their low basal levels and their relative metabolic stability, the formation of phosphatidylalcohols in the presence of primary short-chain alcohols has served as a sensitive and unambiguous marker for PLD activity and thus provided a convenient approach to probe the involvement of PLD in various regulatory processes and functional studies. In contrast, secondary or tertiary alcohols are not utilized by PLD and have been used as negative controls for nonspecific effects of the alcohols.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



Scheme 1.1 Phospholipase D catalyzed reactions.

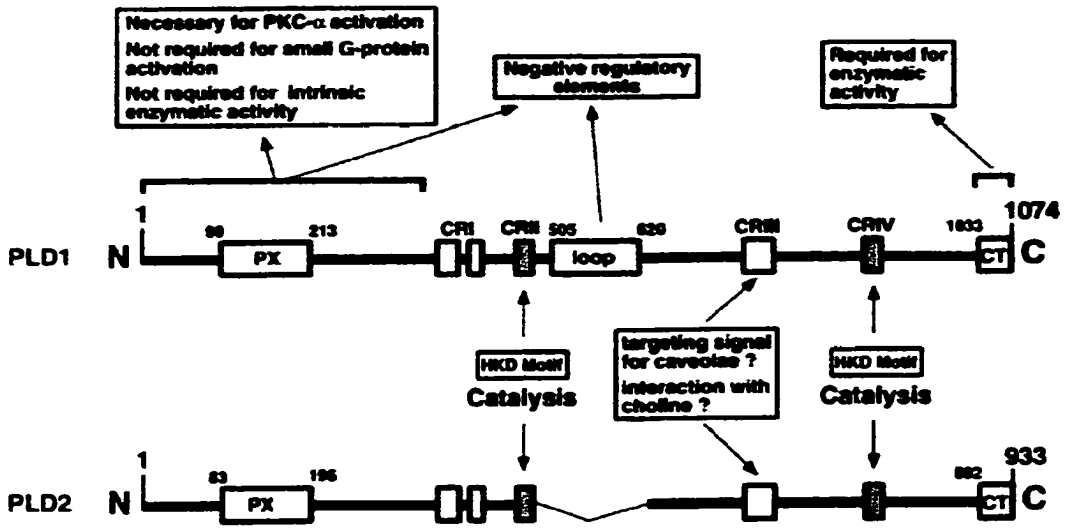
A) PLD hydrolyses the distal phosphodiester bond in phospholipids such as PC, using H_2O as an electron donor to generate PA and choline. B) Through the same mechanism but using short-chain primary alcohols such as 1-butanol (1-BtOH) instead of H_2O as the electron donor, PLD can catalyze the transphosphatidylation reaction and generate phosphatidylalcohol, e.g., phosphatidylbutanol (PBt) instead of PA. Secondary or tertiary alcohols are not preferentially utilized by PLD.

1.2 Mammalian PLD1 and PLD2 belong to a PLD Superfamily of Conserved Motifs.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PLD is an evolutionally well-conserved enzyme, and has been found in a variety of species ranging from bacteria to humans (2). A PLD gene superfamily characterized by a consensus HKD motif (also called transphosphatidylation domain) comprises PLDs from mammals, nematodes, flies, fungi, plants and bacteria, and also the non-PLD phosphatidyltransferases / phospholipid synthases, endonucleases, certain viral envelope proteins and their mammalian counterparts, which all share the HKD motif and a similar mechanism of enzymology (2,4). PLDs of this superfamily have a substrate-specificity for PC and catalyze both hydrolysis and transphosphatidylation reaction (4). To date, several of such PLD genes have been isolated from bacteria, plants and fungi, and recently, two mammalian PLD genes were cloned, named PLD1 (5-11) and PLD2 (10-14). Like all other PC-PLDs from the HKD superfamily, mammalian PLD1 and PLD2 share a relatively conserved catalytic core, which is composed of four conserved regions (CR I-IV) (Scheme 1.2). Both PLD1 and PLD2 also contain a putative Phox (PX) domain (15) and a weakly conserved pleckstrin homology (PH) domain (10,16,17) in the N-terminal region, as well as a conserved C-terminal motif (15). The overall identity between these two isforms is about 50% (9,11-14). Most of the heterogeneity comes from a unique loop region at the center of PLD1 that is not present in PLD2, and otherwise from the less conserved N- and C-terminal regions, which contributes to different enzyme properties of these two isoforms (15,18).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



Scheme 1.2 Conserved and unique features for mammalian PLD1 and PLD2.

The PLD amino acid sequences encode regions that are either unique to PLD1 (loop region) or that are conserved among mammalian PLDs and most PLDs from non-mammalian species (other boxed regions). A putative PH domain (not shown here) is located between the PX domain and CR I. CR II and CR IV are the transphosphatidylation domains also known as consensus HKD motifs. Possible functions that have been proposed or demonstrated for these regions are listed. CR, conserved region; CT, carboxyl terminus; PH, pleckstrin homology domain; PX, phox domain. Adopted from Sung et. al. J Biol Chem 1999 Jan 1;274(1):494-502 (15).

1.3 Intrinsic Properties and Enzymatic Regulation of Mammalian PLD1 and PLD2.

PLD1 is about 120 kDa in size (5,8) with low basal activity (5-8,15,18,19), while PLD2 has a size of 106 kDa (13,14) and is constitutively active *in vitro* (12,14,15). Both PLD1 and PLD2 activities are dependent on phosphoinositides such

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

as phosphoinositol 4,5 bisphosphates (PIP_2) (5-7,12-14,18,19), and inhibited by unsaturated fatty acids such as oleate (5,7,8,13). The two isoforms exhibit very different features in respect to the regulation of enzyme activity, as will be discussed in details in Chapter 3. In mammalian cells, the PIP_2-dependent PLD activity has been found to be activated by a wide variety of extracellular stimuli (2), however, in may cases it is still not known whether the elevated activity is due to PLD1 or PLD2.

1.4 Tissue Distribution and Subcellular Localization of Mammalian PLD1 and PLD2.

PLD is widely distributed in almost all mammalian tissues and cell types (20). The expression of PLD1 and PLD2 varies within tissues and between cell lines. A single cell type can express one, both, or neither isoforms of PLD, although most cells co-express PLD1 and PLD2 (20). The mRNA levels of PLD1 and PLD2 also vary considerably between cell types and are apparently regulated by growth and differentiation factors (20).

Subcellular fractionation and immunocytochemistry studies with either endogenous or overexpressed recombinant PLD1 and PLD2, have revealed that although activities can be sometimes found in cytosol (5,7,13,21-23), both isoforms are predominantly membranes associated. However, the primary sequence of either isoform reveals no obvious transmembrane domain (24). Thus, there must be some membrane-interaction modules that can recruit PLDs to the cellular membranes where their phospholipid substrates are. The identities of such possible modules are still unknown.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PLD1 or PLD1-like activity have been found primarily in various intracellular membranes, including the Golgi apparatus (12,21,25-27), endoplasmic reticulum (12,21,26,28), secretory granules (26,29,30), early endosomes (31), late endosomes (12,32), lysosomes (29,32,33), and nuclei (21,25,34,35); it was also found in the plasma membrane (21,25,26,30,36), and caveolin-enriched light microdomains (37-39). PLD2 exclusively localizes on plasma membrane or caveolin-enriched light microdomains under basal conditions (12,36,39-43); stimulation of cells resulted in redistribution of PLD2 into putative early endosomes (12).

To some degree, the localization in various cellular compartments of the two isoforms reflects their multifarious cellular functions.

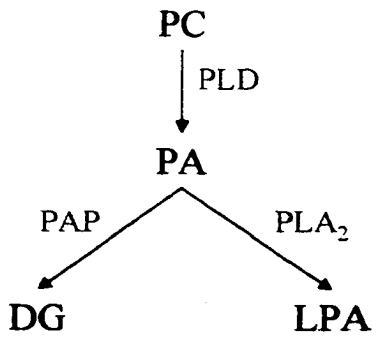
1.5 Physiological Functions of PLD.

Phospholipase D has been implicated in a variety of cellular processes, such as intracellular vesicular trafficking, endocytosis and exocytosis (44-46), proliferation and tumor promotion (47-49), cell survival and apoptosis (50), differentiation (50,51), cell senescence (52) and cytoskeleton reorganization (53). However, in spite of the ubiquitous involvement of this enzyme, the mechanism of how PLD participates these cellular events is not clear.

PLD utilizes PC, the major phospholipid in mammalian cell membranes to produce PA and choline. PA can be dephosphorylated by a PA phosphatase (PAP) to generate diacylglycerol (DG) (54-58); alternatively, it can be converted into lyso-phosphatidic acid (lyso-PA or LPA) by phospholipase A_2 (PLA_2) (59) (Scheme 1.3). PA is also known as an activator of phosphatidylinositol 4-phosphate 5 kinase α (PI4-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

P5Kα) (60) that produces phosphatidylinositol-4,5-bisphosphate (PIP_2). Therefore, PA and the derived DG and LPA, as well as PA-regulated synthesis of PIP_2, are candidates that possibly mediate the effect of PLD in the variety of cellular responses. Consistent with this postulation, all these lipids are bioactive and each has multiple downstream targets. For example, PA activates the GTPase activating proteins (GAP) of small G proteins Rho, Rac and ADP-ribosylation factor (Arf) (61,62), but inhibits Ras GAP (63). PA directly interacts with Raf and thus localizes Raf to the membrane where it is activated (64). DG is known to activate protein kinase C (PKC) (65). LPA binds to and activates its receptor that is coupled to heterotrimeric G protein (66). Like PA, PIP_2 also inhibits Ras GAP (63), but activates Arf GAP (62,67) and Arf guanine nucleotide exchange factor (GEF) (68,69). Hence these lipids are considered as multifunctional second messengers, which elicit many downstream signal transduction pathways (70-78) and may thus carry out the actions of PLD. However, the mechanism of how exactly PLD functions in different cellular events, and which messenger lipids are effectively involved has to be examined on a case-by-case basis.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Scheme 1.3 Metabolism of PA.

The PA produced from PC by PLD is a substrate of PLA_2 (59) and can be converted into LPA. Alternatively, this PA can be also utilized by PAP to generate DG (54-58).

1.6 Overview of this thesis study.

Since PLD was found to respond to a wide variety of extracellular signals, researchers in this field have been intensively studying the regulatory mechanisms of its enzymatic activity. However, a fundamental question remains unanswered, i.e., what is the purpose of the regulation of PLD activity by these signals? And despite the implication of PLD in the cellular processes mentioned above, little is known as to how the elevated PLD activity contributes to these processes.

To date, the function of PLD that has been most scrutinized is its ability to facilitate the formation of intracellular vesicles. Nevertheless, it is not known whether this effect of PLD may provide mechanistic basis for its participation in the various signaling events that lead to mitogenesis, differentiation and so on. To address this question, studies were designed to examine the involvement of PLD in receptor-mediated endocytosis, which employs vesicle transport to transmit extracellular signals. The epidermal growth factor (EGF) receptor (EGFR) has been demonstrated to activate PLD activity (49,58,79) and is also a typical model of ligand-induced endocytosis, thus was chosen for these studies (Chapter 4).

Previous research has suggested a correlation between PLD activity and the invasion of tumor cells (80-82), yet there is no direct evidence that PLD is involved in tumor invasion. Interestingly, activated Src kinase that is closely related to the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

invasiveness of human cancers (83) has been shown to stimulate PLD activity (84,85). Thus there is a possibility that the elevated PLD activity mediates signals of Src to induce tumor invasion. In order to test this hypothesis, v-Src transformed cells and MDA-MB-231 carcinoma cells with increased c-Src level, which are both highly invasive, were used to study the involvement of PLD in Src-induced tumor invasion (Chapter 5).

To provide information for these investigations, the regulation of PLD activity by EGF and v-Src signals was also studied (Chapter 3).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 2 Materials and Methods

2.1 Materials.

The materials used and the sources where they were obtained are listed below.

Table 2.1 Sources of Materials.

Materials	Sources
EGF	Calbiochem
Gö6976	Calbiochem
Rottlerin	Calbiochem
BFA	Sigma
1-BtOH	Sigma
t-BtOH	Sigma
Cytochalasin B	Sigma
Cytochalasin D	Sigma
Phalloidin	Sigma
Colchicine	Sigma
anti-EGFR (LA22)	Upstate Biotechnology
anti-phosphorylated EGFR antibody	Upstate Biotechnology
anti-EGFR (225)	Calbiochem (Oncogene Research Products)
anti-HA	Santa Cruz Biotechnology
anti-Ral	Transduction Laboratories
anti-Na+/K+ ATPase	Transduction Laboratories
anti-MAPK (p44/42)	New England Biolabs (Cell Signaling Technology)
anti-phosphorylated MAPK (P-p44/42)	New England Biolabs (Cell Signaling Technology)
anti-MEK1/2	New England Biolabs (Cell Signaling Technology)
and phosphorylated MEK1/2	New England Biolabs (Cell Signaling Technology)
anti-mouse IgG-horseradish peroxidase	Bio-Rad Laboratories
anti-mouse IgG-Rhodamine Red-X	Jackson ImmunoResearch
anti-rabbit IgG-horseradish peroxidase	Bio-Rad Laboratories
anti-rabbit IgG-cyanine	Jackson ImmunoResearch
[3H]-myristate	New England Nuclear
Bovine calf serum and fetal bovine serum	HyClone Laboratories
Dulbecco's modified Eagle's medium	Gibco BRL (Life Technologies)
Phosphate buffered saline	Gibco BRL (Life Technologies)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LipofectAMINE Plus™	Gibco BRL (Life Technologies)
Antibiotics and other tissue culture reagents	Gibco BRL (Life Technologies)
Western blot reagents	Bio-Rad Laboratories
Enhanced Chemilluminescent™ System	Pierce
Protease inhibitors	Calbiochem
Precoated silica (60A) TLC plates	Whatmen
Organic solvents and other chemicals	Sigma
BIOCOAT™ cell culture inserts, Matrigel™	Becton Dickinson Labware
pCEP4	Invitrogen (Life Technologies)
PLD-encoding plasmids	Dr. Michael A. Frohman in SUNY at Stony Brook

2.2 Cell culture conditions.

3Y1 and NIH3T3 murine fibroblasts, MDA-MB-231 human breast adenocarcinoma cells and the stable or transient transfectants derived from them were maintained at 37°C in a humidified 5% CO_2 atmosphere, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 units/ml penicillin, 50ug/ml streptomycin and 5% (v/v) or 10% (v/v) bovine calf serum (fetal bovine serum for MDA-MB-231 cells derivates) (Table 2.2). Usually, cells were grown to confluency and then made quiescent by replacing fresh DMEM containing 0.5% (v/v) bovine calf serum and 50 units/ml penicillin, 50ug/ml streptomycin one day before experiment. For immunofluorescence staining, morphological studies and motility experiments, cells were made quiescent and used when 50% confluent. For the experiment with mAb225 anti-EGFR antibody treatment (Fig. 4.9), cells were grown to about 70 % confluency but were not made quiescent.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 2.2 Cell culture conditions.

Cell Types	Serum Concentration	Antibiotics Selection
NIH3T3	10%	None
NIH3T3/RalA WT	10%	Neomycin (100 μg/ml)
NIH3T3/RalA S28N	10%	Neomycin (100 μg/ml)
NIH3T3/EGFR	5%	None
NIH3T3/v-Src	10%	None
NIH3T3/v-Src/RalA WT	10%	Neomycin (100 μg/ml)
NIH3T3/v-Src/RalA S28N	10%	Neomycin (100 μg/ml)
3Y1	10%	None
3Y1/PLD1 WT	10%	Hygromycin B (200 μg/ml)
3Y1/PLD1 K898R	10%	Hygromycin B (200 μg/ml)
3Y1/PLD2 WT	10%	Hygromycin B (200 μg/ml)
3Y1/PLD2 K785R	10%	Hygromycin B (200 μg/ml)
3Y1/EGFR/RalA WT	5%	Neomycin (100 μg/ml)
3Y1/EGFR/RalA S28N	5%	Neomycin (100 μg/ml)
3Y1/EGFR/PLD1 WT	5%	Hygromycin B (200 μg/ml)
3Y1/EGFR/PLD1 K898R	5%	Hygromycin B (200 μg/ml)
3Y1/EGFR/PLD2 WT	5%	Hygromycin B (200 μg/ml)
3Y1/EGFR/PLD2 K785R	5%	Hygromycin B (200 μg/ml)
3Y1/v-Src	10%	None
3Y1/v-Src/PLD1 WT	10%	Hygromycin B (200 μg/ml)
3Y1/v-Src/PLD1 K898R	10%	Hygromycin B (200 μg/ml)
3Y1/v-Src/PLD2 WT	10%	Hygromycin B (200 μg/ml)
3Y1/v-Src/PLD2 K785R	10%	Hygromycin B (200 μg/ml)
MDA-MB-231	10%	None
MDA-MB-231/PLD2 K785R	10%	None (transient)

2.3 Transfection and cell line establishment.

NIH3T3 and 3Y1 cell lines stably overexpressing EGFR, v-Src and / or RalA proteins were established previously (49,86,87) and were kept under selection with antibiotics as listed in Table 2.2.

3Y1, 3Y1/EGFR, 3Y1/v-Src and MDA-MB-231 cells that overexpress recombinant PLD proteins were obtained by transfection of plasmids encoding human PLD1, mouse PLD2 or the catalytically inactive mutants human PLD1-K898R or

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

mouse PLD2-K758R, using LipofectAMINE Plus™ reagent according to the vendor's instructions. Transient transfectants were made quiescent 24 hr after transfection and used for experiments 48 hr after transfection. Stable transfectants were obtained by cotransfection with the hygromycin B selection vector pCEP4. Transfected cultures were selected with hygromycin B (200 μg/ml) 24 hr after transfection for 10-14 days. At that time antibiotic-resistant colonies were pooled and expanded under selective conditions (referred as 'pooled stable transfectants') for further use. Alternatively, antibiotic-resistant colonies were picked, amplified under selective conditions (referred as 'established cell lines') and used for experiments. All of the PLD proteins expressed were Flu-tagged and could be detected using anti-HA antibody raised against the Flu epitope.

2.4 Preparation of whole cell lysates and plasma membranes.

2.4.1 Whole cell lysates preparation.

Cells were grown to confluency in 100 mm culture dishes and made quiescent as described above. After treatment as indicated in the figure legends, cells were placed on ice, washed twice with cold phosphate buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4, pH 7.4) and collected in 1 to 1.5 ml buffer A (0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8, 150 nM Aprotinin, 1 μM Leupeptin). Cells were then homogenized with VibraCell™ sonicator at 4°C with maximum output and 20% duty cycle for 1 min. The homogenates were used as whole cell lysates.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2.4.2 Plasma membranes preparation.

3Y1/EGFR, 3Y1/EGFR/RalA WT, and 3Y1/EGFR/RalA S28N cells were each grown in four 150 mm culture dishes to confluency and made quiescent as described above. After 30 min of EGF treatment, cells were placed on ice, washed twice with cold PBS and each sample was collected in buffer A with a total volume of 3 to 4 ml. The preparation of the plasma membrane fractions was carried out using the method developed by Anderson and colleagues (88). Briefly, cells were homogenized in a Wheaton tissue grinder with 25 to 30 strokes followed by centrifugation at 1000 X g for 10 min. The post-nuclear supernatants were layered on top of 23 ml of 30% (v/v) percoll in buffer A and centrifuged at 84,000 X g for 30 min in a Ti 60 rotor (Beckman) at 4°C. The fractions (0.5 ml each) were collected from top to bottom and analyzed for plasma membrane marker Na^+/K^+ ATPase by Western Blot.

2.5 Western blot analysis.

Equal amounts of protein (10 μg) were subjected to SDS-PAGE using an 8% (v/v) acrylamide separating gel, transferred to nitrocellulose filters and blocked at room temperature for 1 hr with 5% (w/v) non-fat dry milk in PBS. The nitrocellulose filters were washed in PBS with 0.05% (v/v) Tween-20 and then incubated with primary antibodies as described in the figure legends. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase was used for detection with the Enhanced

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chemilluminescent™ System. Each antibody incubation was in 5% (w/v) milk / PBS at room temperature for 1 hr and followed by rinse with PBS. Detection reagents-treated nitrocellulose filters were exposed to films, which were developed and then scanned in a densitometer for quantification.

2.6 PLD assays (transphosphatidylation reactions).

PLD activity was measured by the ability of cells to convert the metabolically labeled [^{3}H]-PC into PBt in the presence of exogenously provided 1-BtOH.

Cells were grown in 60 mm culture dishes to confluency and made quiescent as described above. These quiescent cells were labeled for 4 hr with [^{3}H]-myristate (40 Ci/mmol) at final concentration of 1 μCi/ml, and then pretreated with drugs such as BFA, Gö6976 or rottlerin, as described in the figure legends. For the EGFR overexpressing cells, 1% (v/v) 1-BtOH (t-BtOH or no alcohol was used when indicated in Fig. 4.2) were then added in to medium 5 min before EGF treatment. For v-Src expressing cells, this drug pretreatment was followed by 15 min of incubation with 1% (v/v) 1-BtOH. Afterwards, cells were placed on ice, washed twice with cold PBS and collected in 0.5 ml of methanol / 6 M HCl (50:1, v/v). Lipids were extracted by adding 0.5 ml of chloroform. Phase separation was achieved by the addition of 155 μl of 1 M NaCl and the organic phase was recovered after centrifugation. This was followed by reextraction through the addition of 350 μl H_2O, 115 μl 1 M NaCl and 115 μl methanol. An aliquot of the thus obtained organic phase was counted in a liquid scintillation counter and the volume of each sample that had the same intensity of radioactivity was calculated according to the readings. Lipids containing an equal

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

amount of radioactivity were then dried under a stream of nitrogen and redissolved in 50 µl of chloroform / methanol (9:1, v/v). Samples were then spotted on a precoated silica (60A) plates and separated by thin layer chromatography (TLC) with a solvent system of ethylacetate / trimethylpentane / acetic acid / H_2O (9:5:2:10, v/v, upper layer). The transphosphatidylation product PBt (or PA, when t-BtOH or no alcohol was used as in Fig. 4.2) was visualized by autoradiography of the TLC plates and the films were scanned in a densitometer for quantification.

2.7 Microscopy.

2.7.1 Morphological studies.

Cells were grown in 6 well plates to 50% confluency, made quiescent, and then treated as described in the figure legends. The cells were photographed under an Olympus OMT-2 inverted microscope with a Dage MTI CCD 72 video camera and images were obtained using Oncor Image analysis software. .

2.7.2 Immunofluorescence microscopy.

Cells were seeded onto coverslips in 6 well plates. 24 hr later the cells were transiently transfected with PLD-encoding plasmids and were made quiescent 24 hr after transfection. The cells that had been quiescent for 24 hr were then washed twice with PBS, fixed in 3.7% (w/v) paraformaldehyde in PBS for 10 min at room temperature, washed with PBS, and then permeablized by incubation with 0.2% (w/v) Triton X-100 / PBS for 5 min. Cells were then again washed with PBS, incubated with 0.2% (w/v) bovine serum albumin / PBS for 5 min, and subjected to successive

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

incubation with primary and fluorophore-conjugated secondary antibodies. PLD proteins were detected using rabbit polyclonal anti-HA antibody and anti-rabbit IgG conjugated with cyanine (green). EGF receptor was detected by using mouse monoclonal anti-EGFR antibody (LA22) and anti-mouse IgG conjugated with Rhodamine Red-X (red). Each antibody incubation was in 2% (w/v) bovine serum albumin / PBS at room temperature for 1 hr and followed by rinse with PBS. After final rinse, coverslips were mounted onto microscope slides with 50% (w/v) glycerol in PBS and photographed under a Nikon Optiphot 2 upright microscope with a Sony 'Cats Eye' high resolution color video camera and images were obtained using Compix Simple 32 image analysis software.

2.8 Migration and invasion assays.

The assays were carried out using BIOCOAT™ cell culture inserts with polyethelene terephthalate filters (8 μm pore size) on the bottom. For migration assays, inserts were used directly without coating; for invasion assays, the upper surface of the filters were coated with Matrigel™ purified from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins, which closely mimics the basement membrane *in vivo* (89,90). Single cell suspensions in serum-free medium containing 0.1% (w/v) BSA and were added into the inserts. 3Y1/v-Src, 3Y1/v-Src/PLD2 WT and 3Y1/v-Src/PLD2 K758R cells were added 50,000 cell/insert; MDA-MB-231 cells and the transiently transfectants with PLD2 K758R were seeded at 25,000 cells/inserts. The inserts were placed into 24-well plates that held 0.75 ml/well growth medium as a chemoattractant and incubated under normal

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

growth condition for 24 hr. Cells that had not penetrated the filters were wiped away with cotton swabs, and cells that had migrated or invaded to the lower surface of the filters were fixed in methanol and stained with a 0.2% (v/v) solution of crystal violet in 2% (v/v) ethanol. The number of migrated or invaded cells was counted under microscope. The mean of five individual fields in the center of the filter where migration or invasion was the highest was obtained for each well. For assays that used transient transfectants (Fig. 5.7), the efficiency of transfection was verified with immunofluorescence staining for the Flu-tag on PLD proteins, as described earlier in this chapter.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 3 Regulation of Phospholipase D Activity by EGF and v-Src in Murine Fibroblasts

3.1 Introduction

Although PLD activity is distributed in a wide range of species, and may utilize various phospholipids as substrates, studies of its regulation have been mainly focused on the PC-specific PLD in mammalian tissues and cells.

3.1.1 Lipid dependency of PLD activity.

In vitro analysis has revealed the dependency of the mammalian PC-PLD activities on phospholipids and fatty acids, and subdivided them into two classes accordingly. The first class is stimulated by free fatty acids such as oleate, whereas the second class is dependent on phosphoinositides, such as PIP_2 or PIP_3, but usually inhibited by oleate (4). The cloned mammalian PLD1 and PLD2 isoforms both require PIP_2 as a cofactor for activation (5-7,12-14,19) and therefore belong to the second class. Puzzlingly, it has been reported that oleate at low concentration can activate PLD2 synergistically with PIP_2 (91), leaving it unclear as to whether there is any relation between PLD2 and the oleate-dependent PLD, which has not yet been cloned (2).

It is not known how PIP_2 stimulates PLD activity. The discovery of a PH-like domain in both PLD1 and PLD2 suggested a possibility that the regulation is through

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the interaction between PIP_2 and the PH domain. It is thought that this interaction may direct the enzyme to its precise membrane locations and may also induce conformational changes of PLD, thereby regulating its catalytic activity (10,16,17,43). However, there are discrepancies on this point (15,18,92,93) and the exact mechanism of the regulation by PIP_2 remains obscure in spite of the definite requisition of this lipid for PLD activity of the second class.

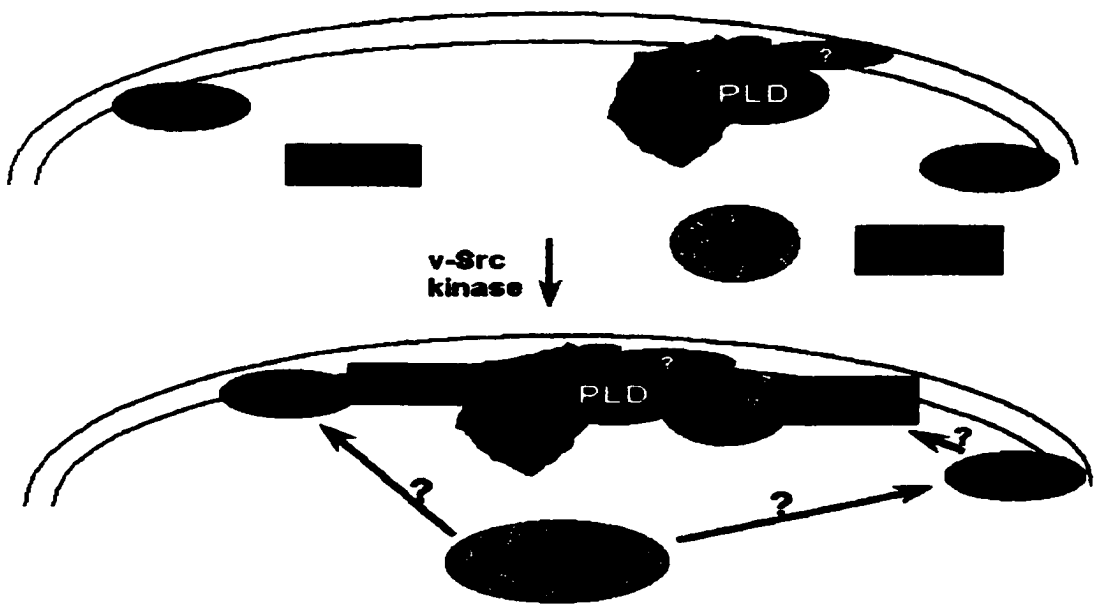
3.1.2 An overview of positive regulators of PLD activity.

The PIP_2-dependent PLD activity is stimulated by numerous extracellular signals, such as hormones, growth factors, cytokines, neurotransmitters, adhesion molecules and other agonists that activate cell surface receptors (2). Studies into the mechanisms of the receptor-PLD coupling have implicated multiple pathways leading to PLD activation, which include non-receptor and receptor protein tyrosine kinases (Src, EGF, PDGF and insulin receptors, etc.); serine/threonine kinases (Raf, Protein kinase C, etc.); trimeric and monomeric GTPases (α subunits of Gi ½, Gi $_3$, G_{13} and Gq; Ras, Ral, Rho and Arf family small G proteins) (2,4,94,95).

It has been recently reported that PLD can be phosphorylated in response to upstream stimulations. PLD1 was found constitutively associated with platelet-derived growth factor (PDGF) receptor and been tyrosine phosphorylated upon stimulations in Swiss 3T3 fibroblasts (96). PLD2 overexpressed in HEK293 cells was shown to be associated in a ligand-independent manner with EGF receptor, and was tyrosine phosphorylated upon EGF treatment (42). However, there are controversies as to whether these phosphorylations are important to the enzyme

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

activity; and the mechanism and functional consequence of such modification on PLD1 and PLD2 is not understood (2,4,95). When stimulated with phorbol ester, PKC alpha (PKCα) translocates onto plasma membranes where it forms complexes with and phosphorylates PLD1 in various cells types as well as observed in cell free systems (18,19,37,38,97-99). Like the tyrosine phosphorylations caused by PDGF and EGF, there are discrepancies upon the relevance of these serine/threonine phosphorylations by PKCα on the enzyme activity and it seems that PKCα could regulate PLD either through phosphorylation-dependent (34,37,97,100) or independent manner (6,8,12,19,101-106). The activation of PLD by receptor tyrosine kinases such as insulin receptor (107) and PDGF receptor (96) were at least partially mediated by PKCα; interestingly, in these studies, PKCα was found constitutively form complexes with PLD2 in HEK293 cells (107) or with PLD1 in Swiss 3T3 cells (96), probably reflecting an instant need of PKCα for PLD activation upon receptor signaling.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Scheme 3.1 Model for the activation of PLD by v-Src via Ras, RalA, and Arf.

It is proposed that in response to the tyrosine kinase activity of v-Src, Ras goes from the inactive GDP form (Ras-GDP) to the active GTP form (Ras-GTP). The activated Ras associates with Ral-GDS (108), resulting in GDP-GTP exchange on RalA and the recruitment of a RalA-PLD1 complex (87). However, because activated RalA is not sufficient to activate PLD1 (87,109), whereas activated Ras is sufficient for PLD1 activation (109), it is proposed that Ras stimulates GDP-GTP exchange on Arf via another mechanism involving an Arf-GDS, leading to the activation of Arf and the association of Arf to RalA-PLD1 complex, which is dependent upon the RalA amino terminus and may engage some unknown factor since the interaction between RalA and Arf is apparently indirect (109). Adopted from Luo et. al. Proc Natl Acad Sci U S A 1998 Mar 31;95(7):3632-7 (109).

The Arf family small GTPases are well known as critical components of vesicular trafficking in eukaryotic cells (110,111) and were later discovered to be activators of PLD (112,113), with different potency of all six Arf isoforms (114). Another small G protein Ral has also emerged as an important regulator of PLD activity. In our lab, PLD was shown to be activated in murine fibroblasts transformed by the oncogenic non-receptor tyrosine kinase v-Src (84,115). This activation is mediated by small GTPase Ras (85,116), RalA and Arf (87,109,117). RalA was found constitutively associated with PLD1 (87) and its amino terminus is crucial for recruiting Arf that would then directly interact with PLD1 (109). Although RalA is required for PLD activation by v-Src (87), RalA cannot activate PLD all by itself (87,109) and the PLD activity associated with RalA is dependent on Arf in the same complex (109). Thus a model was suggested whereas activated Ras mediates v-Src signals and interacts with Ral-GDS (guanine nucleotide dissociation stimulator)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(108), which results in the activation of RalA and the recruitment of RalA-PLD1 complex. Meanwhile, Ras also activates Arf through Arf-GDS. The activated Arf would then join the complex of PLD1 and RalA, and thus activate PLD1 (Scheme 3.1). Consistent with our model, Kim et. al. has demonstrated that Arf1 and RalA each directly interacted with PLD1 on different sites and synergistically activated PLD1 (118). Arf and Ral are also involved in PLD activation by various other signals: Ral is required for EGF, insulin (49,119), phorbol ester (119-121) and oncogenic serine / threonine kinase v-Raf (122) induced PLD activity; Arf is required for insulin (123) and PDGF (124) stimulated PLD activity.

3.1.3 An overview of negative regulators of PLD activity.

In addition to the numerous activators of PLD activity, cells also possess molecules that negatively regulate this enzyme. Several proteins have been identified as inhibitors of PLD and interestingly, all of them fall into two groups. The first group are vesicular trafficking proteins, include amphiphysin (125), AP3 (126), synaptojanin (127), and α/β synucleins (128); the second group comprise cytoskeletal proteins: β-actin (129) and actin-binding proteins α-actinin (130), fodrin (131,132) and gelsolin (133,134). Coincidently, these two categories of inhibitors match well with the major physiological functions of PLD, i.e., vesicle transport and cytoskeleton reorganization (as mentioned in Chapter 1, and will be elaborated in Chapter 4 and 5). Most of these inhibitors act either by direct binding to PLD (125,126,129,130) or by sequestering PIP_2 that is essential for PLD activity (127,131,132). Calphostin-C, a fungal metabolite previously known as a PKC inhibitor, has now been discovered to

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

independently inhibit PLD activity by an irreversible modification of the enzyme but does not affect its binding with PIP_2 (105). The inhibition of both PLD and PKC by the same compound may imply a common involvement of these two enzymes in cellular processes. PLD is also inhibited by ceramide, a product of sphingomyelinase activity. The fact that ceramide can induce cell cycle arrest, apoptosis and cell senescence (135,136), implicating a possible involvement of PLD in these processes as well (52,137).

3.1.4 Differential regulation of PLD1 and PLD2 activity.

Regulators such as PKCα, Arf, Ral and Rho family small GTPases have all been shown to elevate PLD1 activity from its low basal level, and these factors often act in a synergistical manner with each other. Contrarily, PLD2 does not respond to most of these regulators as observed *in vitro*; it has a high basal activity and thus is thought to be negatively regulated *in vivo* under basal conditions but relieved from inhibition upon signaling (2,4,94,95). However, the discovery that PLD2 can be indeed weakly activated by Arf (14,15,101,107,118,123,124) and by PKCα or phorbol ester (12,93,105,107) both *in vitro* and *in vivo*, suggested that positive regulation on PLD2 activity may also exist in intact cells.

3.1.5 Orchestration of PLD regulation by cells.

Mammalian PLDs are generally membrane-bound enzymes, as discussed in Chapter 1. The regulation of PLD activity is thought to be subject to the availability of positive and negative regulators as well as the substrates in the membrane location

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

of the enzyme, which is dynamically administered by cells in both regional and temporal manner. The complex regulation of PLD activity is believed to reflect the intricacy of its functions.

3.1.6 Purpose of the studies in this chapter.

In order to explore the involvement of PLD in various physiological processes, the understanding of its regulatory mechanism is a fundamental issue. The studies in this chapter focused on the activation of PLD by EGF in murine fibroblasts, as well as the involvement of Ral, Arf and PKC in the regulation of EGF-induced PLD activity. It was also aimed to answer the questions: (i) does EGF activate PLD1 or PLD2 or both? (ii) are the two isforms differentially regulated if they are both activated by EGF? (iii) is there any relationship between the induction of these two isoforms? Besides, the regulation of v-Src-induced PLD activity was also studied. The purpose of these studies was to provide evidence for the investigation of PLD's involvement in the EGF-induced receptor endocytosis (Chapter 4) and in v-Src-induced invasive migration (Chapter 5).

3.2 Results

3.2.1 EGF-induced PLD activity.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.2.1.1 EGF induces endogenous PLD activity in NIH3T3/EGFR and 3Y1/EGFR cells.

In both NIH3T3 and 3Y1 murine fibroblasts, EGF receptor is not detectable by Western blot analysis (Fig. 3.1.A), neither is the PLD activation in response to EGF as measured by the production of PBt in the presence of 1-BtOH (data not shown). In order to study the EGF-induced PLD activity, these cells were stably transfected with human EGF receptor ErbB1 as reported previously (49) (Fig. 3.1.A). The basal PLD activities (Fig. 3.1.B, lanes 1 and 3) in these cell lines (NIH3T3/EGFR, 3Y1/EGFR) were increased by the overexpression of EGFR *per se*, when compared with the parental cell lines (data not shown), conceivably due to the default activity of the overexpressed EGF receptors. Once treated with EGF, the levels of PBt were substantially enhanced (Fig. 3.1.B, lanes 2 and 4), indicating that the EGFR transmitted agonistic signals to stimulate PLD activity.

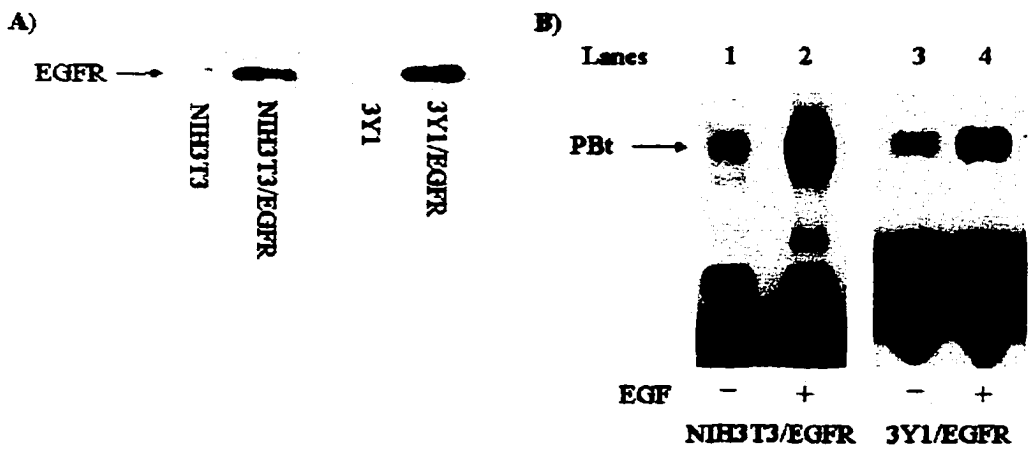


Fig. 3.1 EGF induces endogenous PLD activity in NIH3T3/EGFR and 3Y1/EGFR cells.

A) NIH3T3 and 3Y1 cells and these cells stably overexpressing EGFR were harvested, lysed and subjected to Western Blot analysis using anti-EGFR antibody (LA22). B) Cells overexpressing EGFR

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

were prelabeled with [^{3}H]-myristate for 4 hr and then treated without or with EGF (100 ng/ml) for 10 min in the presence of 1-BtOH (1% v/v) which was added 5 min before EGF. The transphosphatidylation product PBt were separated by TLC and visualized by autoradiography of TLC plates as described in Chapter 2. The data presented are representative results of experiments that were repeated for several times.

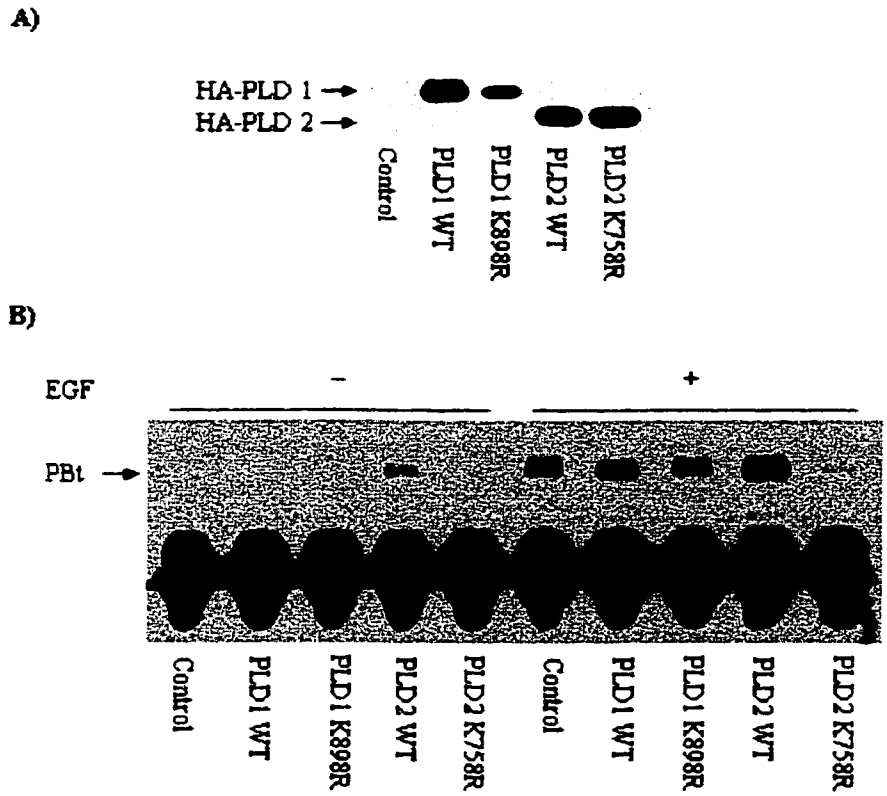


Fig. 3.2 EGF induces both PLD1 and PLD2 activity in 3Y1/EGFR cells.

A) 3Y1/EGFR and the derived cells stably overexpressing PLD proteins were harvested, lysed and the expression level of PLD was analyzed with Western Blot using anti-HA antibody that detects the Flu epitope tagged on all of these recombinant PLD proteins. B) Cells were prelabeled [^{3}H]-myristate for 4 hr and treated without or with EGF (100 ng/ml) for 10 min in the presence of 1-BtOH (1% v/v) which was added 5 min before EGF, and PLD activity was assayed in the same way as in Fig. 3.1.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Control: 3Y1/EGFR cells; PLD1 WT, PLD2 WT, PLD1 K898R, PLD2 K758R: 3Y1/EGFR cell lines stably overexpressing these proteins. The data presented are representative results of experiments that were repeated for three times.

3.2.1.2 EGF induces both PLD1 and PLD2 activity in 3Y1/EGFR cells.

To investigate whether EGF induces PLD1, PLD2 or both, 3Y1/EGFR cells were stably transfected with either human PLD1 (hPLD1) (5), mouse PLD2 (mPLD2) (12), or their catalytically inactive mutants, hPLD1 K898R or mPLD2 K758R (18,138) (Fig. 3.2.A); and the corresponding cell lines (3Y1/EGFR/PLD1 WT, 3Y1/EGFR/PLD1 K898R, 3Y1/EGFR/PLD2 WT and 3Y1/EGFR/PLD2 K785R) were established as described in Chapter 2. The EGF-induced PLD activity was strongly elevated by the overexpression of either PLD1 or PLD2 wild type (PLD1 WT, PLD2 WT), but reduced by the two catalytically inactive mutants (Fig. 3.2.B), suggesting that both PLD1 and PLD2 activities could be stimulated by EGF in 3Y1/EGFR cells.

3.2.1.3 Expression of endogenous Ral is correlated with PLD activity.

Interestingly, the level of endogenous Ral in 3Y1/EGFR cells is elevated upon the overexpression of either PLD1 or PLD2 wild type, and decreased upon the overexpression of the catalytically inactive mutants (Fig. 3.3), suggesting that the expression of Ral is possibly dependent on PLD activity.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

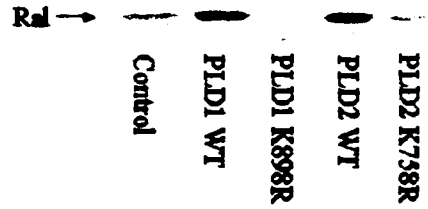


Fig. 3.3 Expression of endogenous Ral is correlated with PLD activity.

3Y1/EGFR cells stably overexpressing PLD1 WT, PLD2 WT, PLD1 K898R or PLD2 K758R as in Fig. 3.2 were harvested, lysed and subjected to Western Blot analysis using anti-Ral antibody. The data presented are representative results of an experiment that was repeated twice.

3.2.1.4 EGF-induced endogenous PLD activity in 3Y1/EGFR cells is dependent on RalA.

To investigate the regulation of EGF-induced PLD activity by Ral, 3Y1/EGFR cell lines that stably overexpress RalA (3Y1/EGFR/RalA WT) and its dominant negative mutant RalA S28N (3Y1/EGFR/RalA S28N) were established as reported previously (49) (Fig. 3.4.A). As shown in Figure 3.4.B, the EGF-induced PLD activity was dramatically increased by RalA wild type (RalA WT), but inhibited by RalA S28N. Thus the EGF-induced endogenous PLD activity in these cells is dependent on RalA.

Taken together with the data that Ral expression may be modulated by PLD activity (Fig. 3.3), it is possible that a positive feed back loop of regulation between PLD and Ral exists in these cells.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

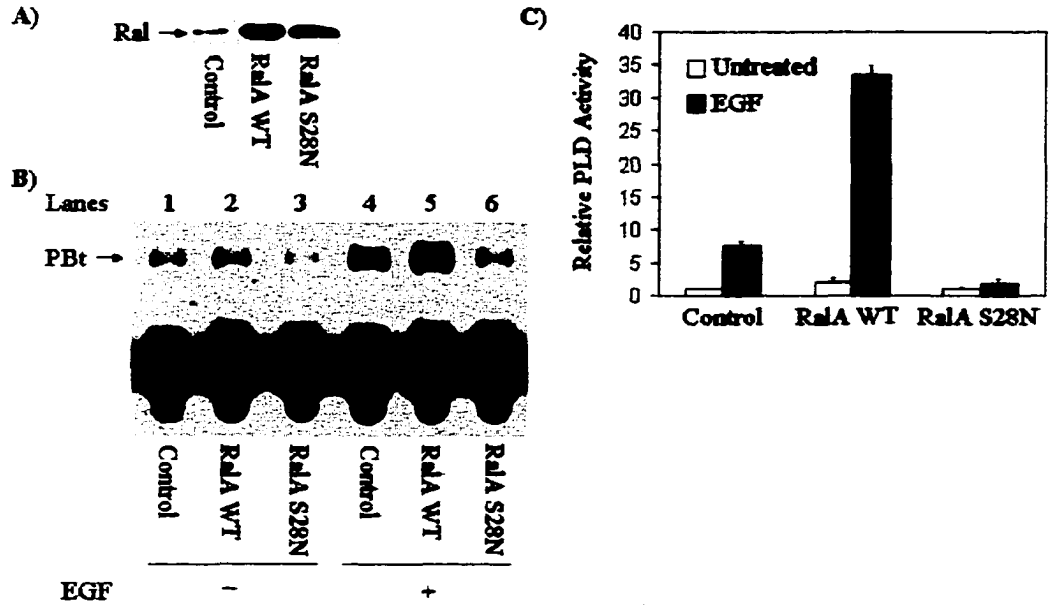


Fig. 3.4 EGF-induced endogenous PLD activity in 3Y1/EGFR cells is dependent on RalA.

A) 3Y1/EGFR and the derived cells stably overexpressing RalA WT or RalA S28N were harvested and the lysates were subjected to Western Blot analysis using anti-Ral antibody. B) Cells were prelabeled [^{3}H]-myristate for 4 hr and treated without or with EGF (100 ng/ml) for 10 min in the presence of 1-BtOH (1% v/v) which was added 5 min before EGF, and PLD activity was assayed in the same way as in Fig. 3.1. The data presented are representative results of experiments that were repeated for three times. C) The result in B) was quantified by densitometry and normalized with the PLD activity in untreated 3Y1/EGFR cells. Error bars represent the mean +/- standard of three independent experiments performed in duplicate. Control: 3Y1/EGFR cells; RalA WT: 3Y1/EGFR/RalA WT cells, RalA S28N: 3Y1/EGFR/RalA S28N cells.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.2.1.5 EGF-induced endogenous PLD activity in 3Y1/EGFR cells is insensitive to brefeldin A.

As discussed above, Arf family small GTPases are well-documented activators of PLD activity. Brefeldin A (BFA), a fungal metabolite that prevents nucleotide exchange on Arf by inhibiting Arf-GEF, has been widely used to probe the functional dependence of conventional Arfs. Surprisingly, treatment with BFA did not affect the EGF-induced endogenous PLD activity in 3Y1/EGFR cells (Fig. 3.5). Therefore, EGF-induced PLD activation in these cells either does not involve Arf or involves some Arf isoform(s) with BFA-insensitive GEF(s).

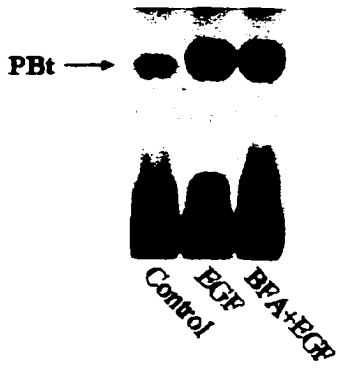


Fig. 3.5 EGF-induced endogenous PLD activity in 3Y1/EGFR cells is insensitive to BFA. 3Y1/EGFR cells were prelabeled with [^{3}H]-myristate for 4 hr then pretreated without or with BFA (20 μg/ml) for 15 min. After this, cells were incubated in the presence of 1-BtOH (1% v/v) for 5 min before treated without or with EGF (100 ng/ml) for 10 min. The produced PBt was isolated and visualized in the same way as in Fig. 3.1. The data presented are representative results of an experiment that was repeated for three times.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.2.1.6 Levels of endogenous PKC isoforms are affected by PLD expression.

Like that of Ral, the levels of PKC isoforms are also affected by the expression of PLD. 3Y1/EGFR cell lines were established carrying either PLD1 or PLD2 wild type that was constructed in an inducible expression vector (unpublished data of Troy Joseph). As shown in Fig. 3.6, upon induced expression of either PLD1 WT (Fig. 3.6.A) or PLD2 WT (Fig. 3.6.B), the level of endogenous PKCα was significantly upregulated, conversely, PKCδ level was downregulated.

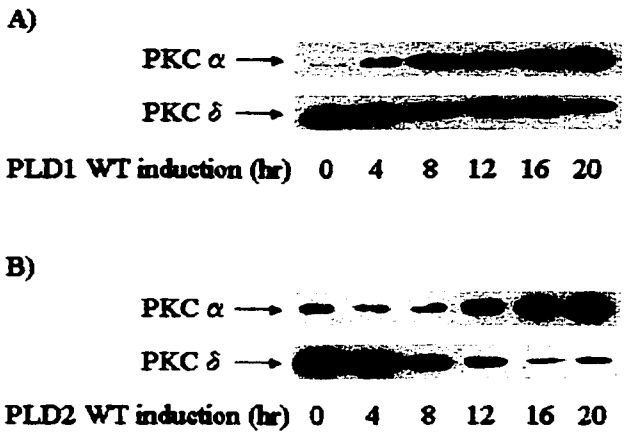


Fig. 3.6 Levels of endogenous PKC isoforms are affected by PLD expression.

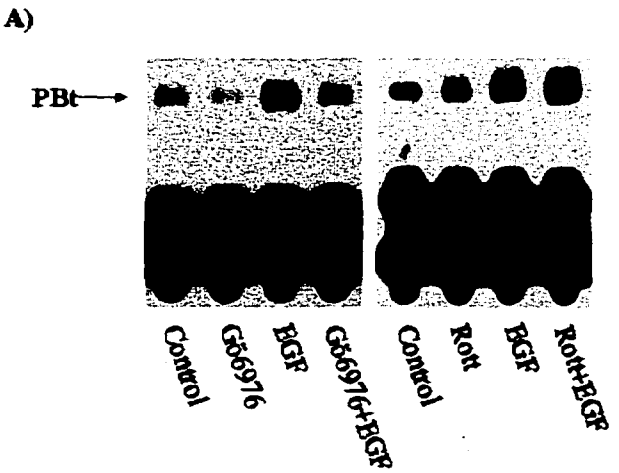
3Y1/EGFR cells carrying inducible PLD1 WT or PLD2 WT were stimulated to turn on the expression. At the indicated time after induction of A) PLD1 WT and B) PLD2 WT, cells were harvested and the lysates were subjected to Western Blot analysis using anti-PKCα and anti-PKCδ antibody. The data presented are representative results of experiments that were repeated for three times. These data were kindly provided by Troy Joseph.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.2.1.7 EGF-induced endogenous PLD activity in 3Y1/EGFR cells is regulated by PKC isoforms.

To examine to role for PKC isoforms, the EGF-induced PLD activation was tested in the presence of PKC inhibitors, Gö6976, at an effective concentration specific to PKCα, or rottlerin at an effective concentration specific to PKCδ. As shown in Figure 3.7.A, the EGF-induced endogenous PLD activity in 3Y1/EGFR cells was abolished by Gö6976, indicating a dependence on PKCα. Contrarily, inhibiting PKCδ by rottlerin did not reduce, but increased the activation of PLD, revealing a complex regulation of PLD activity by multiple PKC isoforms in response to EGF signals.

Data in Figure 3.6 and Figure 3.7.A showed that expression of PLD correlates with increased level of PKCα and suppressed level of PKCδ, and that PKCα and PKCδ are positive and negative regulators of the EGF-induced PLD activity respectively. Thus, these data may suggest a dual positive feed back regulation between PLD and the PKC isoforms, one through PKCα and the other through PKCδ.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

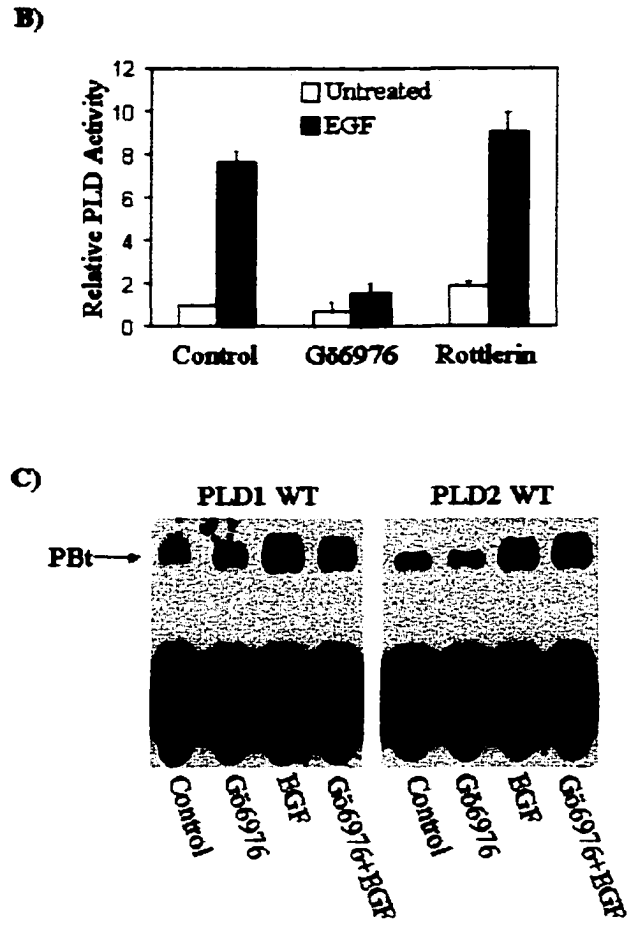


Fig. 3.7 Effects of PKC inhibitors on PLD activity.

A) 3Y1/EGFR cells were prelabeled with [^{3}H]-myristate for 4 hr and pretreated with DMSO (0.1% v/v) (control), Gö6976 (0.5 μM in DMSO) or rottlerin (10 μM in DMSO) for 30 min. Cells were then incubated in the presence of 1-BtOH (1% v/v) for 5 min before treated without or with EGF (100 ng/ml) for 10 min. The produced PBt was isolated and visualized in the same way as in Fig. 3.1. B) The result in A) was quantified by densitometry and normalized with the PLD activity in untreated 3Y1/EGFR cells (control). C) The same experiment with Gö6976 was done in 3Y1/EGFR/PLD1 WT and 3Y1/EGFR/PLD2 WT cells. The data presented are representative results of experiments that were repeated for three times. Error bars represent the mean +/- standard of three independent experiments performed in duplicate.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.2.1.8 EGF-induced PLD1 but not PLD2 activity in 3Y1/EGFR cells is dependent on PKCα.

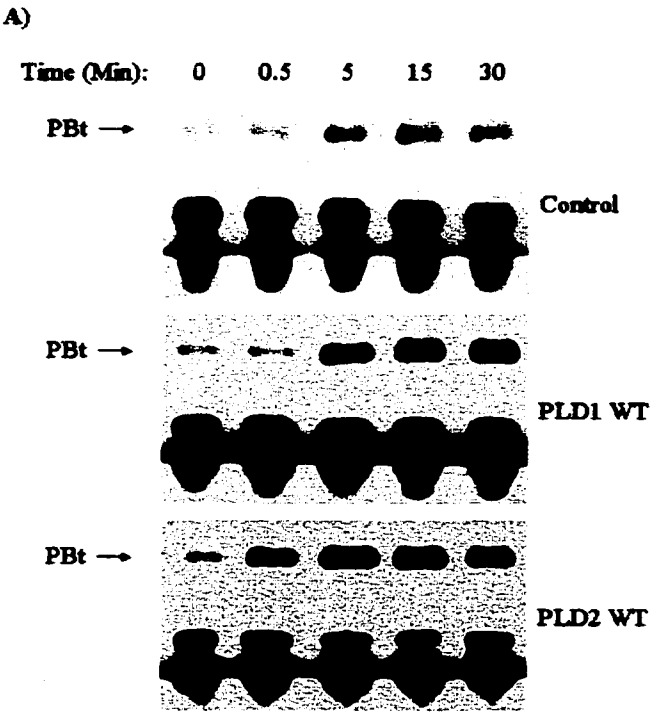
The effect of PKCα inhibitor Gö6976 was also tested on the activation of PLD1 and PLD2 by EGF. As shown in Fig. 3.2, both PLD1 and PLD2 can be activated in response to EGF. Pretreatment with Gö6976 lessened the EGF induction of PLD1 activity, but had no effect on PLD2 (Fig. 3.7.C). These data suggest that PKCα is involved in the EGF-induced activation of PLD1 but not PLD2. Taken together with the fact that Gö6976 almost abolished the EGF-induced PLD activity in the parental 3Y1/EGFR cells (Fig. 3.7.A), it is possible that PLD1 accounts for most of the EGF-induced endogenous PLD activity. The partial inhibition of Gö6976 on EGF-induced PLD1 activity may be explained by the overwhelming effect of the expressed PLD1 that cannot be counteracted by the drug at the concentration used; however using Gö6976 at higher concentration will lose its specificity to PKCα and also inhibit other isoforms including PKCδ that has an opposite effect on PLD activity, and thus the result will be difficult to interpret.

3.2.1.9 EGF-induced PLD1 and PLD2 activation in 3Y1/EGFR cells have different kinetics.

Once stimulated with EGF, the activation of the overexpressed PLD1 and PLD2 showed different kinetics. 3Y1/EGFR and the derived wild type PLD overexpressing cells (as in Fig. 3.2 and Fig. 3.6) were exposed to EGF for various length of time before assayed for PLD activity. The EGF-induced PLD2 activity was quickly elevated within 1 minute of EGF treatment, reached a peak between 2 to 10

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

minutes and started to fade after 15 minutes (Fig. 3.8.A, bottom panel, curved as in Fig. 3.8.B). In contrast, PLD1 activity started to respond much later but did not significantly drop at 30 minutes of treatment (Fig. 3.8.A, middle panel, curved as in Fig. 3.8.B). Hence the EGF-induced PLD2 activation was more prompt and transient than that of PLD1. Interestingly, the activation of endogenous PLD (Fig. 3.8.A, top panel, curved as in Fig. 3.8.B) had very similar kinetics to that of overexpressed PLD1, but not PLD2 (compare the panels in Fig. 3.8.A, curved as in Fig. 3.8.B), thus it supports the speculation that the EGF-activated endogenous PLD activity is mostly PLD1 activity.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

B)

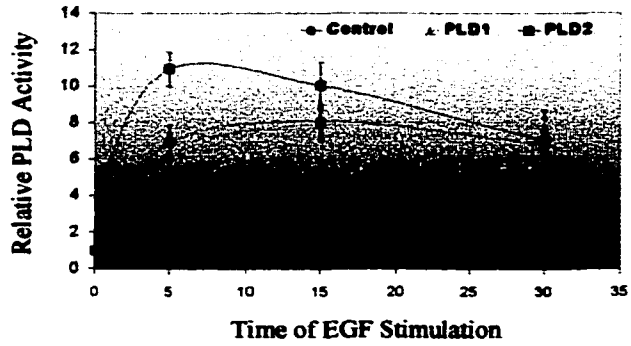


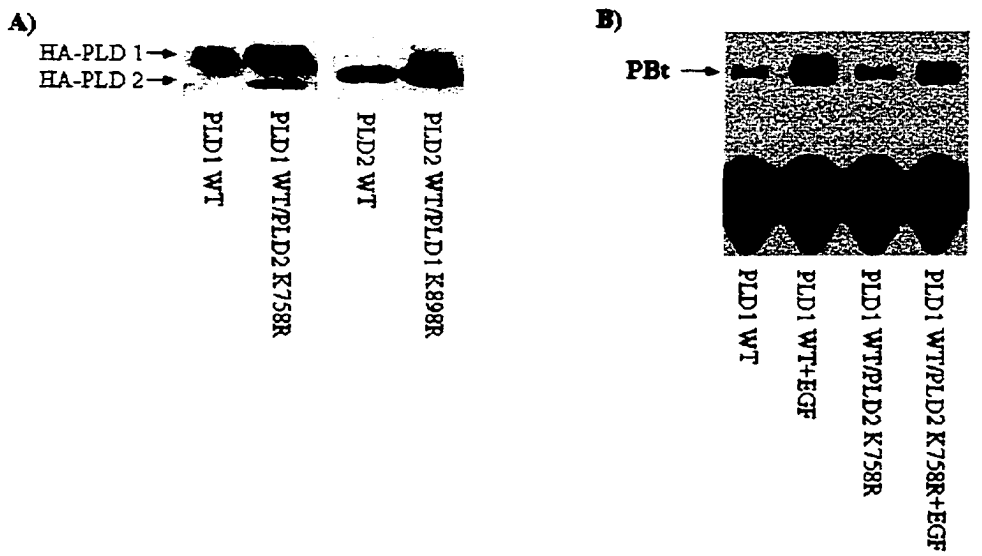
Fig. 3.8 EGF-induced PLD1 and PLD2 activation in 3Y1/EGFR cells with different kinetics. A) 3Y1/EGFR, 3Y1/EGFR/PLD1 WT and 3Y1/EGFR/PLD2 WT cells were prelabeled with [^{3}H]-myristate for 4 hr and incubated with1-BtOH (1% v/v) for 5 min before exposed to EGF (100 ng/ml) for the indicated length of time. The produced PBt was isolated and visualized in the same way as in Fig. 3.1. The data presented are representative results of experiments that were repeated for three times. B) The results of these experiments were quantified by densitometry and normalized with the PLD activity in the untreated samples of each cell lines. Error bars represent the mean +/- standard of three independent experiments performed in duplicate.

3.2.1.10 EGF-induced PLD1 activity in 3Y1/EGFR cells is dependent on PLD2.

To further investigate the relationship between the activation of PLD1 and PLD2, the wild type PLD overexpressing cells (as in Fig. 3.2, Fig. 3.6) were transiently transfected with the catalytically inactive mutant of the other isoform, i.e., PLD2 K758R into PLD1 WT cells and PLD1 K898R into PLD2 WT cells (Fig. 3.9.A), and the induction of PLD activity by EGF in these cells was analyzed. Introducing PLD2 catalytically inactive mutant into cells overexpressing PLD1 WT significantly decreased EGF-induced PLD activity to about 50% (Fig. 3.9.B), while

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PLD1 catalytically inactive mutant had no effect on the EGF-induced PLD activity in PLD2 WT overexpressing cells (Fig. 3.9.C). Noticing that when expressed at high level, PLD2 K758R can almost completely block the EGF-induced endogenous PLD activity (presumably mostly PLD1 activity) (Fig. 3.2), the partial inhibition of PLD2 K758R in PLD1 WT overexpressing cells may be explained by its low expression level (Fig. 3.9.A) and it is conceivable that when the amount of PLD2 K758R is enough to counteract that of PLD1 WT, it may likely render a complete inhibition. Thus the activation of PLD1 by EGF is (at least partially) dependent on PLD2 activity in these cells. Taken together with the data that PLD2 is activated in a much quicker fashion than PLD1 (Fig. 3.8), it is intriguing to postulate that EGF induces sequential and causal activation of PLD2 and PLD1.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

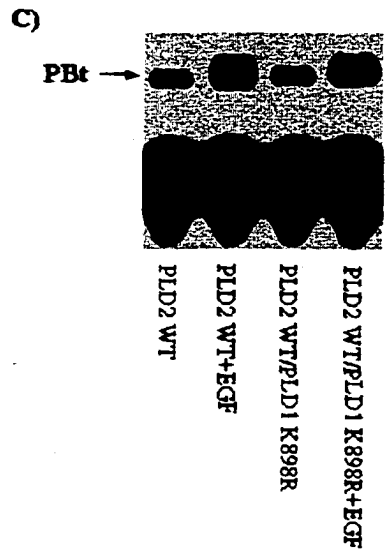


Fig. 3.9 EGF-induced PLD activity in 3Y1/EGFR/PLD1 WT cells is inhibited by catalytically inactive PLD2; EGF-induced PLD activity in 3Y1/EGFR/PLD2 WT cells is not affected by catalytically inactive PLD1.

3Y1/EGFR/PLD1 WT and 3Y1/EGFR/PLD2 WT were transiently transfected with the catalytically inactive mutant of the other isoform. A) At 48 hr after transfection, the expression of the HA-tagged PLD proteins was detected with Western Blot analysis using anti-HA antibody. B), C) At the same time point, cells that had been prelabeled [^{3}H]-myristate for 4 hr were treated without or with EGF (100 ng/ml) for 10 min in the presence of 1-BtOH (1% v/v) which was added 5 min before EGF, and PLD activity in these cells was assayed in the same way as in Fig. 3.1. The data presented are representative results of experiments that were repeated for three times.

3.2.2 v-Src-induced PLD activity.

3.2.2.1 v-Src induces PLD activity in NIH3T3 cells.

A NIH3T3 cell line stably expressing the constitutively active non-receptor tyrosine kinase v-Src (NIH3T3/v-Src) was established previously (86). The overexpressed v-Src stimulated endogenous PLD activity, as shown in Figure 3.10

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(lower panel, compare lanes 1 and 4). Similar but less effect was also observed in 3Y1 cells overexpressing v-Src (data not show).

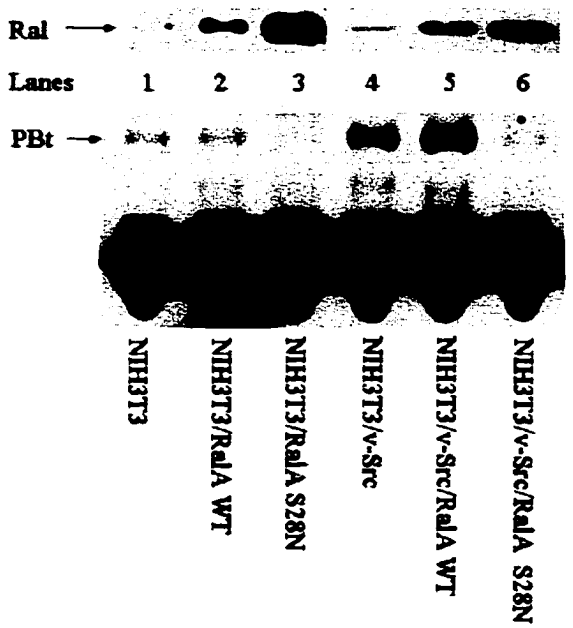


Fig. 3.10 v-Src-induced PLD activity in NIH3T3 cells is RalA-dependent.

Upper panel: NIH3T3 the derived cells stably expressing v-Src, RalA proteins or both were harvested and the lysates were subjected to Western Blot analysis using anti-Ral antibody. Lower panel: these cells were prelabeled with [^{3}H]-myristate for 4 hr and then incubated with 1-BtOH (1% v/v) for 15 min. The produced PBt was isolated and visualized in the same way as in Fig. 3.1. The data presented are representative results of an experiment that was repeated twice.

3.2.2.2 v-Src-induced PLD activity in NIH3T3 cells is dependent on RalA.

To study the involvement of Ral in the activation of PLD by v-Src, NIH3T3/v-Src cell lines that stably overexpressing either RalA WT (NIH3T3/v-Src/RalA WT) or dominant negative mutant RalA S28N (NIH3T3/v-Src/RalA S28N)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

were established previously (87). Like that induced by EGF, v-Src-induced PLD activity is also dependent on RalA, as it was strongly enhanced by RalA WT and completely blocked by RalA S28N (Fig. 3.10, lower panel, lanes 4,5,6). However, overexpression of RalA WT in NIH3T3 cells did not increase PLD activity (Fig. 3.10, lower panel, compare lanes 1 and 2), indicating that RalA alone is not sufficient for PLD activation and that some other factor(s) must be required to mediate the signals from upstream v-Src.

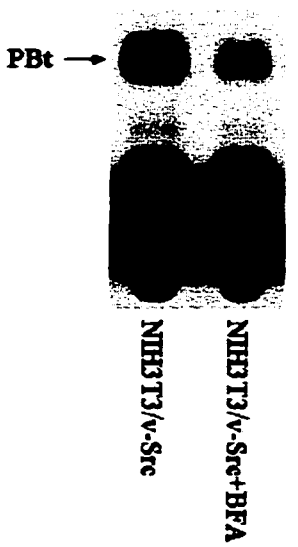


Fig. 3.11 v-Src-induced PLD activity in NIH3T3 cells is Arf-dependent.

NIH3T3/v-Src cells were prelabeled with [^{3}H]-myristate for 4 hr and then treated without or with BFA (20 μg/ml) for 15 min before incubated with 1-BtOH (1% v/v) for 15 min. The produced PBt was isolated and visualized in the same way as in Fig. 3.1. The data presented are representative results of an experiment that was repeated twice.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3.2.2.3 v-Src-induced PLD activity in NIH3T3 cells is dependent on Arf.

Unlike that induced by EGF, v-Src-induced PLD activity in NIH3T3 cells was inhibited by BFA (Fig. 3.11) and thus is dependent on Arf(s) with BFA-sensitive GEF(s). It is therefore possible for Arf to be a candidate that cooperates with Ral and facilitate the activation of PLD by v-Src.

3.3 Discussion

EGF induces endogenous PLD activities in 3Y1/EGFR and NIH3T3/EGFR cells, but whether these PLD activities are due to PLD1 or PLD2 was not clear. By introducing exogenous PLD1 and PLD2 into 3Y1/EGFR cells and comparing the kinetics of the enzyme activation as well as the dependency on PKCα, data presented in this chapter suggest that both PLD1 and PLD2 can be stimulated by EGF in 3Y1/EGFR cells and that PLD1 contributes to most of the EGF-induced endogenous PLD activity in these cells.

Consistent with these data, both PLD1 and PLD2 were reported to be activated by EGF receptor (42) and insulin receptor (107). However, there have been also other reports showing that insulin and PDGF receptors activate PLD2 rather than PLD1, based on the observation that catalytically inactive mutant PLD1 K898R did not block the agonist-induced PLD activity (123,124). It is noteworthy that in the established 3Y1/EGFR/PLD1 K898R cells, the PLD induction by EGF was not completely inhibited by this mutant (Fig. 3.2.B); and the effect could be much less in transient transfectants or even in stable pools if the transfection efficiency is low,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

which might be similar as observed by those authors who used transient transfectants and found it ineffective. This may argue that PLD1 K898R does not act like a dominant negative mutant, but to simply draw the conclusion that PLD1 is not activated by the receptors is somehow insufficient. In fact, as will be discussed in Chapter 4 (4.2.7), the steady state mass of EGFR in 3Y1/EGFR cells is substantially increased by the catalytically inactive mutant PLDs, but reduced by the wild type PLDs (Fig. 4.7.C). Thus, when normalized with the amount of cell surface receptor that is essential for the cells' response to the agonist, the EGF-induced PLD activity became efficiently inhibited by PLD1 K898R (Fig. 4.7.D). Since it is possible that PLD1 K898R also increases the basal levels of PDGF receptor and insulin receptor, normalization of PLD activity in those cases may uncover considerable inhibition by this mutant.

The dependency of the EGF-induced PLD1 on the activity of PLD2 and the timing of their activation strongly support the hypothesis that upon EGF stimulation, PLD2 is activated first and this may facilitate the later activation of PLD1. However, additional evidence will be required to make such conclusion. Interestingly, it was found that PLD2, but not PLD1 constitutively associated with EGFR in HEK293 cells (42), suggesting that the activation of PLD2 by EGFR is more instantly than PLD1 in those cells as well.

It has been shown that the insulin- and PDGF-induced PLD activities were inhibited by the treatment of BFA (123,124). However, the EGF-induced endogenous PLD activity in 3Y1/EGFR cells is insensitive to this fungal metabolite. This may suggest that Arf is not involved, or alternatively, Arf1 or Arf6 that has BFA-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

insensitive GEF (68,139-142) mediates the EGF signals to activate PLD in these cells. Arf1 is predominantly localized in Golgi membranes and cytosol, with a small amount on plasma membranes and intracellular vesicles (124,143-145), and is a critical component of intracellular vesicle transportation (44,144,146,147). Arf6 is exclusively localized on the plasma membrane, early endosomes and secreting granules that are close to the plasma membrane region (124,144,145,148-151), and is involved in endocytosis and exocytosis (144,148-150,152,153). Interestingly, the distribution of Arf1 and Arf6 corresponds closely with that of PLD1 and PLD2 respectively (124), suggesting possible regulation of PLD1 by Arf1 and PLD2 by Arf6. Consistent with this speculation, it was found that Arf1 activated PLD1 but not PLD2 *in vitro* (data not shown), leaving Arf6 as the candidate to regulate PLD2.

RalA is required for the endogenous PLD activity in 3Y1/EGFR cells (Fig. 3.4). This small GTPase has also been shown to form signaling complexes with PLD1 and Arf, and to potentiate the activation of PLD1 by Arf (109,117,118). On the other hand, there is a correlation of increased level of Ral and increased PLD1 activity in 3Y1/EGFR cells (Fig. 3.3), suggesting that Ral expression may be regulated by PLD1 activity and thus there may be a positive feed back loop between PLD1 and RalA. However, the fact that RalA failed to associate with purified PLD2 (unpublished data of Jingqing Luo) in the way it interacts with PLD1 has made it questionable whether it regulates PLD2, although its expression level is also correlated with PLD2 activity (Fig. 3.3). One strategy to explore this issue is to introduce dominant negative RalA S28N into PLD1 and PLD2 overexpressing cells

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and test the effect on the EGF-induced PLD1 and PLD2 activity, which is currently under investigation.

PKCα is required for PLD1 activation by EGF (Fig. 3.7.C), and its expression level is upregulated upon the induction of PLD1 activity (Fig. 3.6). Thus like that between RalA and PLD1, PKCα is also possibly engaged in a positive feed back loop of regulation with PLD1. EGF induced PLD2 activation does not rely on PKCα (Fig. 3.7.C), however, induction of PLD2 activity is accompanied by increased expression level of PKCα just as that of PLD1 does (Fig. 3.6). The differential responses of PLD1 and PLD2 to PKCα and the dependency of the EGF-induced PLD1 activity on PLD2 have made us wondering if PKCα could mediate signals from PLD2 to PLD1. To answer this question, we are currently investigating whether PLD2 activity can lead to a prompt activation of PKCα, hopefully this would help to understand the signaling among PLD1, PLD2 and PKCα.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 4 A Role for Phospholipase D in EGF Receptor-Mediated Endocytosis

4.1 Introduction

Phospholipase D has been implicated in numerous cellular functions, among which the most intensively studied is the involvement of this enzyme in intracellular vesicle transport and membrane traffic. The first indication that PLD might play a role in these processes has to be retraced to the small GTPase Arf. The Arf family is well-defined regulators of vesicular transport in various cellular compartments (110). The discovery that Arf is an activator of PLD has implicated PLD as a participant in membrane trafficking events (112,113). Evidence in support of this speculation has emerged during recent years. PA production by PLD-mediated hydrolysis of PC has been reported to be required for the formation of Golgi vesicles (154) and for the vesicle transport from the endoplasmic reticulum to the Golgi complex (155). PLD has been shown to stimulate the release of nascent secretory vesicles from the trans-Golgi network (146,156-158), and to be essential for exocytosis (36). Moreover, it has been demonstrated the adaptor recruitment to endosomes (159) and lysosomes (33) are also dependent on PLD activity. In most these reports, PLD either mediates the effect of Arf, or its activation by Arf correlates with vesicle transport; therefore it was proposed that the role that Arf plays in vesicle transport is to regulate PLD

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

activity and PA production (2,44,45,160). However, there is controversy on this point (161-163) and it is still not clear how PLD and its primary metabolite PA might contribute to vesicle formation.

As discussed in Chapter 3, PLD activity is elevated in response to many extracellular signals including growth factors, such as PDGF (96,124,164,165), insulin (107,123,166), and EGF (42,58,167,168). Receptors of these growth factors are internalized from the cell surface upon ligand binding (169-171) and the internalization is a process that involves endocytic vesicles. The concurrence of PLD activation and receptor endocytosis after ligand stimulation, and the fact that PLD has been implicated in vesicle transport, has led us to hypothesize that PLD might play a role in receptor-mediated endocytosis.

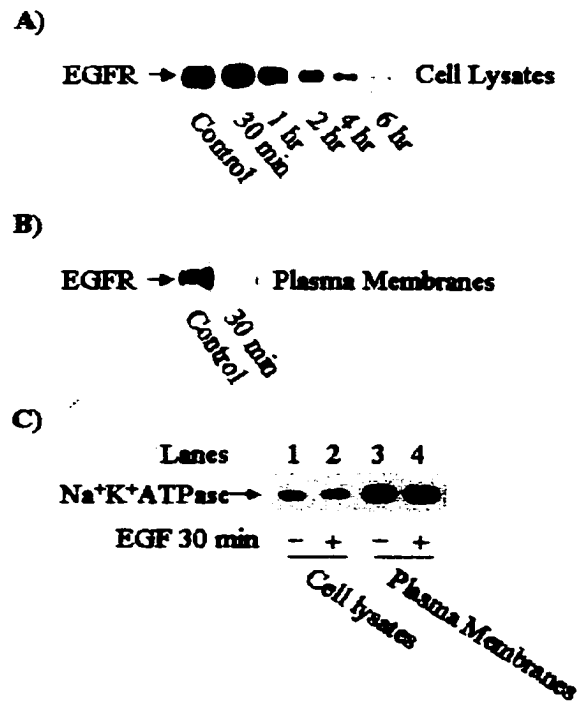
4.2 Results

4.2.1 EGFR is internalized and degraded after binding to its ligand.

The EGF receptor has served as a model to study the ligand-induced receptor endocytosis for many years. It was well established that EGF binding to the surface receptors results in down-regulation of EGFR (172,173). This down-regulation is attributed to rapid internalization of the activated receptors via endocytic vesicles followed by the efficient sorting of the internalized receptors to the lysosome degradation pathway (174). This ligand-induced receptor internalization and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

degradation was studied in 3Y1/EGFR cells. After stimulation of EGF, the degradation of EGFR was detected in the whole cell lysates by Western Blot analysis using anti-EGFR antibody (Fig. 4.1.A). To monitor the internalization of the EGF receptor, plasma membranes were isolated by cell fractionation as described in Chapter 2. The presence of marker protein Na^+/K^+ ATPase (Fig. 4.1.C) and the amount of EGFR (Fig. 4.1.B) in the plasma membrane fractions was then analyzed by Western Blot. Within hours of EGF treatment, the amount of EGFR gradually decreased due to the proteolytic degradation of the receptor (Fig. 4.1.A). At 30 minutes of stimulation, before the degradation was detectable (Fig. 4.1.A), the receptor was almost abolished from the plasma membrane (Fig. 4.1.B), indicating that it has been internalized at this moment and the loss of plasma membrane-bound receptor was not due to proteolysis. As a control, the plasma membrane marker Na^+/K^+ ATPase was not lost from this fraction after EGF treatment (Fig. 4.1.C, lanes 3 and 4).



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. 4.1 EGFR is internalized and degraded after binding to its ligand.

A) 3Y1/EGFR cells were treated without or with EGF (100 ng/ml) for the indicated time before harvested. The amount of EGFR in the whole cell lysates was analyzed with Western Blot using anti-EGFR antibody (LA22). B) 3Y1/EGFR cells were treated without or with EGF (100 ng/ml) for 30 min. Cells were harvested and plasma membranes were isolated as described in Chapter 2. The plasma membrane fractions were then subjected to Western Blot analysis using anti-EGFR antibody (LA22). C) 3Y1/EGFR cells were treated in the same way as in B). The whole cell lysates and the plasma membrane fractions were then subjected to Western Blot analysis using anti-Na^+/K^+ ATPase antibody. The data presented are representative results of experiments that were repeated for three times.

4.2.2 EGF-induced PA production by PLD is blocked by primary alcohol, but not tertiary alcohol.

When treated with EGF, 3Y1/EGFR cells produced increased amount of PA due to elevated PLD activity (Fig. 4.2.A, lanes 1 and 2). In the presence of primary alcohol 1-BtOH, the production of PA was inhibited, concurrent with an increase in the transphosphatidylation product PBt (Fig. 4.2.A, lanes 3 and 4; densitometry quantified as in Fig. 4.2.B). As a negative control, tertiary alcohol t-butanol (t-BtOH), which is not utilized by PLD, did not block PA production (Fig. 4.2.A, lanes 5 and 6).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

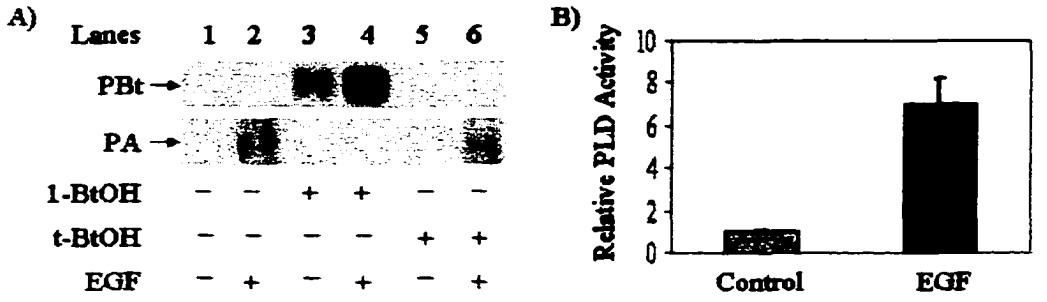


Fig. 4.2 EGF-induced PA production by PLD is blocked by primary alcohol, but not tertiary alcohol.

A) 3Y1/EGFR cells that had been prelabeled with [^{3}H]-myristate for 4 hr were incubated without or with the indicated alcohols (1% v/v) for 5 min. Cells were then treated without or with EGF (100 ng/ml) for 10 min before collected. The produced PA and PBt were separated by TLC and visualized by autoradiography of TLC plates as described in Chapter 2. The exposure of PA and PBt were from the same TLC plate, however PA bands were exposed longer than PBt bands. The data presented are representative results of an experiment that was repeated for several times. B) The production of PBt (lanes 3, 4) in the presence of 1-BtOH (1% v/v) was quantified by densitometry and normalized with the value of the unstimulated sample (lane 3). Error bars represent the mean +/- standard of three independent experiments performed in duplicate.

4.2.3 EGF-induced receptor degradation is inhibited by primary, but not tertiary alcohol.

The ability of PLD to discriminate between primary and tertiary alcohols has been widely used as an indicator for its involvement in many functional studies. As shown in Figure 4.1.A, the EGF receptor is nearly depleted after 4 hours of EGF treatment in 3Y1/EGFR cells. The loss of receptor in response to EGF was strongly inhibited by 1-BtOH, but not by the tertiary alcohol t-BtOH (Fig. 4.3.A). However,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1-BtOH had no effect upon autophosphorylation of the receptor induced by EGF (Fig. 4.3.B), indicating that the inhibitory effect was not due to interference of the alcohol with receptor dimerization or kinase activity. Taken together with the data that PA production is blocked by 1-BtOH, but not t-BtOH, these data suggest a requirement of PA production or PLD activity for the EGF-induced receptor degradation.

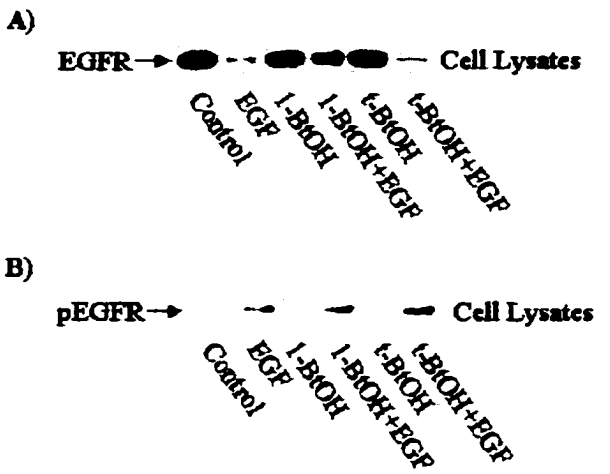


Fig. 4.3 EGF-induced receptor degradation is inhibited by primary, but not tertiary alcohol. 3Y1/EGFR cells were preincubated without or with the indicated alcohols (1% v/v) for 5 min, followed by 4 hr of treatment without or with EGF (100 ng/ml) before harvested. The whole cell lysates were subjected to Western Blot analysis using A) anti-EGFR antibody (LA22) or B) anti-phosphorylated EGFR antibody. The data presented are representative results of experiments that were repeated for several times.

4.2.4 EGF-induced receptor internalization is inhibited by primary, but not tertiary alcohol.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The effect of alcohols was also tested on receptor internalization. Within 30 min of EGF treatment, the EGF receptor was internalized from the plasma membrane (Fig. 4.4.A, compare lanes 1 and 4). This was inhibited by 1-BtOH, but not by t-BtOH (Fig. 4.4.A, lanes 5 and 6), suggesting that PLD activity is also required for the EGF-induced receptor internalization. At this time point, there was no detectable loss of the EGF receptor in whole cell lysates (Fig. 4.4.B), which confirms that the loss was due to internalization but not degradation, also suggests that it is at the level of internalization that PLD is critical.

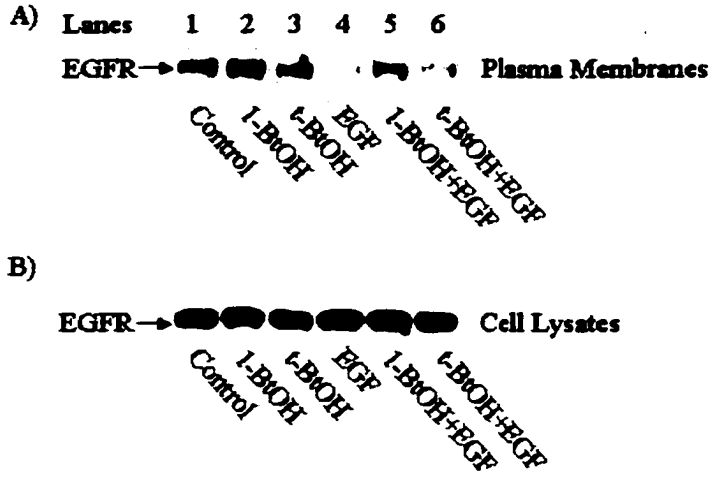


Fig. 4.4 EGF-induced receptor internalization is inhibited by primary, but not tertiary alcohol. 3Y1/EGFR cells were preincubated without or with the indicated alcohols (1% v/v) for 5 min, followed by 30 min of treatment without or with EGF (100 ng/ml) before harvested. A) Plasma membranes were isolated as in Fig. 4.1.B and subjected to Western Blot analysis using anti-EGFR antibody (LA22). B) The whole lysates of these cells (from which plasma membranes were isolated) were subjected to Western Blot analysis using anti-EGFR antibody (LA22). The data presented are representative results of experiments that were repeated for three times.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4.2.5 EGF-induced receptor internalization and degradation are dependent on RalA.

As described in Chapter 3, EGF-induced endogenous PLD activity in 3Y1/EGFR cells is dependent on the small GTPase RalA (Fig. 3.4). Thus the effect of RalA was also examined on the EGF-induced receptor internalization and degradation. As shown in Figure 4.5.A, overexpression of RalA WT increased the rate of receptor loss from the plasma membrane in response to EGF, whereas overexpression of the dominant negative RalA S28N significantly reduced the EGF-induced receptor internalization. Similarly, the degradation of EGFR was substantially accelerated by RalA WT, and retarded by RalA S28N (Fig. 4.5.B). Thus, higher level of induced PLD activity seen in the cells overexpressing RalA WT (Fig. 3.4) corresponded with an increased rate of internalization and degradation of the EGF receptor. Furthermore, this increased turnover of EGFR in 3Y1/EGFR/RalA WT cells was inhibited by 1-BtOH, but not t-BtOH (Fig. 4.5.C), indicating that the effect of RalA is dependent upon PLD activity. Collectively, these data show that RalA, which is required for EGF-induced endogenous PLD activity, is also required for the EGF-induced receptor internalization and degradation.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

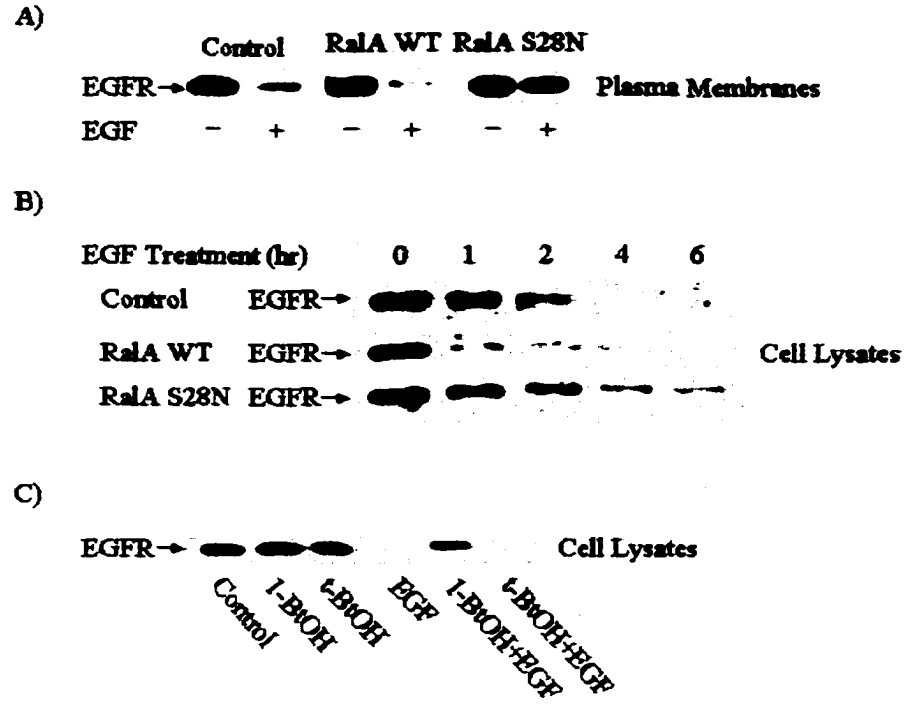


Fig. 4.5 EGF-induced receptor internalization and degradation are dependent on RalA.

A) 3Y1/EGFR, 3Y1/EGFR/RalA WT and 3Y1/EGFR/RalA S28N cells (as in Fig. 3.4) were treated without or with EGF (100 ng/ml) for 30 min before harvested. Plasma membranes were then isolated and subjected to Western Blot analysis using anti-EGFR antibody (LA22). B) These cells were treated without or with EGF (100 ng/ml) for the indicated time before harvested. The whole cell lysates were subjected to Western Blot analysis using anti-EGFR antibody (LA22). C) 3Y1/EGFR/RalA WT cells were treated without or with EGF (100 ng/ml) for 2 hr in the absence or presence of the indicated alcohols (1% v/v), which was added 5 min before EGF. The whole cell lysates were subjected to Western Blot analysis using anti-EGFR antibody (LA22). The data presented are representative results of experiments that were repeated for three times.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4.2.6 EGF-induced receptor internalization and degradation are regulated by PKC isoforms.

It was also demonstrated in Chapter 3 that PKCα is required for EGF-induced endogenous PLD activity in 3Y1/EGFR cells, while PKCδ has an inhibitory effect on this PLD activity (Fig. 3.7.A). When cells were treated with inhibitors of these PKC isoforms, the EGF-induced receptor internalization, as measured by the loss of EGFR from plasma membranes, was inhibited by Gö6976, but enhanced by rottlerin (Fig. 4.6.A). In these experiments, 30 min of EGF treatment typically reduced more than 80% (densitometry quantification, not shown) of membrane-bound EGFR, while in the presence of Gö6976, the receptor level was only reduced by 35%. Conversely, the PKCδ inhibitor rottlerin increased the loss to more than 90%, consistent with its ability to elevate PLD activity (Fig. 3.7.A). Similarly, Gö6976 (Fig. 4.6.B) inhibited, but rottlerin (Fig. 4.6.C) slightly accelerated the EGF-induced receptor degradation. Thus, inhibiting PKCα, which is required for EGF-induced endogenous PLD activity, inhibited the EGF-induced receptor internalization and degradation; on the other hand, elevating EGF-induced PLD activity by inhibiting PKCδ, increased the rate of these processes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

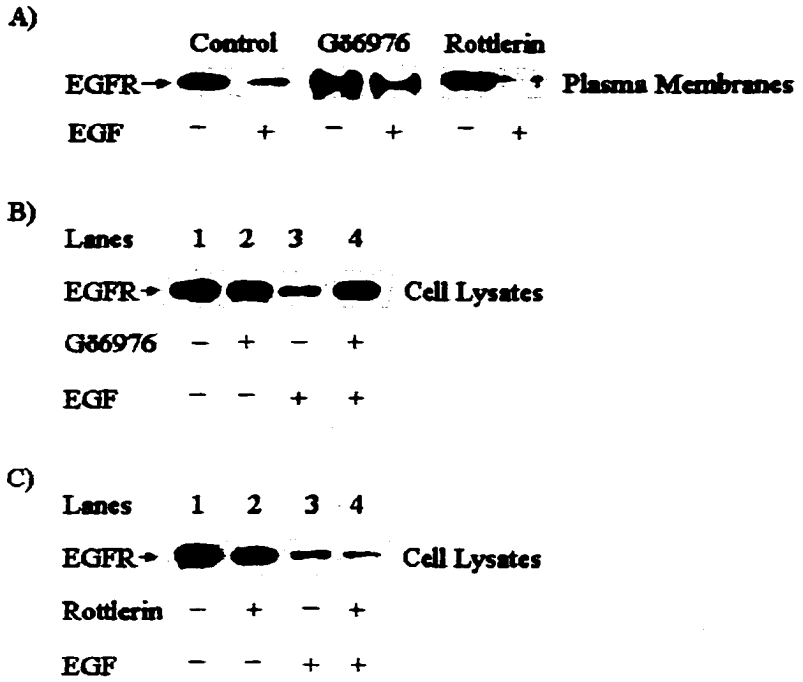


Fig. 4.6 EGF-induced receptor internalization and degradation are dependent on PKCα. 3Y1/EGFR cells were preincubated with DMSO (0.1% v/v), Gö6976 (0.5 μM in DMSO) or rottlerin (10 μM in DMSO) for 30 min, followed by treatment without or with EGF (100 ng/ml). A) After 30 min of EGF stimulation, cells were harvested and plasma membranes were isolated. The plasma membrane fractions were subjected to Western Blot analysis using anti-EGFR antibody (LA22). B), C) After 2 hr of EGF stimulation, cells were harvested and the whole cell lysates were subjected to Western Blot analysis using anti-EGFR antibody (LA22). The data presented are representative results of experiments that were repeated for three times.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4.2.7 Overexpression of PLD proteins influences EGFR degradation.

Data in Figure 4.5 and Figure 4.6 suggest that elevated PLD activity leads to increased receptor internalization and degradation. To test this hypothesis more directly, studies were done in 3Y1/EGFR cells overexpressing PLD proteins.

The stable transfectants (pooled) with PLD1 WT, PLD2 WT, PLD1 K898R or PLD2 K758R were prepared as described in Chapter 2 and the expression of PLD in these cells was verified by Western Blot analysis (Fig. 4.7.A). The subcellular distribution of the overexpressed PLD1 and PLD2 was also examined by cell fractionation and was found to have essentially the same pattern as observed for endogenous PLD1 and PLD2 (unpublished data of Lizhong Xu).

Both the EGF-induced PLD activity and receptor degradation were examined in these cells. As shown in Figure 4.7.B, unlike in the established cell lines (Fig. 3.2.B), the EGF-stimulated PBt production in these pooled transfectants was only weakly elevated by PLD1 WT or inhibited by PLD1 K898R (discussed below). Nevertheless, each of these PLD proteins had remarkable effect on the EGF-induced receptor degradation (Fig. 4.7.C). When treated with EGF, more than 90% (densitometry quantitation) of EGFR was degraded before 2 hr in cells overexpressing PLD1 WT or PLD2 WT, whereas in the parental 3Y1/EGFR cells, it took about 4 hr to reach the same level of depletion. However, in cells overexpressing PLD1 K898R or PLD2 K758R, EGF treatment hardly decreased the receptor level by 4 hr (Fig. 4.7.C). Thus it confirms that higher PLD activity correlates with faster endocytosis.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

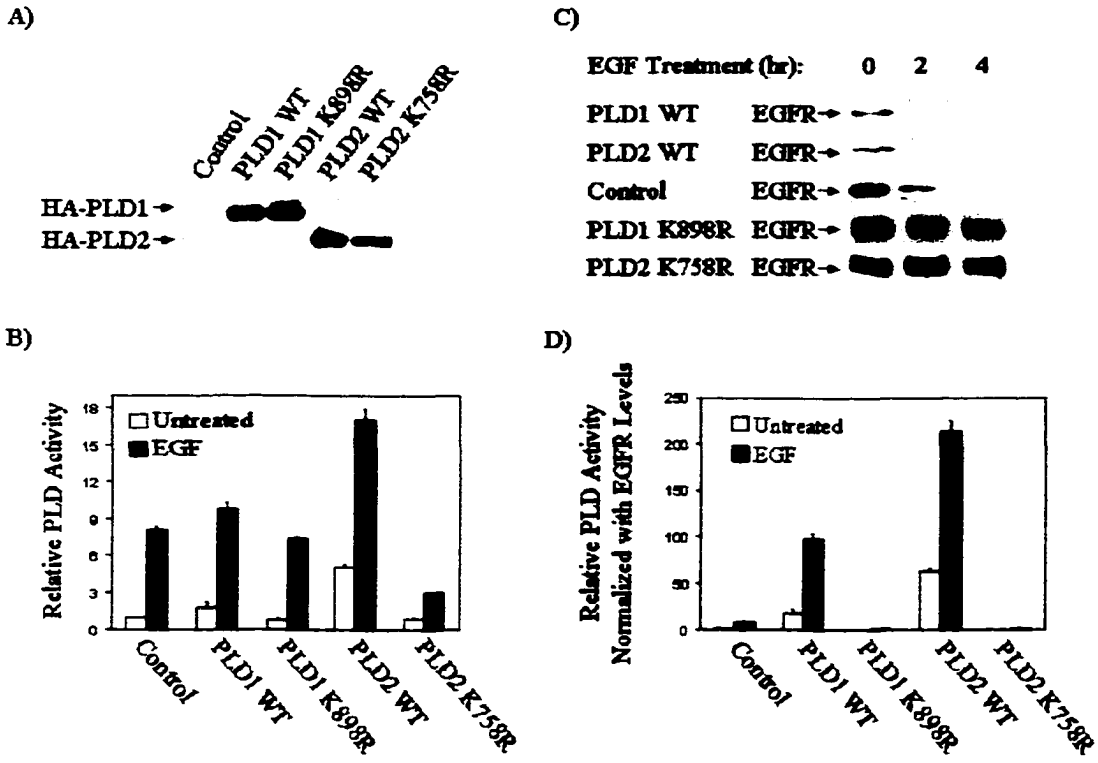


Fig. 4.7 Overexpression of PLD proteins influences EGFR degradation.

3Y1/EGFR cells stably overexpressing PLD1 WT, PLD2 WT, PLD1 K898R or PLD2 K758R were pooled as described in Chapter 2. A) Cells were harvested and the expression level of PLD was analyzed with Western Blot using anti-HA antibody. B) PLD activity in these cells was assayed in the same way as in Fig. 3.2.B. C) These cells were treated with EGF (100 ng/ml) for the indicated time before harvested. The whole cell lysates were subjected to Western Blot analysis using anti-EGFR antibody (LA22). D) PLD activity in each type of these cells as shown in B) was normalized with the cells' basal level of EGFR as shown in C). Control: 3Y1/EGFR cells; PLD1 WT, PLD2 WT, PLD1 K898R, PLD2 K758R: stable pools of 3Y1/EGFR cells overexpressing these proteins. Error bars represent the mean +/- standard of three independent experiments performed in duplicate. The data presented in A) and C) are representative results of experiments that were repeated for three times.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

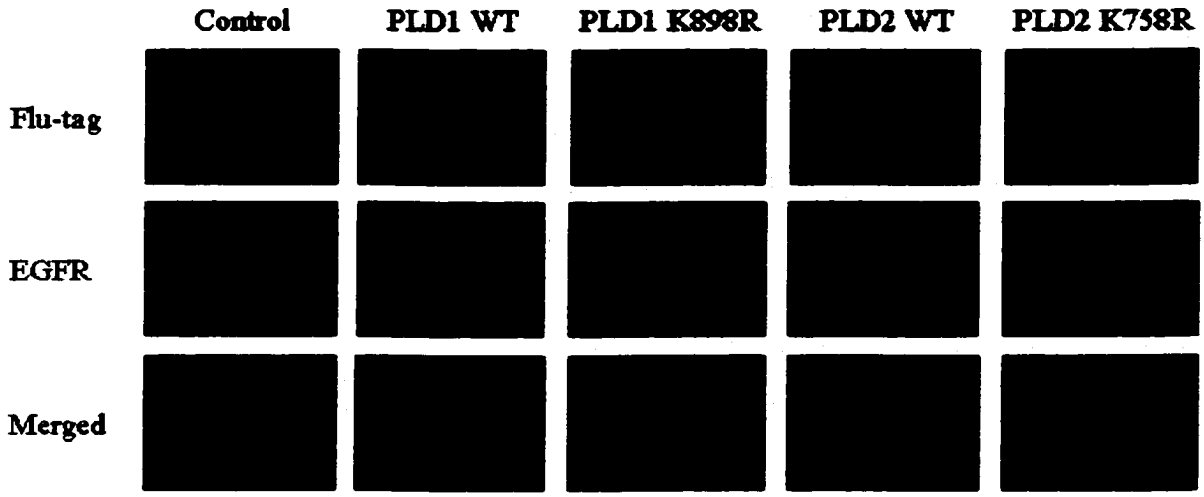


Fig. 4.8 Immunocytochemistry study of PLDs' influence on the basal EGFR level.

3Y1/EGFR cells were transiently transfected with PLD1 WT, PLD2 WT, PLD1-K898R or PLD2-K758R as described in Chapter 2. 48 hr after transfection, cells were prepared and double stained for the Flu-tagged PLD and EGFR with fluorophore-conjugated antibodies as described in Chapter 2. Upper panels: the Flu-tagged PLD proteins were stained with green fluorophore. Middle panels: EGF receptor was stained with red fluorophore. Bottom panels: the images in upper and middle panels were merged. Images shown are representative results that were observed in the majority of the treated cells in two independent experiments.

Moreover, the basal level of EGFR was significantly reduced by wild type PLDs but dramatically increased by the catalytically inactive mutants (Fig. 4.7.C, compare the EGFR levels of the untreated cells). This was confirmed by immunofluorescent staining. PLD proteins were transiently transfected into 3Y1/EGFR cells. The expression of the Flu-tagged PLD, as seen by green fluorescence (Fig. 4.8, upper panels), and the level of EGF receptor, as seen by red fluorescence (Fig. 4.8, middle panels) exhibited an interesting pattern of relation.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Cells with higher expression of PLD1 WT or PLD2 WT had lower level of EGF receptor (compared with adjacent cells), whereas cells with higher level of the mutant PLDs also had higher level of EGFR. When images of the two staining were merged (Fig. 4.8, bottom panels), the red and green stain did not overlap in wild type PLD-expressing cells; but overlapped in mutant PLD-expressing cells. Thus, these data are consistent with those in Figure 4.7.C.

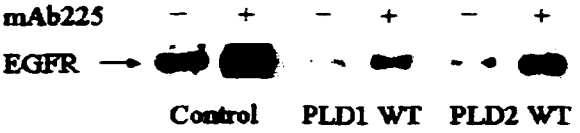


Fig. 4.9 Antagonistic anti-EGFR antibody reverses the effects of wild type PLDs on the basal level of EGFR.

3Y1/EGFR cells and the derived stable pools of PLD1 WT and PLD2 WT overexpressing cells (as in Fig. 4.7) were treated without or with mAb225 (2 μg/ml) for 2 hr. Cells were then collected and the lysates were subjected to Western Blot analysis using anti-EGFR antibody (LA22). Control: 3Y1/EGFR cells; PLD1 WT, PLD2 WT: stable pools of 3Y1/EGFR cells overexpressing these proteins. The data presented are representative results of an experiment that was repeated for several times.

To test if the decrease of basal EGFR level is an effect of accelerated default endocytosis, cells overexpressing PLD1 WT and PLD2 WT were treated with antagonistic anti-EGFR antibody mAb225. The monoclonal antibody mAb225 inhibits ligand binding of EGFR and its kinase activity, therefore was used to block the receptor signaling (175). As shown in Figure 4.9, mAb225 significantly increased

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the amount of EGFR in 3Y1/EGFR cells, indicating that this antagonistic antibody inhibited the default endocytosis of EGFR. Moreover, this antibody reversed the effect of wild type PLDs on the basal receptor level, and almost restored the EGFR level in PLD2 WT overexpressing cells to the level in the parental 3Y1/EGFR cells (Fig. 4.9). Thus these data suggest that not only the EGF-induced, but also the default receptor endocytosis is regulated by PLD. The fact that overexpression of PLD1 WT or PLD2 WT *per se* was enough to decrease the receptor level to that seen in the parental 3Y1/EGFR cells only after hours of EGF treatment (Fig. 4.7.C), indicating that elevated PLD activity is sufficient to induce receptor endocytosis in these cells.

In order to understand the puzzling ineffectiveness of both PLD1 wild type and the catalytically inactive PLD1 K898R on cellular PLD activity, the EGF-induced PBt production (shown in Figure 4.7.B) was normalized with the basal level of EGFR of each of these cells (densitometry quantitation for Fig. 4.7.C, not shown), since the EGF receptor is a key for cells' response to EGF. As shown in Figure 4.7.D, the relative PLD activity adjusted with the amount of EGFR was strongly elevated by both PLD1 WT, and remarkably inhibited by PLD1 K898R; meanwhile the effects of PLD2 WT and PLD2 K758R after such normalization were also enhanced. Given the fact that the basal level of EGFR is dramatically different among these cells, the normalized value should reflect the *bona fide* PLD activity under the condition that the same intensity of extracellular signal is received.

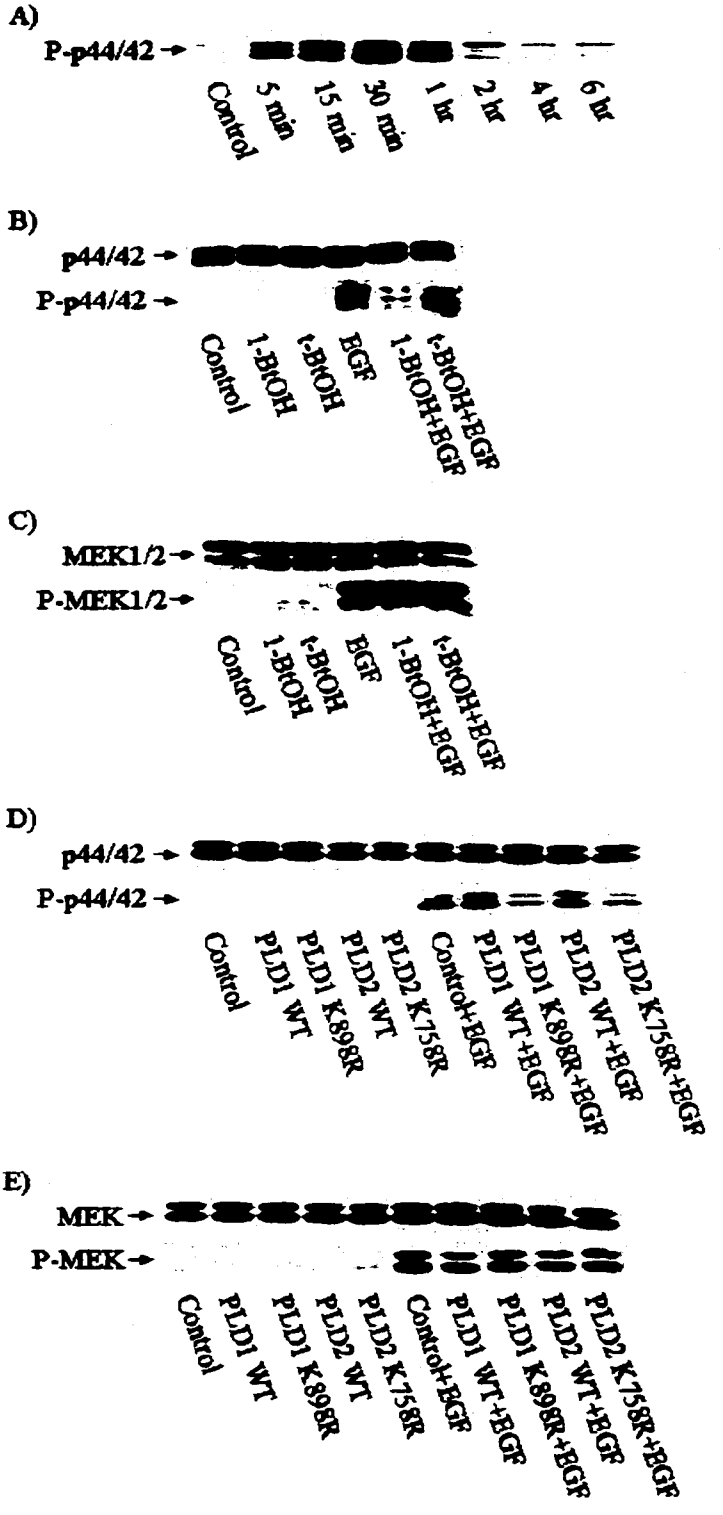
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

4.2.8 EGF-induced MAPK, but not MEK activation is dependent upon PLD.

Besides the events of receptor internalization and degradation, the signal transduction originated from EGFR was also evaluated for the dependency on PLD. Ligand binding triggers dimerization and autophosphorylation of the EGF receptor, followed by a cascade of signaling responses. A key downstream event after EGF receptor ligation is the activation of the mitogen activated protein kinase (MAPK) / extracellular signal regulated kinase (ERK) pathway (176,177). The EGF-induced MAP kinase activation has been shown to be dependent on receptor endocytosis (178,179). However the activation of MAPK kinase (MEK) is independent of this process (179). Therefore the effect of inhibiting PA production on the EGF-induced activation of MAPK and MEK was examined. As shown in Figure 4.10.A, p42/p44 MAPK was phosphorylated within 5 minutes after EGF treatment. 1-BtOH, but not t-BtOH prevented EGF-induced phosphorylation of MAPK (Fig. 4.10.B, lower panel). This treatment did not affect the level of MAPK protein (Fig. 4.10.B, upper panel). In contrast, the phosphorylation of MEK was not affected by 1-BtOH (Fig. 4.10.C), indicating that MEK activation is independent of PLD activity. To confirm these results, the activation of these signals was also examined in cells overexpressing PLD proteins. The EGF-induced MAPK phosphorylation was enhanced by both PLD1 WT and PLD2 WT, and inhibited by the catalytically inactive mutants (Fig. 4.10.D); however, no effect of these PLD proteins was observed on the phosphorylation of MEK (Fig. 4.10.E). Thus, the EGF-induced MAPK phosphorylation, which is dependent on EGFR internalization, is dependent on PLD activity; EGF-induced

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

MEK activation, which is independent of EGFR internalization, is not dependent on PLD activity.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. 4.10 EGF-induced MAPK activation is dependent on PLD; EGF-induced MEK activation is not dependent on PLD.

A) 3Y1/EGFR cells were treated without or with EGF (100 ng/ml) for the indicated time. Cells were then harvested and the lysates were subjected to Western Blot analysis using an anti-phosphorylated MAPK (P-p42/44) antibody. B) 3Y1/EGFR cells were treated without or with EGF (100 ng/ml) for 30 min, in the absence or presence of the indicated alcohols (1% v/v), which were added 5 min prior to EGF treatment. Cells were then harvested and the lysates were subjected to Western Blot analysis using either anti-MAPK (p44/42) antibody (upper panel) or anti-phosphorylated MAPK (P-p44/42) antibody (lower panel). (C) The samples from B) were also analyzed for MEK1/2 and phosphorylated MEK1/2 by Western Blot analysis using either anti-MEK1/2 (upper panel) or anti-phosphorylated MEK1/2 antibodies (lower panel). D) 3Y1/EGFR cells were transiently transfected with PLD1 WT, PLD2 WT, PLD1-K898R or PLD2-K758R as in Fig. 4.8. 48 hr after transfection, cells were harvested and the lysates were subjected to Western Blot analysis using either anti-MAPK (p44/42) antibody (upper panel) or anti-phosphorylated MAPK (P-p44/42) antibody (lower panel). E) The samples from D) were also analyzed for MEK1/2 and phosphorylated MEK1/2 by Western Blot analysis using either anti-MEK1/2 (upper panel) or anti-phosphorylated MEK1/2 antibodies (lower panel). The data presented are representative results of experiments that were repeated for at least twice.

4.3 Discussion

PLD activity is elevated in response to wide variety of mitogenic signals. Yet, in spite of the apparent ubiquitous involvement of PLD, little is known about the purpose of its activation, or what are the effects of PLD and its primary metabolite PA on the transduction of intracellular signals. Data presented in this chapter suggested that a role PLD plays in EGF signaling is to facilitate receptor-mediated

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

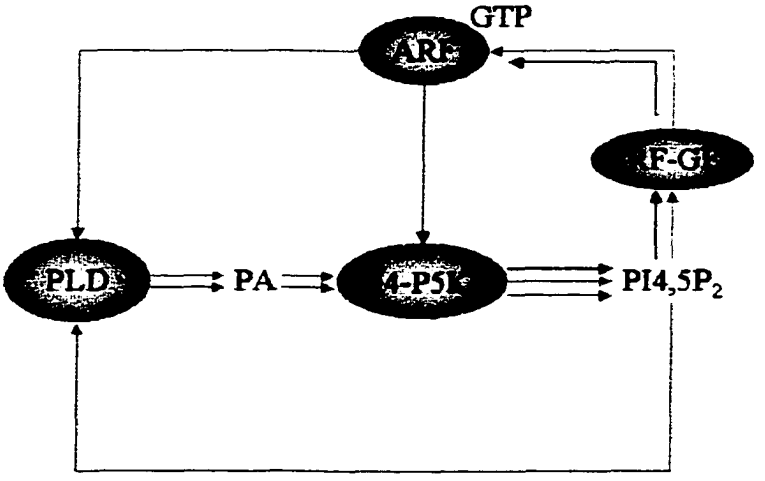
endocytosis. The mechanism of how PLD regulates endocytosis is unclear. However, it is possible that PLD may contribute to this process in a way similar to its functions in other intracellular vesicle transports, and that PLD may be involved in multiple steps of vesicle transportation, which include the stimulation of membrane fission and fusion, and the cytoskeleton reorganization which provides tracks for vesicle movement (2).

PLD converts PC to PA; and as discussed in Chapter 1, PA can itself be metabolized into other lipids such as DG or LPA (Scheme 1.3); PA can also activate PI4-P5Kα (60) that produces PIP_2. Thus it is a question whether the observed effect of PLD in the receptor endocytosis is due to PA *per se*, or is caused by these other lipids whose synthesis are secondary to PA formation. It was found that neither DG nor LPA is effective in Golgi-dependent secretion (156,180); however, whether this also applies to the receptor endocytosis has to be evaluated. One approach to explore this issue is to test the requirement of the enzymes that generate these lipids, i.e., PAP that produces DG, PLA_2 that makes LPA, or PI4-P5Kα that synthesizes PIP_2. Alternatively, the influence of exogenously supplied lipids should also help to clarify which of these lipids is (are) functionally in charge of the endocytosis.

There are currently different models regarding how PA enhances vesicle budding, which differ as to whether PA functions directly or through PIP_2 (33,36,149,154,156,157,159). Interestingly, whereas PA stimulates the biosynthesis of PIP_2, PIP_2 itself is an essential cofactor for the activation of PLD that generates PA (181), thus it provides a positive feedback; alternatively, PIP_2 can stimulate Arf GEF (68,69) that promotes GTP-binding of Arf, the activated Arf would in turn stimulate

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PLD activity and thus it provides another positive feedback between PIP_2 and PA (Scheme 4.1). Because of this, it is difficult to dissociate the effects of these two lipids. Both PA and PIP_2 are negatively charged acidic phospholipids, the predicted effect of a local elevation of such lipids would be increased membrane curvature due to the physical properties of these lipids (182,183), and the recruitment of coat proteins, dynamin and Arf (33,154,159,184-188) that ultimately lead to vesicle budding and membrane fission. Thus it is unlikely that either PA or PIP_2 is the only lipid responsible for the vesiculation, but rather there is a collaboration of these two.



Scheme 4.1 Two positive feed back loops between PA (PLD) and PIP_2.

PLD hydrolyses PC to generate PA and choline. PA is known as an activator of PI 4-P5Kα (60) that produces PIP_2. PIP_2 itself is an essential cofactor for the activation of PLD (181), thus there is a positive feedback between PA (PLD) and PIP_2 (red loop). PIP_2 also stimulates Arf GEF activity (68,69), which promotes GTP-binding of Arf. The activated Arf in turn stimulates PLD activity and that provides another positive feedback between PIP_2 and PA (PLD) (blue loop). Arf activates PI 4-P5Kα (189,190) and leads a positive feedback to PIP_2 (black loop).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Data present in this chapter demonstrate that PLD activity is both required and sufficient for the EGF receptor endocytosis in 3Y1/EGFR cells. Moreover, the overexpression of PLD proteins suggested that both PLD1 and PLD2 are involved. However, it is not clear if there are differences in the contribution of these two isoforms. Since PLD1 and PLD2 respond differently to PKCα (Fig. 3.7.C), the effect of PKCα on the receptor endocytosis may help to differentiate the involvement of the two isoforms. Interestingly, PKCα inhibitor Gö6976 partially inhibited on the receptor internalization (Fig. 4.6.A), but almost completely blocked the receptor degradation in response to EGF (Fig. 4.6.B), indicating that different PLD activities are involved in these two separate steps of endocytosis. Because PLD2 is not regulated by PKCα in these cells (does not respond to Gö6976) (Fig. 3.7.C), it is likely that PLD2 participates in the step of internalization but not degradation. PLD1 is regulated by PKCα (Fig. 3.7.C), and thus is probably responsible for the endosomal / lysosomal fusion that leads to proteolysis, and also partially involved the receptor internalization. This speculation is consistent with the subcellular localization of these two isoforms. As discussed in Chapter 1, PLD1 predominantly localizes in intracellular membranes (2,25), among which are early endosomes (31), late endosomes (12,32) and lysosomes (29,32,33); it was also found in plasma membrane (21,25,26,30,36) and caveolin-enriched light microdomains (37-39). PLD2 exclusively localizes on plasma membrane or caveolin-enriched light microdomains under basal conditions (12,36,39-43), and may redistribute into putative early endosomes after stimulation (12). The presence of PLD1 but not PLD2 in late

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

endosomes and lysosomes suggests that the step of lysosomal fusion is regulated by PLD1. In addition, PLD2 was found to constitutively form complexes with EGFR on the plasma membrane (42) while PLD1 was reported to colocalize with internalized EGFR in endosomes (31). Further more, PLD2 is turned on by EGF more promptly than PLD1 (Fig. 3.8) and this timing of activation also supports the role for PLD2 that act on the plasma membrane to facilitate the receptor internalization, which has to happen prior to the step of lysosomal fusion regulated by PLD1. However, to verify this hypothesis, more direct evidence is required.

Consistent with the observation that RalA is required for EGFR endocytosis in 3Y1/EGFR cells (Fig. 4.5), Nakashima et al. have recently shown that Ral is required for EGF and insulin-induced receptor endocytosis in A431 and CHO-IR cells respectively (191). After activated by EGF, Ral and its downstream molecules were found to form complexes with EGFR, clathrin and clathrin adaptor protein complex AP2 (191).

It is well established that EGFR is internalized through clathrin-coated vesicles and the assembly of AP2 on plasma membrane has been found insensitive to BFA (192). Consistently, in 3Y1/EGFR cells, neither the EGF-induced PLD activity (Fig. 3.5) nor the EGF receptor endocytosis (data not shown) was affected by this drug, indicating that the regulatory machinery either doesn't involve an Arf, or involves some Arf whose GEF is insensitive to BFA, e.g. Arf1 or Arf6. In agreement with the later possibility, dominant negative Arf6 T27N was found to block the default turnover of EGFR (unpublished data of Lizhong Xu), which is similar to the effect of the catalytically inactive mutants of PLDs. Taken together with the plasma

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

membrane / early endosome localization of Arf6 and its function in endocytosis (144,148,153), it is possible that Arf6 mediates EGF signals to activate of PLD2 (also discussed in Chapter 3) and receptor internalization in these cells.

Although degradation is the ultimate fate of internalized EGFR, the rate of degradation is much slower than the rate of internalization (Fig. 4.1); the substantial lag period between these two steps suggests that the receptor is still on duty during this time. It is shown here that PLD is not only involved in the receptor-mediated endocytosis, but also required for the signals that are dependent on endocytosis, in other words, PLD is essential for the signals that are transmitted through the endocytic vesicles. Thus these data have strengthened the hypothesis of 'signaling endosomes' whereby receptor internalization is not merely a negative feedback pathway leading to its downregulation, but also an important aspect of intracellular signal transduction (193). Evidence is growing that supports such hypothesis: the internalization of nerve growth factor (NGF) and its receptor TrkA is required for transmission of NGF-mediated signals from distal axons to the cell body (194); EGF receptor remains phosphorylated and associated with signaling molecules after being internalized into endosomes (195,196); endocytosis of the β-adrenergic receptor and EGF receptor is required for ligand-induced MAPK activation (178,179,197,198). The last observation is consistent with the data in Figure 4.10 that EGF-induced MAPK activation is dependent upon PLD activity, which is required for the EGF receptor endocytosis.

BFA, which prevents PLD activation by insulin, inhibited insulin receptor internalization (123), suggesting that PLD activity may be critical in regulating

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

endocytosis of insulin receptor as well. At this point, we don't know if the effect of PLD on receptor endocytosis could be universal, but it will be interesting to evaluate the involvement of this enzyme in the endocytosis of other receptor tyrosine kinases and G protein-coupled receptors (174,199-202) that also activate PLD (101,124,203-206).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 5 A Role for Phospholipase D 2 in Src-Induced Cell Protrusion and Tumor Invasion

5.1 Introduction

Both PLD1 and PLD2 were found overexpressed and hyperactivated in human cancers (207,208). Consistent with these findings, it has been shown that PLD facilitates oncogenic transformation (47,49,79) and is involved in cell survival (50). PLD activity has also been implicated in the process of tumor invasion whereby tumor cells migrate from their primary location into adjacent tissues (80-82).

Tumor invasion is a compulsory step in metastasis and is based on dysregulated cell motility (209). Cell motility is essential for many physiological processes such as embryogenesis, neurite outgrowth and wound healing; however, when improperly increased, it can cause calamities such as the spreading of cancers.

Cytoskeleton networks, which include actin microfilaments, microtubules and intermediate filaments, play a central role in cell motility (210-216). Actin filaments are pivotal in cell polarity, contractile and migration processes, and have been subject to intense scrutiny in the studies of tumor invasion where their dynamics are a fundamental issue (209,215,217,218). Microtubules are vital for intracellular trafficking of vesicles, organelles and proteins, function in chromosome alignment and segregation during mitosis, and have also been found critical for cell polarity and the direction of cell migration (211,213). The integrity of microtubules is essential

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

for tumor invasion and there have been numerous reports that microtubule disrupting drugs arrest tumor invasion in both tissue culture and tumor-transplanted animals (219-225). To achieve well organized locomotion, a precise coordination among these distinct networks is a necessity. It is thought that actin cytoskeletons generate the driving force for the motile cells, while microtubules steer the cells and guide their movement in a directed manner; misregulation may cause either deficiency or aggressiveness in cell motility (210-216).

Extracellular signals such as growth factors, cytokines, cell-cell and cell-matrix interactions, regulate cell motility through modulations on cytoskeleton components and their associated proteins and motors. Many of these signals are mediated by the non-receptor tyrosine kinase Src (226). Src was found localized along actin filaments and microtubules, as well as at focal adhesions and adherens junctions where both of these cytoskeleton networks interface (83); at these sites, Src interacts with a broad range of downstream effectors to coordinate these cytoskeletal systems and control the processes of adhesion and migration (226,227). Src kinase activity and sometimes Src protein level were found elevated in many types of cancers, with a correlation often observed between the increase in Src kinase activity and the degree of malignancy / invasiveness (83).

v-Src, a constitutively active form of Src, efficiently transforms cells (83), and this is usually accompanied by an induction of cellular PLD activity (84,115). Thus it is speculated that PLD may mediate some of the Src signals in developing malignant phenotypes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

It was found that exogenously provided bacterial PLD can induce tumor invasion (80,81) and that PLD activity is inhibited by anti-invasion drugs (82). However, there is no direct evidence that cellular PLD participates in this process; neither is it understood how PLD activity could be associated with elevated cell motility. Interestingly, PLD activity has been shown to be involved in the reorganization of actin cytoskeleton (12,153,228-232); however, so far, it has not been reported whether microtubule dynamics is also under influence of this enzyme. Data presented in this chapter demonstrate that PLD2 activity is necessary for v-Src-induced microtubule protrusion, and moreover, PLD2 is also required for the invasiveness of v-Src transformed cells as well as other tumor cells.

5.2 Results

5.2.1 PLD2, but not PLD1, is required for v-Src-induced cell protrusion in 3Y1 fibroblasts.

Cultured 3Y1 rat fibroblasts are well spread and have flat morphology (Fig. 5.1.A). However, upon introducing of v-Src, cells (3Y1/v-Src) exhibit transformed phenotypes, become less spread and extend filopodia-like protrusions or pseudopods (Fig. 5.1.D). As discussed in Chapter 3, the expression of v-Src elevates PLD activity in these cells. To explore if PLD activity mediates v-Src effects on cell protrusion, PLD proteins were stably transfected into 3Y1/v-Src cells (as described in Chapter 2) and the morphological changes were studied in these established cell lines (3Y1/v-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Src/PLD1 WT, 3Y1/v-Src/PLD1 K898R, 3Y1/v-Src/PLD2 WT, 3Y1/v-Src/PLD2 K758R). Interestingly, the protrusions in 3Y1/v-Src cells were strongly elongated by PLD2 WT (Fig. 5.1.E), but abolished by PLD2 K758R, which made cells round up (Fig. 5.1.F), indicating that PLD2 is required for the cell protrusion induced by v-Src. However, PLD2 WT alone did not cause protrusion, nor did PLD2 K758R make any obvious morphological change in the parental 3Y1 cells (Fig. 5.1.B, C). Thus, PLD2 is not sufficient to induce protrusion, but rather mediates v-Src signals to trigger this phenomenon. Overexpression of PLD1 proteins had no effect on the shape of 3Y1 cells either; however, unlike PLD2, PLD1 did not affect the protrusion of 3Y1/v-Src cells but caused numerous vesicle-like structures accumulated at the periphery of these cells (data not shown).

When PLD activity was measured in 3Y1/v-Src and those cells overexpressing PLD2 proteins (Fig. 5.2), it was found correlated with the level of cell protrusion (Fig. 5.1). Moreover, primary alcohol 1-BtOH, which blocks PA production by PLD (Fig. 4.2.A), retracted the protrusions in both 3Y1/v-Src and 3Y1/v-Src/PLD2 WT cells; as a control, t-BtOH has no effect (Fig. 5.3). Thus these data confirm that the activity of PLD2 is necessary for v-Src-induced cell protrusion.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

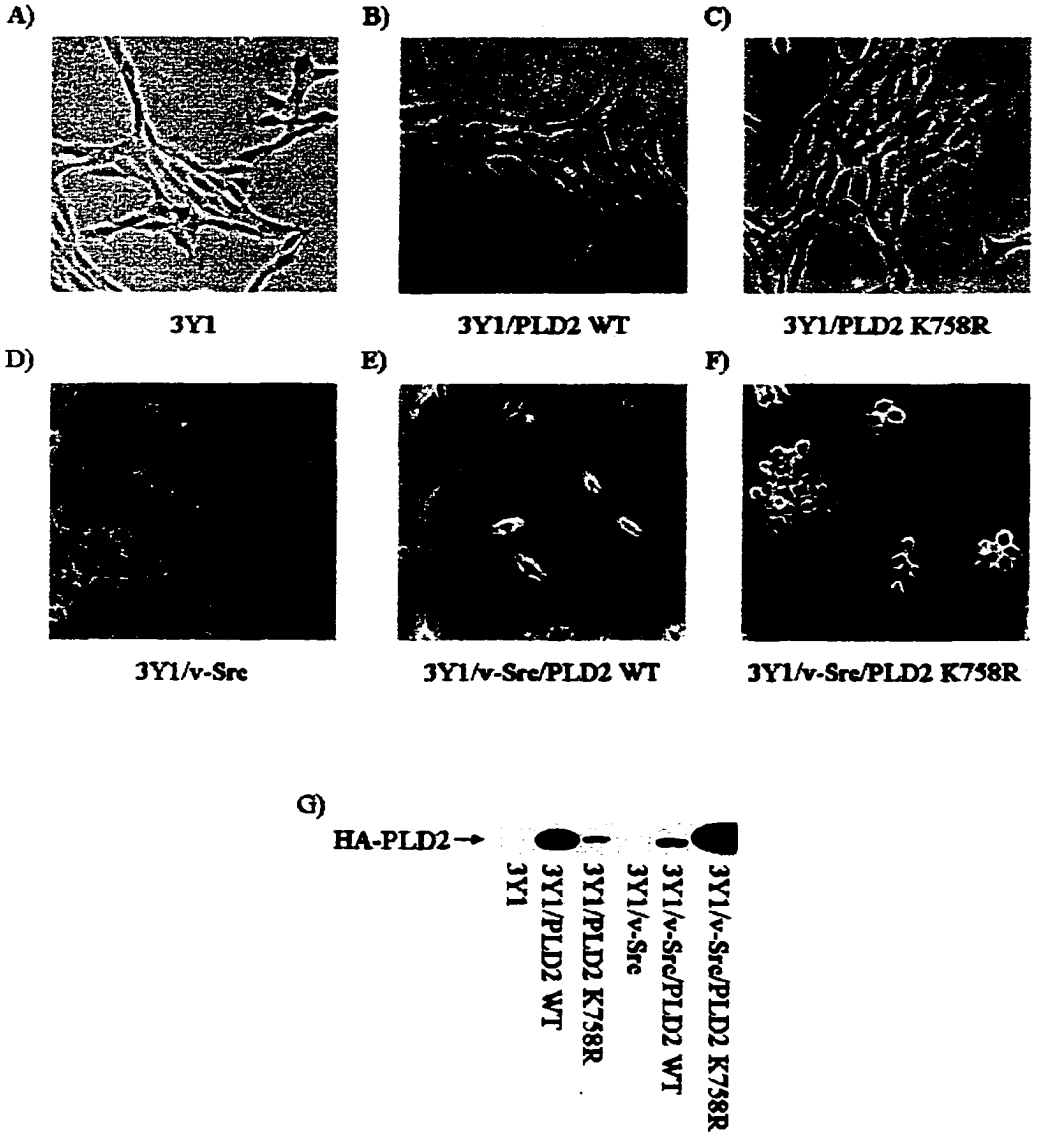


Fig. 5.1 PLD2 is required for v-Src-induced protrusion in 3Y1 cells.

A-F) 3Y1, 3Y1/v-Src and the derived cells stably overexpressing PLD2 WT or PLD2 K758R were prepared as described in Chapter 2. The morphology that represents the majority of each type of these cells was photographed under microscope. For each type of transfectants, two additional independent clones were also observed with the same results. G) The cells shown in A-F) were harvested and the lysates were subjected to Western Blot analysis using anti-HA antibody. The data presented are representative results of an experiment that was repeated for several times.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

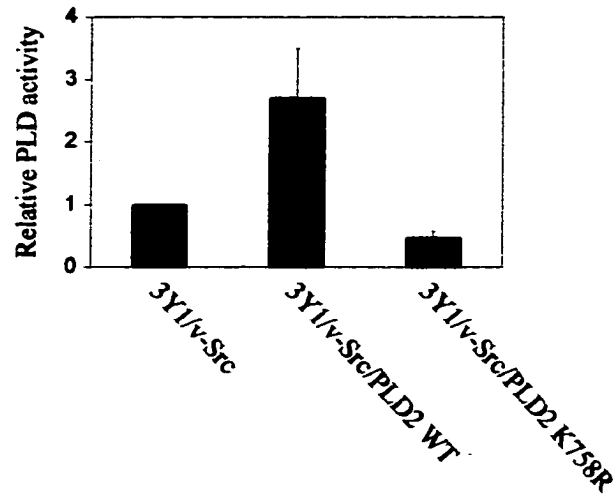
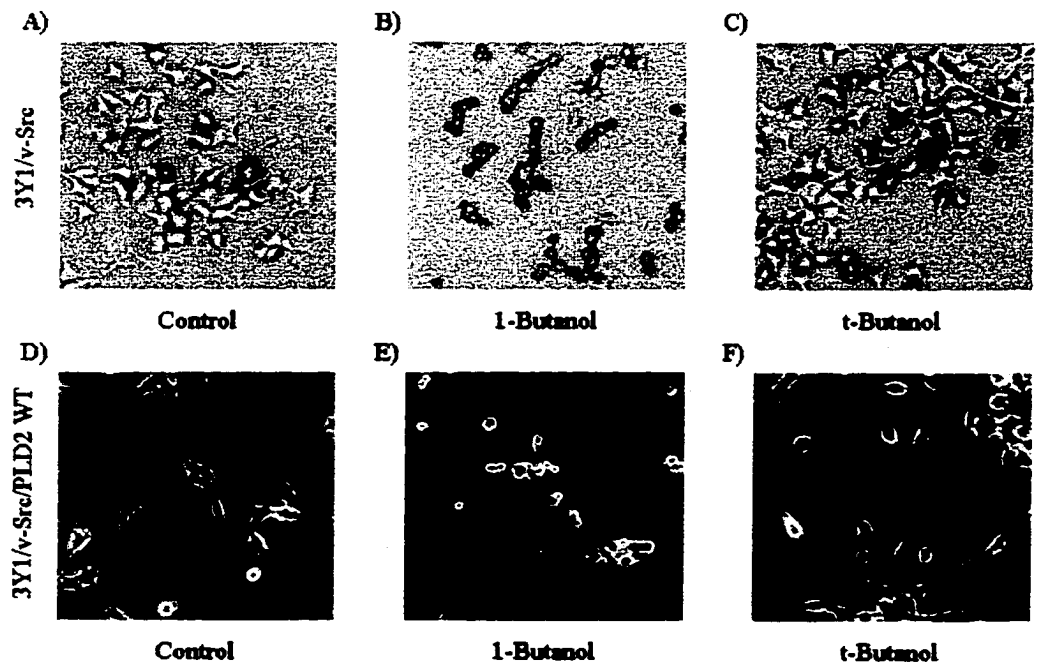


Fig. 5.2 PLD activity in 3Y1/v-Src/PLD2 cells.

3Y1/v-Src, 3Y1/v-Src/PLD2 WT and 3Y1/v-Src/PLD2 K758R cells were prelabeled with [^{3}H]-myristate for 4 hr and then incubated with 1-BtOH (1% v/v) for 15 min. The produced PBt was isolated by TLC, visualized by autoradiography and quantified by densitometry as described in Chapter 2, the values were normalized with the PLD activity in 3Y1/v-Src cells. Error bars represent the mean +/- standard of three independent experiments performed in duplicate.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. 5.3 Protrusions in 3Y1/v-Src and 3Y1/v-Src/PLD2 WT cells are abolished by primary, but not tertiary alcohol.

3Y1/v-Src and 3Y1/v-Src/PLD2 WT cells were treated without or with the indicated alcohols (1% v/v) for 15 min before the morphology that represents the majority of the cells was photographed under microscope. A), B), C): 3Y1/v-Src cells; D), E), F): 3Y1-v-Src/PLD2 WT cells. The data presented are representative results of an experiment that was repeated for several times.

5.2.2 v-Src-induced protrusions are dynamic microtubule-based structures.

To identify the cytoskeleton components of the protrusions, 3Y1/v-Src and 3Y1/v-Src/PLD2 WT cells were treated with drugs that interfere the polymerization of actin filaments or microtubules. Cytochalasin B, which blocks actin polymerization, rounded cell bodies but did not retract the protrusions in either 3Y1/v-Src or 3Y1/v-Src/PLD2 WT cells (Fig. 5.4). The same effects were observed with cytochalasin D, which also prevents actin polymerization (data not shown). In contrast, colchicine, an inhibitor of microtubule polymerization, completely abrogated the protrusions without rounding up cell bodies (Fig. 5.5.B, E). Furthermore, pretreatment with phalloidin, a compound that stabilizes actin filaments, did not prevent the retraction by colchicine (Fig. 5.5.C, F). Consistent with these data, *in situ* staining of both actin filaments and microtubules revealed that the structures are predominantly based on microtubules, although actin was also stained at the periphery and the tips of the protrusions (data not shown). Therefore, the major cytoskeletal components of v-Src-induced protrusions are microtubules, rather than actin filaments.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Interestingly, in 3Y1/v-Src/PLD2 WT cells, the length of protrusions changes dynamically and periodically, which is probably synchronized with the cell cycle (data not shown).

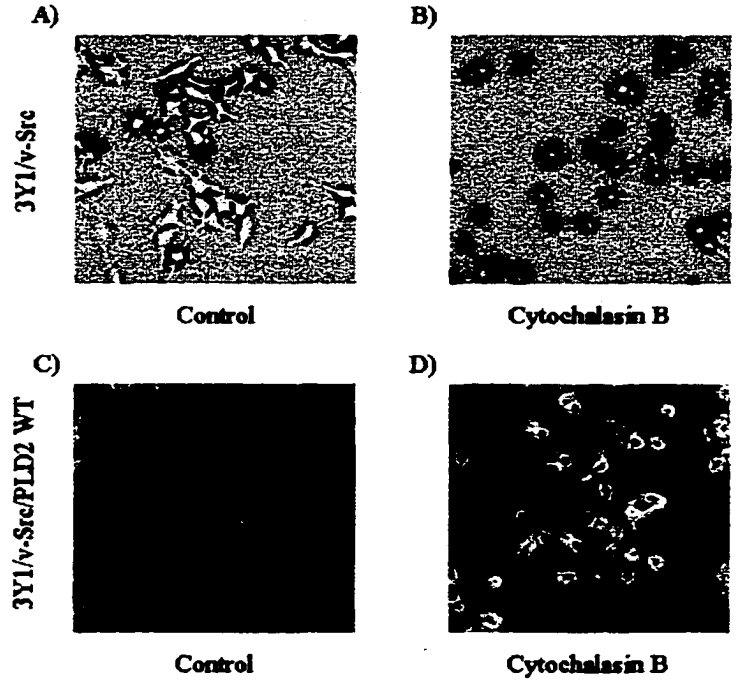


Fig. 5.4 Protrusions in 3Y1/v-Src and 3Y1/v-Src/PLD2 WT cells are not retracted by the inhibitor of actin polymerization.

3Y1/v-Src and 3Y1/v-Src/PLD2 WT cells were treated either with DMSO (0.1% v/v) or with cytochalasin B (15 μM in DMSO) for 30 min before the morphology that represents the majority of the cells was photographed under microscope. A), B): 3Y1/v-Src cells; C), D): 3Y1-v-Src/PLD2 WT cells. The data presented are representative results of an experiment that was repeated for several times.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

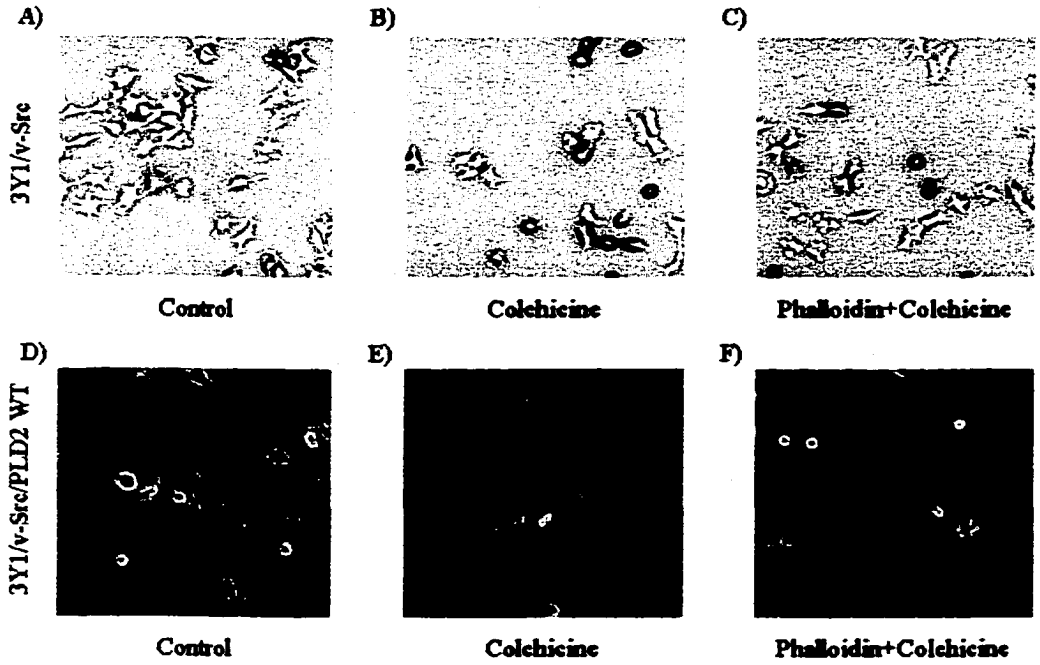


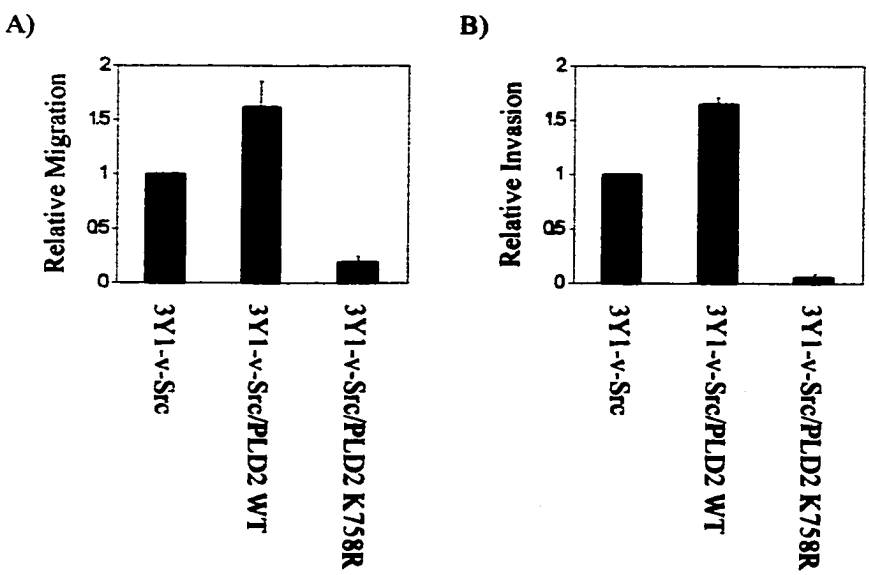
Fig. 5.5 Protrusions in 3Y1/v-Src and 3Y1/v-Src/PLD2 WT cells are abolished by the inhibitor of microtubule.

3Y1/v-Src and 3Y1/v-Src/PLD2 WT cells were either untreated, or treated without or with phalloidin (3.2 mM) for 1 hr followed by colchicine (50 μM) for 30 min before the morphology that represents the majority of the cells was photographed under microscope. A), B), C): 3Y1/v-Src cells; D), E), F): 3Y1-v-Src/PLD2 WT cells. The data presented are representative results of an experiment that was repeated for several times.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

5.2.3 PLD2 is also required for the motility of 3Y1/v-Src cells and MDA-MB-231 breast cancer cells.

Cytoskeleton dynamics is a prerequisite for cell motility (211-213,215,217) and the protrusion of pseudopodial structures has been shown to be a characteristic of invasive tumors (215). To test whether the observed protrusions are related to increased cell motility, 3Y1/v-Src, 3Y1/v-Src/PLD2 WT and 3Y1/v-Src/PLD2 K758R cells were subjected to an *in vitro* migration assay that assesses their intrinsic motility. Since migration is a mechanistic basis of invasion, the invasiveness of these cells was also examined. As shown in Figure 5.6, the ability of both migration and invasion of 3Y1/v-Src cells was enhanced by PLD2 WT, but almost abolished by the catalytically inactive mutant PLD2 K758R, exhibiting a pattern that correlated with the level of protrusion (Fig. 5.1), as well as the PLD activity in these cells (Fig. 5.2). Hence, the protrusions are motion-associated structures; and more importantly, PLD2 is not only required for v-Src-induced cell protrusion, but is also required for the related cell motility and invasiveness.



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. 5.6 PLD2 is required for the migration and invasion of 3Y1/v-Src cells.

3Y1/v-Src, 3Y1/v-Src/PLD2 WT and 3Y1/v-Src/PLD2 K758R cells were prepared and seeded into BIOCOAT™ cell culture inserts which have microporous filters on the bottom (as describe in Chapter 2). A) The number of cells that reached the lower surface of the filters after 24 hr was counted and normalized with the sample of 3Y1/v-Src cells. B) The experiment was performed and evaluated in the same way as in A) except that the microporous filters were coated with Matrigel™ before used. Error bars represent the mean +/- standard of three independent experiments performed in duplicate.

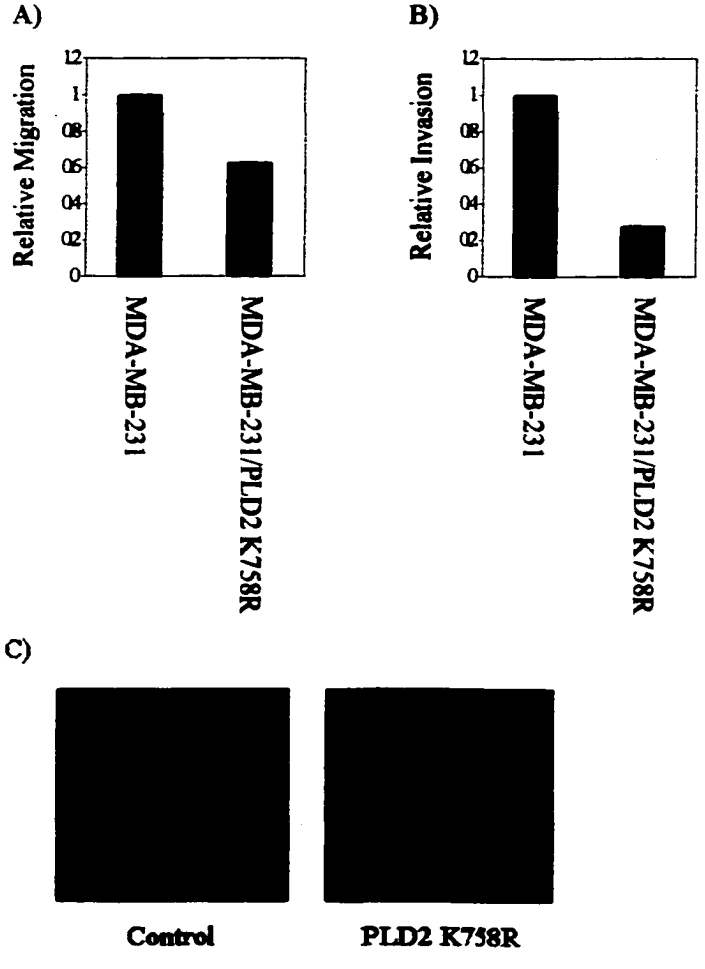


Fig. 5.7 PLD2 is required for the migration and invasion of MDA-MB-231 cells.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

MDA-MB-231 cells were transiently transfected without or with PLD2 K758R as described in Chapter 2. 48 hr after transfection, cells were suspended and seeded in BIOCOAT™ cell culture inserts as in Fig. 5.6. A) The migration ability of these cells was measured in the same way as in Fig. 5.6.A. B) The invasion ability of these cells was measured in the same way as in Fig 5.6.B. Values were normalized with the sample of the parental MDA-MB-231 cells. C) At the same time point, cells were fixed and stained for the Flu-tagged PLDs with fluorophore-conjugated antibody as described in Chapter 2. The data presented are representative results of experiments that were repeated for three times.

Because the oncogenic v-Src is sufficient to transform cells and that Src is found to be overexpressed and highly activated in a wide variety of human cancers (83,226), this phenomenon of PLD2-dependent motility in the v-Src transformed cells may imply a general involvement of PLD2 in the invasiveness of Src-related tumors. To examine such a possibility, the motility and invasiveness of MDA-MB-231 human breast cancer cells were studied. These adenocarcinoma cells overexpress c-Src as well as EGF and TGFα receptors and are highly invasive (233,234). Overexpression of PLD2 K758R inhibited both migration and invasion of these cells (Fig. 5.7), supporting the speculation that PLD2 may be crucial for the invasiveness of Src-related tumors.

5.3 Discussion

Data presented in this chapter demonstrate that PLD2 is required for v-Src-induced invasion of 3Y1 fibroblasts. The generality of the role for PLD2 in tumor

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

invasion is supported by fact that MDA-MB-231 breast cancer cells, which have increased level of c-Src, are also dependent on this isozyme for the invasiveness. In addition, these data suggest that the PLD2-dependent cytoskeleton (microtubule) reorganization may contribute to the elevated cell motility and invasion.

However, PLD2 is only required but not sufficient in this case. Consistently, it has been recently reported that PLD is also required but not sufficient for Arf GEF-induced morphological changes and migratory behavior (232). Moreover, Ral has been shown to be required for Ras-induced tumor invasion (235). Given that Ras and Ral hierarchically mediate PLD activation by v-Src (87,116), this observation and the data presented here may very likely reveal different aspects of the same event, which together suggest a signaling pathway elicited from Src and sequentially transmitted through Ras, Ral and PLD to the downstream phenomenon.

In order for v-Src to enhance cell motility, it has to orchestrate the dynamics of multiple cytoskeletons that each carries out distinct functions required for locomotion (210-216). Since the role microtubules play in cell motility is rather passive compared with the driving force provided by actin filaments, it is a question as to how v-Src-induced microtubule protrusions associate with the increased cell motility. Although the mechanism is not clear, there is no doubt that this has to go through the coordination between microtubule and actin cytoskeletons (210-216). Interestingly PLD2 has been shown to provoke the reorganization of actin networks (12) and it is demonstrated here that this isoform also regulates microtubules. Thus PLD2 is very likely a coordinator of these two cytoskeletal systems and this may

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

provide the mechanistic basis for it to manipulate the morphological and migratory behavior.

Among 3Y1, 3Y1/EGFR and 3Y1/v-Src cells, only the v-Src transformed cells exhibit dramatic morphological changes upon overexpression of PLD2. 3Y1 and 3Y1/EGFR cells essentially keep their relatively flat and well spread morphology when transfected with either PLD2 WT or PLD2 K758R. Stimulation of 3Y1/PLD2 WT cells with PDGF or treatment of 3Y1/EGFR/PLD2 WT cells with EGF for hours does not mimic the effect of v-Src (data not shown), indicating that PLD2 is selectively involved in Src signals that cause cell protrusion. Moreover, when examined with SDS-PAGE, PLD2 WT overexpressed in 3Y1/v-Src cells has apparently faster gel mobility (Fig. 5.1.G) compared with that expressed in 3Y1 and 3Y1/EGFR cells (data not shown), which is verified in all independent clones of these transfectants. However, v-Src does not affect the gel mobility of the catalytically inactive mutant PLD2 K758R (Fig. 5.1.G) nor PLD1 proteins (data not shown). Thus it seems that v-Src specifically induces posttranslational modification of PLD2 and this modification is also dependent on the activity of PLD2 itself. The fact that among all the PLD2 transfectants, 3Y1/v-Src/PLD2 WT cells are the only type of cells that have such modification of PLD2 and the only type of cells that have elongated morphology, has made us wondering whether this modification is possibly related to the morphological changes.

Interestingly, it was found that overexpression of PLD2 K758R in 3Y1/v-Src cells, which inhibits invasiveness, does not affect cell growth (data not shown). In agreement with this, a dominant negative Ral was reported to have the same effects in

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Ras-transformed cells (235). Moreover, colchicine that abolishes PLD2-dependent cell protrusion (Fig. 5.5) specifically blocked invasion without inhibiting tumor growth when used at therapeutic doses (236). This similarity of the influence of Ral, PLD2 and colchicine, again supports the postulated pathway that Src and Ras signals sequentially relay through Ral, PLD and microtubules to cause aggressive cell motility. And obviously, it also suggests that tumor growth and invasion are two independent issues.

Tumor invasion is a multiple step process. First, tumor cells must recognize and interact with the surrounding extracellular matrix (ECM); second, they must degrade or remodel the ECM; and finally can these tumor cells actually migration through the dissolved ECM to reach adjacent tissues (209). Interestingly, PLD has been implicated in each of these steps. PLD activity was shown to correlate with the production of CD44, a transmembrane hyaluronan receptor that mediates cell-matrix interaction (237,238). PLD was also reported to be required for the secretion of matrix metalloproteinases and urokinase plasminogen activator, which are proteases that dissolve the interstitial matrix and basement membranes to allow invasion (180,239-242). Furthermore, PLD was found to be involved in the reorganization of actin cytoskeletons (12,153,228-232), and to mediate v-Src-induced microtubule formation as well as the related cell motility (as demonstrated here). Collectively, the mechanisms by which PLD participates in tumor invasion might be through the remodeling of ECM and the increasing of cell motility.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 6 Summary

PLD is a signal-activated phospholipase and is conceptually grouped with other phospholipases, sphingomyelinases and lipid kinases, whose activation upon signaling result in the modification of various lipid constituents in cellular membranes and the generation of one or more products (messengers) that are able to recruit or modulate specific target proteins, thus altogether representing the molecular basis for rapidly growing field of lipid signaling (2).

The two isoforms of mammalian PLD, PLD1 and PLD2, are both activated by EGF stimulation. Based on the diverse kinetics of their activation by EGF (Fig. 3.8) and their different dependency on each other (Fig. 3.9), it is suggested that EGF sequentially activates PLD2 and PLD1, and that the activation of PLD1 by EGF is (at least partially) dependent on PLD2 activity. The fact that PLD1 and PLD2 respond differently to regulators such as PKC (Fig. 3.7.C), Arf (data not shown) and Ral (unpublished data of Jingqing Luo), reflects the heterogeneity in their molecular structure (4). On the other hand, this may also reflect their different subcellular localizations where different regulators are available, e.g., PLD1 on Golgi membranes is subjected to the regulation of Arf1 in this vicinity (94), while PLD2 is possibly modulated by Arf6 as both of them have very similar membrane location profiles in the plasma membrane region (12,36,40-43,124,144,145,148-153).

Settled in different membrane compartments and under regulation of different factors, PLD1 and PLD2 execute different functions. PLD1 causes vesicle-like

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

structures accumulated at the periphery of 3Y1/v-Src cells (data not shown), while PLD2 potentiates the microtubule-based cell protrusions in these cells (Fig. 5.1). Whereas both isoforms are involved in the EGF-induced receptor endocytosis in 3Y1/EGFR cells (Fig. 4.7.C), it is likely that the step of endosomal / lysosomal fusion is controlled by PLD1 but not PLD2.

However, despite all these distinctions, PLD1 and PLD2 still must crosstalk to each other since the EGF-induced PLD1 activation partially relies on PLD2 activity (Fig. 3.9.B). Moreover, these two isforms also have to cooperate to accomplish functions such as the EGF receptor endocytosis where inhibition of either one would block the turnover of EGFR (Fig. 4.7.C, Fig. 4.8).

Intriguingly, PLD not only facilitates vesicle formation and membrane fusion (as in endocytosis), it also contributes to build the cytoskeletal highways along which the vesicles traverse. In cells, actin filaments and microtubules are bridged through their associated proteins and motors to form continuous tracks that connect from the cortical to the cell body (210,213). Both PLD1 and PLD2 have been implicated in the regulation of actin cytoskeletons in response to extracellular stimuli (12,153,228-231) and PLD2 was shown here to be involved in v-Src-induced microtubule formation (Fig. 5.5). Therefore, PLD is a multifunctional traffic regulator: once activated by upstream signals, PLD stimulates vesicle budding and fission from the donor membranes, it also participates in the construction of the traffic roadways which lead to the destinations, and finally it facilitates the fusion of vesicles with the target membranes. Vesicle transportation is a process through which cells translocate signal molecules from one location to the other, thus the maneuver of vesicle

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

trafficking by PLD must be at least part of the mechanistic basis of its contribution in signal transduction.

The altered cytoskeleton systems (microtubules as observed here) that are regulated by PLD can also lead to increased cell motility (Fig. 5.6), which is a prerequisite for tumor invasion. Thus taken together with previous reports, this mitogenic signal-regulated enzyme is not only involved in tumorigenesis (47,49,50,79), but also participates in processes of metastasis such as ECM remodeling (180,237-242), as well as the invasive migration that is demonstrated here; and for this reason, this enzyme should provide a potential target for therapeutic intervention.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter 7 References

1. Holbrook, P. G., Pannell, L. K., and Daly, J. W. (1991) *Biochim Biophys Acta* **1084**(2), 155-8.

2. Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000) *Biochem J* **345 Pt 3**, 401-15.

3. Yang, S. F., Freer, S., and Benson, A. A. (1967) *J Biol Chem* **242**(3), 477-84.

4. Frohman, M. A., Sung, T. C., and Morris, A. J. (1999) *Biochim Biophys Acta* **1439**(2), 175-86.

5. Hammond, S. M., Altshuller, Y. M., Sung, T. C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) *J Biol Chem* **270**(50), 29640-3

6. Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G. D., Frohman, M. A., and Morris, A. J. (1997) *J Biol Chem* **272**(6), 3860-8.

7. Katayama, K., Kodaki, T., Nagamachi, Y., and Yamashita, S. (1998) *Biochem J* **329**(Pt 3), 647-52.

8. Park, S. K., Provost, J. J., Bae, C. D., Ho, W. T., and Exton, J. H. (1997) *J Biol Chem* **272**(46), 29263-71

9. Colley, W. C., Altshuller, Y. M., Sue-Ling, C. K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Branch, K. D., Tsirka, S. E., Bollag, R. J., Bollag, W. B., and Frohman, M. A. (1997) *Biochem J* **326**(Pt 3), 745-53.

10. Steed, P. M., Clark, K. L., Boyar, W. C., and Lasala, D. J. (1998) *Faseb J* **12**(13), 1309-17.

11. Nakashima, S., Matsuda, Y., Akao, Y., Yoshimura, S., Sakai, H., Hayakawa, K., Andoh, M., and Nozawa, Y. (1997) *Cytogenet Cell Genet* **79**(1-2), 109-13

12. Colley, W. C., Sung, T. C., Roll, R., Jenco, J., Hammond, S. M., Altshuller, Y., Bar-Sagi, D., Morris, A. J., and Frohman, M. A. (1997) *Curr Biol* **7**(3), 191-201

13. Kodaki, T., and Yamashita, S. (1997) *J Biol Chem* **272**(17), 11408-13

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

14. Lopez, I., Arnold, R. S., and Lambeth, J. D. (1998) *J Biol Chem* **273**(21), 12846-52

15. Sung, T. C., Altshuller, Y. M., Morris, A. J., and Frohman, M. A. (1999) *J Biol Chem* **274**(1), 494-502

16. Holbrook, P. G., Geetha, V., Beaven, M. A., and Munson, P. J. (1999) *FEBS Lett* **448**(2-3), 269-72.

17. Hodgkin, M. N., Masson, M. R., Powner, D., Saqib, K. M., Ponting, C. P., and Wakelam, M. J. (2000) *Curr Biol* **10**(1), 43-6.

18. Sung, T. C., Zhang, Y., Morris, A. J., and Frohman, M. A. (1999) *J Biol Chem* **274**(6), 3659-66.

19. Min, D. S., Park, S. K., and Exton, J. H. (1998) *J Biol Chem* **273**(12), 7044-51.

20. Meier, K. E., Gibbs, T. C., Knoepp, S. M., and Ella, K. M. (1999) *Biochim Biophys Acta* **1439**(2), 199-213.

21. Provost, J. J., Fudge, J., Israelit, S., Siddiqi, A. R., and Exton, J. H. (1996) *Biochem J* **319**(Pt 1), 285-91.

22. Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R. G., Symons, M., and Exton, J. H. (1995) *J Biol Chem* **270**(15), 8466-73.

23. Vinggaard, A. M., Provost, J. J., Exton, J. H., and Hansen, H. S. (1997) *Cell Signal* **9**(2), 189-96.

24. Liscovitch, M., Czarny, M., Fiucci, G., Lavie, Y., and Tang, X. (1999) *Biochim Biophys Acta* **1439**(2), 245-63

25. Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M., and Shields, D. (2001) *Mol Biol Cell* **12**(4), 943-55.

26. Whatmore, J., Morgan, C. P., Cunningham, E., Collison, K. S., Willison, K. R., and Cockcroft, S. (1996) *Biochem J* **320**(Pt 3), 785-94.

27. Ktistakis, N. T., Brown, H. A., Sternweis, P. C., and Roth, M. G. (1995) *Proc Natl Acad Sci U S A* **92**(11), 4952-6.

28. Decker, C., Miro Obradors, M. J., Sillence, D. J., and Allan, D. (1996) *Biochem J* **320**(Pt 3), 885-90.

29. Brown, F. D., Thompson, N., Saqib, K. M., Clark, J. M., Powner, D., Thompson, N. T., Solari, R., and Wakelam, M. J. (1998) *Curr Biol* **8**(14), 835-8.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

30. Morgan, C. P., Sengelov, H., Whatmore, J., Borregaard, N., and Cockcroft, S. (1997) *Biochem J* **325**(Pt 3), 581-5.

31. Hughes, W. E., and Parker, P. J. (2001) *Biochem J* **356**(Pt 3), 727-36.

32. Toda, K., Nogami, M., Murakami, K., Kanaho, Y., and Nakayama, K. (1999) *FEBS Lett* **442**(2-3), 221-5.

33. Arneson, L. S., Kunz, J., Anderson, R. A., and Traub, L. M. (1999) *J Biol Chem* **274**(25), 17794-805.

34. Balboa, M. A., and Insel, P. A. (1995) *J Biol Chem* **270**(50), 29843-7.

35. Banno, Y., Tamiya-Koizumi, K., Oshima, H., Morikawa, A., Yoshida, S., and Nozawa, Y. (1997) *J Biol Chem* **272**(8), 5208-13.

36. Vitale, N., Caumont, A. S., Chasserot-Golaz, S., Du, G., Wu, S., Sciorra, V. A., Morris, A. J., Frohman, M. A., and Bader, M. F. (2001) *Embo J* **20**(10), 2424-2434.

37. Kim, Y., Han, J. M., Han, B. R., Lee, K. A., Kim, J. H., Lee, B. D., Jang, I. H., Suh, P. G., and Ryu, S. H. (2000) *J Biol Chem* **275**(18), 13621-7.

38. Kim, J. H., Han, J. M., Lee, S., Kim, Y., Lee, T. G., Park, J. B., Lee, S. D., Suh, P. G., and Ryu, S. H. (1999) *Biochemistry* **38**(12), 3763-9.

39. Xu, L., Shen, Y., Joseph, T., Bryant, A., Luo, J. Q., Frankel, P., Rotunda, T., and Foster, D. A. (2000) *Biochem Biophys Res Commun* **273**(1), 77-83.

40. Czarny, M., Lavie, Y., Fiucci, G., and Liscovitch, M. (1999) *J Biol Chem* **274**(5), 2717-24

41. Czarny, M., Fiucci, G., Lavie, Y., Banno, Y., Nozawa, Y., and Liscovitch, M. (2000) *FEBS Lett* **467**(2-3), 326-32.

42. Slaaby, R., Jensen, T., Hansen, H. S., Frohman, M. A., and Seedorf, K. (1998) *J Biol Chem* **273**(50), 33722-7

43. Emoto, M., Klarlund, J. K., Waters, S. B., Hu, V., Buxton, J. M., Chawla, A., and Czech, M. P. (2000) *J Biol Chem* **275**(10), 7144-51.

44. Jones, D., Morgan, C., and Cockcroft, S. (1999) *Biochim Biophys Acta* **1439**(2), 229-44

45. Roth, M. G., Bi, K., Ktistakis, N. T., and Yu, S. (1999) *Chem Phys Lipids* **98**(1-2), 141-52

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

46. Kahn, R. A., Yucel, J. K., and Malhotra, V. (1993) *Cell* **75**(6), 1045-8.

47. Daniel, L. W., Sciorra, V. A., and Ghosh, S. (1999) *Biochim Biophys Acta* **1439**(2), 265-76.

48. Boarder, M. R. (1994) *Trends Pharmacol Sci* **15**(2), 57-62.

49. Lu, Z., Hornia, A., Joseph, T., Sukezane, T., Frankel, P., Zhong, M., Bychenok, S., Xu, L., Feig, L. A., and Foster, D. A. (2000) *Mol Cell Biol* **20**(2), 462-7

50. Nakashima, S., and Nozawa, Y. (1999) *Chem Phys Lipids* **98**(1-2), 153-64.

51. Ohguchi, K., Nakashima, S., and Nozawa, Y. (1999) *Biochim Biophys Acta* **1439**(2), 215-27.

52. Venable, M. E., and Obeid, L. M. (1999) *Biochim Biophys Acta* **1439**(2), 291-8.

53. Kanaho, Y., Nogami, M., and Honda, A. (2000) *Tanpakushitsu Kakusan Koso* **45**(7), 1219-28.

54. Martin, T. W. (1988) *Biochim Biophys Acta* **962**(3), 282-96.

55. Billah, M. M., Eckel, S., Mullmann, T. J., Egan, R. W., and Siegel, M. I. (1989) *J Biol Chem* **264**(29), 17069-77.

56. Anthes, J. C., Krasovsky, J., Egan, R. W., Siegel, M. I., and Billah, M. M. (1991) *Arch Biochem Biophys* **287**(1), 53-9.

57. Sciorra, V. A., and Morris, A. J. (1999) *Mol Biol Cell* **10**(11), 3863-76.

58. Song, J., Jiang, Y. W., and Foster, D. A. (1994) *Cell Growth Differ* **5**(1), 79-85

59. Kitatani, K., Akiba, S., and Sato, T. (2000) *Biol Pharm Bull* **23**(12), 1430-3.

60. Jenkins, G. H., Fisette, P. L., and Anderson, R. A. (1994) *J Biol Chem* **269**(15), 11547-54.

61. Ahmed, S., Lee, J., Kozma, R., Best, A., Monfries, C., and Lim, L. (1993) *J Biol Chem* **268**(15), 10709-12.

62. Randazzo, P. A., and Kahn, R. A. (1994) *J Biol Chem* **269**(14), 10758-63.

63. Tsai, M. H., Yu, C. L., Wei, F. S., and Stacey, D. W. (1989) *Science* **243**(4890), 522-6.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

64. Ghosh, S., Strum, J. C., Sciorra, V. A., Daniel, L., and Bell, R. M. (1996) *J Biol Chem* **271**(14), 8472-80.

65. Nishizuka, Y. (1992) *Science* **258**(5082), 607-14.

66. Moolenaar, W. H., and van Corven, E. J. (1990) *Ciba Found Symp* **150**, 99-106

67. Brown, M. T., Andrade, J., Radhakrishna, H., Donaldson, J. G., Cooper, J. A., and Randazzo, P. A. (1998) *Mol Cell Biol* **18**(12), 7038-51.

68. Chardin, P., Paris, S., Antonny, B., Robineau, S., Beraud-Dufour, S., Jackson, C. L., and Chabre, M. (1996) *Nature* **384**(6608), 481-4.

69. Paris, S., Beraud-Dufour, S., Robineau, S., Bigay, J., Antonny, B., Chabre, M., and Chardin, P. (1997) *J Biol Chem* **272**(35), 22221-6.

70. Waggoner, D. W., Xu, J., Singh, I., Jasinska, R., Zhang, Q. X., and Brindley, D. N. (1999) *Biochim Biophys Acta* **1439**(2), 299-316.

71. English, D., Cui, Y., and Siddiqui, R. A. (1996) *Chem Phys Lipids* **80**(1-2), 117-32.

72. Hodgkin, M. N., Pettitt, T. R., Martin, A., Michell, R. H., Pemberton, A. J., and Wakelam, M. J. (1998) *Trends Biochem Sci* **23**(6), 200-4.

73. Huijbregts, R. P., Topalof, L., and Bankaitis, V. A. (2000) *Traffic* **1**(3), 195-202.

74. Moolenaar, W. H. (1995) *J Biol Chem* **270**(22), 12949-52.

75. Swarthout, J. T., and Walling, H. W. (2000) *Cell Mol Life Sci* **57**(13-14), 1978-85.

76. Czech, M. P. (2000) *Cell* **100**(6), 603-6.

77. Toker, A. (1998) *Curr Opin Cell Biol* **10**(2), 254-61.

78. De Camilli, P., Emr, S. D., McPherson, P. S., and Novick, P. (1996) *Science* **271**(5255), 1533-9.

79. Hornia, A., Lu, Z., Sukezane, T., Zhong, M., Joseph, T., Frankel, P., and Foster, D. A. (1999) *Mol Cell Biol* **19**(11), 7672-80

80. Imamura, F., Horai, T., Mukai, M., Shinkai, K., Sawada, M., and Akedo, H. (1993) *Biochem Biophys Res Commun* **193**(2), 497-503.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

81. Mukai, M., Shinkai, K., Yoshioka, K., Imamura, F., and Akedo, H. (1993) *Hum Cell* **6**(3), 194-8.

82. Pai, J. K., Frank, E. A., Blood, C., and Chu, M. (1994) *Anticancer Drug Des* **9**(4), 363-72.

83. Bjorge, J. D., Jakymiw, A., and Fujita, D. J. (2000) *Oncogene* **19**(49), 5620-35.

84. Song, J., and Foster, D. A. (1993) *Biochem J* **294**(Pt 3), 711-7.

85. Jiang, H., Alexandropoulos, K., Song, J., and Foster, D. A. (1994) *Mol Cell Biol* **14**(6), 3676-82.

86. Qureshi, S. A., Cao, X. M., Sukhatme, V. P., and Foster, D. A. (1991) *J Biol Chem* **266**(17), 10802-6.

87. Jiang, H., Luo, J. Q., Urano, T., Frankel, P., Lu, Z., Foster, D. A., and Feig, L. A. (1995) *Nature* **378**(6555), 409-12.

88. Smart, E. J., Ying, Y. S., Mineo, C., and Anderson, R. G. (1995) *Proc Natl Acad Sci U S A* **92**(22), 10104-8

89. Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. (1987) *Cancer Res* **47**(12), 3239-45.

90. Albini, A. (1998) *Pathol Oncol Res* **4**(3), 230-41

91. Kim, J. H., Kim, Y., Lee, S. D., Lopez, I., Arnold, R. S., Lambeth, J. D., Suh, P. G., and Ryu, S. H. (1999) *FEBS Lett* **454**(1-2), 42-6.

92. Hoer, A., Cetindag, C., and Oberdisse, E. (2000) *Biochim Biophys Acta* **1481**(1), 189-201.

93. Sciorra, V. A., Rudge, S. A., Prestwich, G. D., Frohman, M. A., Engebrecht, J., and Morris, A. J. (1999) *Embo J* **18**(21), 5911-21.

94. Exton, J. H. (1999) *Biochim Biophys Acta* **1439**(2), 121-33

95. Houle, M. G., and Bourgoin, S. (1999) *Biochim Biophys Acta* **1439**(2), 135-49.

96. Min, D. S., Kim, E. G., and Exton, J. H. (1998) *J Biol Chem* **273**(45), 29986-94.

97. Kim, Y., Han, J. M., Park, J. B., Lee, S. D., Oh, Y. S., Chung, C., Lee, T. G., Kim, J. H., Park, S. K., Yoo, J. S., Suh, P. G., and Ryu, S. H. (1999) *Biochemistry* **38**(32), 10344-51.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

98. Min, D. S., and Exton, J. H. (1998) *Biochem Biophys Res Commun* **248**(3), 533-7.

99. Lee, T. G., Park, J. B., Lee, S. D., Hong, S., Kim, J. H., Kim, Y., Yi, K. S., Bae, S., Hannun, Y. A., Obeid, L. M., Suh, P. G., and Ryu, S. H. (1997) *Biochim Biophys Acta* **1347**(2-3), 199-204.

100. Lopez, I., Burns, D. J., and Lambeth, J. D. (1995) *J Biol Chem* **270**(33), 19465-72.

101. Du, G., Altshuller, Y. M., Kim, Y., Han, J. M., Ryu, S. H., Morris, A. J., and Frohman, M. A. (2000) *Mol Biol Cell* **11**(12), 4359-68.

102. Conricode, K. M., Brewer, K. A., and Exton, J. H. (1992) *J Biol Chem* **267**(11), 7199-202.

103. Singer, W. D., Brown, H. A., Jiang, X., and Sternweis, P. C. (1996) *J Biol Chem* **271**(8), 4504-10.

104. Ohguchi, K., Banno, Y., Nakashima, S., and Nozawa, Y. (1995) *Biochem Biophys Res Commun* **211**(1), 306-11.

105. Sciorra, V. A., Hammond, S. M., and Morris, A. J. (2001) *Biochemistry* **40**(8), 2640-6.

106. Melendez, A. J., Harnett, M. M., and Allen, J. M. (2001) *Curr Biol* **11**(11), 869-74.

107. Slaaby, R., Du, G., Altshuller, Y. M., Frohman, M. A., and Seedorf, K. (2000) *Biochem J* **351 Pt 3**, 613-9.

108. Urano, T., Emkey, R., and Feig, L. A. (1996) *Embo J* **15**(4), 810-6.

109. Luo, J. Q., Liu, X., Frankel, P., Rotunda, T., Ramos, M., Flom, J., Jiang, H., Feig, L. A., Morris, A. J., Kahn, R. A., and Foster, D. A. (1998) *Proc Natl Acad Sci U S A* **95**(7), 3632-7

110. Rothman, J. E. (1994) *Nature* **372**(6501), 55-63

111. Moss, J., and Vaughan, M. (1998) *J Biol Chem* **273**(34), 21431-4.

112. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) *Cell* **75**(6), 1137-44

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

113. Cockcroft, S., Thomas, G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) *Science* **263**(5146), 523-6

114. Brown, H. A., Gutowski, S., Kahn, R. A., and Sternweis, P. C. (1995) *J Biol Chem* **270**(25), 14935-43.

115. Song, J. G., Pfeffer, L. M., and Foster, D. A. (1991) *Mol Cell Biol* **11**(10), 4903-8

116. Jiang, H., Lu, Z., Luo, J. Q., Wolfman, A., and Foster, D. A. (1995) *J Biol Chem* **270**(11), 6006-9.

117. Luo, J. Q., Liu, X., Hammond, S. M., Colley, W. C., Feig, L. A., Frohman, M. A., Morris, A. J., and Foster, D. A. (1997) *Biochem Biophys Res Commun* **235**(3), 854-9.

118. Kim, J. H., Lee, S. D., Han, J. M., Lee, T. G., Kim, Y., Park, J. B., Lambeth, J. D., Suh, P. G., and Ryu, S. H. (1998) *FEBS Lett* **430**(3), 231-5.

119. Voss, M., Weernink, P. A., Haupenthal, S., Moller, U., Cool, R. H., Bauer, B., Camonis, J. H., Jakobs, K. H., and Schmidt, M. (1999) *J Biol Chem* **274**(49), 34691-8

120. Schmidt, M., Voss, M., Thiel, M., Bauer, B., Grannass, A., Tapp, E., Cool, R. H., de Gunzburg, J., von Eichel-Streiber, C., and Jakobs, K. H. (1998) *J Biol Chem* **273**(13), 7413-22.

121. El Hadj, N. B., Popoff, M. R., Marvaud, J. C., Payrastre, B., Boquet, P., and Geny, B. (1999) *J Biol Chem* **274**(20), 14021-31.

122. Frankel, P., Ramos, M., Flom, J., Bychenok, S., Joseph, T., Kerkhoff, E., Rapp, U. R., Feig, L. A., and Foster, D. A. (1999) *Biochem Biophys Res Commun* **255**(2), 502-7.

123. Rizzo, M. A., Shome, K., Vasudevan, C., Stolz, D. B., Sung, T. C., Frohman, M. A., Watkins, S. C., and Romero, G. (1999) *J Biol Chem* **274**(2), 1131-9

124. Shome, K., Rizzo, M. A., Vasudevan, C., Andresen, B., and Romero, G. (2000) *Endocrinology* **141**(6), 2200-8.

125. Lee, C., Kim, S. R., Chung, J. K., Frohman, M. A., Kilimann, M. W., and Rhee, S. G. (2000) *J Biol Chem* **275**(25), 18751-8.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

126. Lee, C., Kang, H. S., Chung, J. K., Sekiya, F., Kim, J. R., Han, J. S., Kim, S. R., Bae, Y. S., Morris, A. J., and Rhee, S. G. (1997) *J Biol Chem* **272**(25), 15986-92.

127. Chung, J. K., Sekiya, F., Kang, H. S., Lee, C., Han, J. S., Kim, S. R., Bae, Y. S., Morris, A. J., and Rhee, S. G. (1997) *J Biol Chem* **272**(25), 15980-5.

128. Jenco, J. M., Rawlingson, A., Daniels, B., and Morris, A. J. (1998) *Biochemistry* **37**(14), 4901-9.

129. Lee, S., Park, J. B., Kim, J. H., Kim, Y., Shin, K. J., Lee, J. S., Ha, S. H., Suh, P. G., and Ryu, S. H. (2001) *J Biol Chem* **23**, 23

130. Park, J. B., Kim, J. H., Kim, Y., Ha, S. H., Yoo, J. S., Du, G., Frohman, M. A., Suh, P. G., and Ryu, S. H. (2000) *J Biol Chem* **275**(28), 21295-301.

131. Lukowski, S., Lecomte, M. C., Mira, J. P., Marin, P., Gautero, H., Russo-Marie, F., and Geny, B. (1996) *J Biol Chem* **271**(39), 24164-71.

132. Lukowski, S., Mira, J. P., Zachowski, A., and Geny, B. (1998) *Biochem Biophys Res Commun* **248**(2), 278-84.

133. Banno, Y., Fujita, H., Ono, Y., Nakashima, S., Ito, Y., Kuzumaki, N., and Nozawa, Y. (1999) *J Biol Chem* **274**(39), 27385-91.

134. Steed, P. M., Nagar, S., and Wennogle, L. P. (1996) *Biochemistry* **35**(16), 5229-37.

135. Hannun, Y. A. (1996) *Science* **274**(5294), 1855-9.

136. Hannun, Y. A., Luberto, C., and Argraves, K. M. (2001) *Biochemistry* **40**(16), 4893-903.

137. Spiegel, S., and Milstien, S. (1996) *Chem Phys Lipids* **80**(1-2), 27-36.

138. Sung, T. C., Roper, R. L., Zhang, Y., Rudge, S. A., Temel, R., Hammond, S. M., Morris, A. J., Moss, B., Engebrecht, J., and Frohman, M. A. (1997) *Embo J* **16**(15), 4519-30

139. Someya, A., Sata, M., Takeda, K., Pacheco-Rodriguez, G., Ferrans, V. J., Moss, J., and Vaughan, M. (2001) *Proc Natl Acad Sci U S A* **98**(5), 2413-8.

140. Franco, M., Peters, P. J., Boretto, J., van Donselaar, E., Neri, A., D'Souza-Schorey, C., and Chavrier, P. (1999) *Embo J* **18**(6), 1480-91.

141. Ogasawara, M., Kim, S. C., Adamik, R., Togawa, A., Ferrans, V. J., Takeda, K., Kirby, M., Moss, J., and Vaughan, M. (2000) *J Biol Chem* **275**(5), 3221-30.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

142. Meacci, E., Tsai, S. C., Adamik, R., Moss, J., and Vaughan, M. (1997) *Proc Natl Acad Sci U S A* **94**(5), 1745-8.

143. Galas, M. C., Helms, J. B., Vitale, N., Thierse, D., Aunis, D., and Bader, M. F. (1997) *J Biol Chem* **272**(5), 2788-93.

144. Peters, P. J., Hsu, V. W., Ooi, C. E., Finazzi, D., Teal, S. B., Oorschot, V., Donaldson, J. G., and Klausner, R. D. (1995) *J Cell Biol* **128**(6), 1003-17.

145. Cavenagh, M. M., Whitney, J. A., Carroll, K., Zhang, C., Boman, A. L., Rosenwald, A. G., Mellman, I., and Kahn, R. A. (1996) *J Biol Chem* **271**(36), 21767-74.

146. Chen, Y. G., Siddhanta, A., Austin, C. D., Hammond, S. M., Sung, T. C., Frohman, M. A., Morris, A. J., and Shields, D. (1997) *J Cell Biol* **138**(3), 495-504

147. Monier, S., Chardin, P., Robineau, S., and Goud, B. (1998) *J Cell Sci* **111**(Pt 22), 3427-36.

148. D'Souza-Schorey, C., Li, G., Colombo, M. I., and Stahl, P. D. (1995) *Science* **267**(5201), 1175-8.

149. Caumont, A. S., Galas, M. C., Vitale, N., Aunis, D., and Bader, M. F. (1998) *J Biol Chem* **273**(3), 1373-9.

150. Radhakrishna, H., and Donaldson, J. G. (1997) *J Cell Biol* **139**(1), 49-61.

151. Millar, C. A., Powell, K. A., Hickson, G. R., Bader, M. F., and Gould, G. W. (1999) *J Biol Chem* **274**(25), 17619-25.

152. Donaldson, J. G., and Radhakrishna, H. (2001) *Methods Enzymol* **329**, 247-56

153. Schafer, D. A., D'Souza-Schorey, C., and Cooper, J. A. (2000) *Traffic* **1**(11), 892-903.

154. Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1996) *J Cell Biol* **134**(2), 295-306

155. Bi, K., Roth, M. G., and Ktistakis, N. T. (1997) *Curr Biol* **7**(5), 301-7

156. Siddhanta, A., and Shields, D. (1998) *J Biol Chem* **273**(29), 17995-8.

157. Siddhanta, A., Backer, J. M., and Shields, D. (2000) *J Biol Chem* **275**(16), 12023-31.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

158. Tuscher, O., Lorra, C., Bouma, B., Wirtz, K. W., and Huttner, W. B. (1997) *FEBS Lett* **419**(2-3), 271-5.

159. West, M. A., Bright, N. A., and Robinson, M. S. (1997) *J Cell Biol* **138**(6), 1239-54.

160. Roth, M. G., and Sternweis, P. C. (1997) *Curr Opin Cell Biol* **9**(4), 519-26

161. Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R., and Orci, L. (1998) *Proc Natl Acad Sci U S A* **95**(19), 11199-204.

162. Stamnes, M., Schiavo, G., Stenbeck, G., Sollner, T. H., and Rothman, J. E. (1998) *Proc Natl Acad Sci U S A* **95**(23), 13676-80.

163. Bremser, M., Nickel, W., Schweikert, M., Ravazzola, M., Amherdt, M., Hughes, C. A., Sollner, T. H., Rothman, J. E., and Wieland, F. T. (1999) *Cell* **96**(4), 495-506.

164. Ben-Av, P., and Liscovitch, M. (1989) *FEBS Lett* **259**(1), 64-6.

165. Plevin, R., Cook, S. J., Palmer, S., and Wakelam, M. J. (1991) *Biochem J* **279**(Pt 2), 559-65.

166. Donchenko, V., Zannetti, A., and Baldini, P. M. (1994) *Biochim Biophys Acta* **1222**(3), 492-500.

167. Fisher, G. J., Henderson, P. A., Voorhees, J. J., and Baldassare, J. J. (1991) *J Cell Physiol* **146**(2), 309-17.

168. Cook, S. J., and Wakelam, M. J. (1992) *Biochem J* **285**(Pt 1), 247-53.

169. Schlessinger, J., Shechter, Y., Willingham, M. C., and Pastan, I. (1978) *Proc Natl Acad Sci U S A* **75**(6), 2659-63.

170. Nilsson, J., Thyberg, J., Heldin, C. H., Westermark, B., and Wasteson, A. (1983) *Proc Natl Acad Sci U S A* **80**(18), 5592-6.

171. Haigler, H., Ash, J. F., Singer, S. J., and Cohen, S. (1978) *Proc Natl Acad Sci U S A* **75**(7), 3317-21

172. Carpenter, G., and Cohen, S. (1976) *J Cell Biol* **71**(1), 159-71.

173. Beguinot, L., Lyall, R. M., Willingham, M. C., and Pastan, I. (1984) *Proc Natl Acad Sci U S A* **81**(8), 2384-8.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

174. Sorkin, A., and Waters, C. M. (1993) *Bioessays* **15**(6), 375-82.

175. Gill, G. N., Kawamoto, T., Cochet, C., Le, A., Sato, J. D., Masui, H., McLeod, C., and Mendelsohn, J. (1984) *J Biol Chem* **259**(12), 7755-60.

176. Avruch, J., Zhang, X. F., and Kyriakis, J. M. (1994) *Trends Biochem Sci* **19**(7), 279-83.

177. Robinson, M. J., and Cobb, M. H. (1997) *Curr Opin Cell Biol* **9**(2), 180-6.

178. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) *Science* **274**(5295), 2086-9

179. Kranenburg, O., Verlaan, I., and Moolenaar, W. H. (1999) *J Biol Chem* **274**(50), 35301-4

180. Williger, B. T., Ho, W. T., and Exton, J. H. (1999) *J Biol Chem* **274**(2), 735-8.

181. Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L. C. (1995) *J Biol Chem* **270**(10), 5130-5.

182. Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A. V., Witke, W., Huttner, W. B., and Soling, H. D. (1999) *Nature* **401**(6749), 133-41.

183. Weigert, R., Silletta, M. G., Spano, S., Turacchio, G., Cericola, C., Colanzi, A., Senatore, S., Mancini, R., Polishchuk, E. V., Salmona, M., Facchiano, F., Burger, K. N., Mironov, A., Luini, A., and Corda, D. (1999) *Nature* **402**(6760), 429-33.

184. Takei, K., Haucke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H., and De Camilli, P. (1998) *Cell* **94**(1), 131-41.

185. Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S. Y., Hamamoto, S., Schekman, R., and Yeung, T. (1998) *Cell* **93**(2), 263-75.

186. Randazzo, P. A. (1997) *J Biol Chem* **272**(12), 7688-92.

187. Manifava, M., Thuring, J. W., Lim, Z. Y., Packman, L., Holmes, A. B., and Ktistakis, N. T. (2001) *J Biol Chem* **276**(12), 8987-94.

188. Burger, K. N., Demel, R. A., Schmid, S. L., and de Kruijff, B. (2000) *Biochemistry* **39**(40), 12485-93.

189. Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999) *Cell* **99**(5), 521-32

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

190. Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisci, C., Luini, A., Corda, D., and De Matteis, M. A. (1999) *Nat Cell Biol* **1**(5), 280-7.

191. Nakashima, S., Morinaka, K., Koyama, S., Ikeda, M., Kishida, M., Okawa, K., Iwamatsu, A., Kishida, S., and Kikuchi, A. (1999) *Embo J* **18**(13), 3629-42

192. Robinson, M. S., and Kreis, T. E. (1992) *Cell* **69**(1), 129-38.

193. Wiley, H. S., and Burke, P. M. (2001) *Traffic* **2**(1), 12-8.

194. Riccio, A., Pierchala, B. A., Ciarallo, C. L., and Ginty, D. D. (1997) *Science* **277**(5329), 1097-100

195. Burke, P., Schooler, K., and Wiley, H. S. (2001) *Mol Biol Cell* **12**(6), 1897-910.

196. Oksvold, M. P., Skarpen, E., Wierod, L., Paulsen, R. E., and Huitfeldt, H. S. (2001) *Eur J Cell Biol* **80**(4), 285-94.

197. Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S., Caron, M. G., and Lefkowitz, R. J. (1998) *J Biol Chem* **273**(2), 685-8.

198. Ahn, S., Maudsley, S., Luttrell, L. M., Lefkowitz, R. J., and Daaka, Y. (1999) *J Biol Chem* **274**(3), 1185-8.

199. McCune, D. F., Edelmann, S. E., Olges, J. R., Post, G. R., Waldrop, B. A., Waugh, D. J., Perez, D. M., and Piascik, M. T. (2000) *Mol Pharmacol* **57**(4), 659-66.

200. Roseberry, A. G., and Hosey, M. M. (1999) *J Biol Chem* **274**(47), 33671-6.

201. Pierce, K. L., Maudsley, S., Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (2000) *Proc Natl Acad Sci U S A* **97**(4), 1489-94.

202. Bunemann, M., and Hosey, M. M. (1999) *J Physiol* **517**(Pt 1), 5-23.

203. Pronin, A. N., Morris, A. J., Surguchov, A., and Benovic, J. L. (2000) *J Biol Chem* **275**(34), 26515-22.

204. Rumenapp, U., Asmus, M., Schablowski, H., Woznicki, M., Han, L., Jakobs, K. H., Fahimi-Vahid, M., Michalek, C., Wieland, T., and Schmidt, M. (2001) *J Biol Chem* **276**(4), 2474-9.

205. Llahi, S., and Fain, J. N. (1992) *J Biol Chem* **267**(6), 3679-85.

206. MacNulty, E. E., McClue, S. J., Carr, I. C., Jess, T., Wakelam, M. J., and Milligan, G. (1992) *J Biol Chem* **267**(4), 2149-56.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

207. Noh, D. Y., Ahn, S. J., Lee, R. A., Park, I. A., Kim, J. H., Suh, P. G., Ryu, S. H., Lee, K. H., and Han, J. S. (2000) *Cancer Lett* **161**(2), 207-14.

208. Zhao, Y., Ehara, H., Akao, Y., Shamoto, M., Nakagawa, Y., Banno, Y., Deguchi, T., Ohishi, N., Yagi, K., and Nozawa, Y. (2000) *Biochem Biophys Res Commun* **278**(1), 140-3.

209. Kassis, J., Lauffenburger, D. A., Turner, T., and Wells, A. (2001) *Semin Cancer Biol* **11**(2), 105-17.

210. Goode, B. L., Drubin, D. G., and Barnes, G. (2000) *Curr Opin Cell Biol* **12**(1), 63-71.

211. Small, J. V., Kaverina, I., Krylyshkina, O., and Rottner, K. (1999) *FEBS Lett* **452**(1-2), 96-9.

212. Elbaum, M., Chausovsky, A., Levy, E. T., Shtutman, M., and Bershadsky, A. D. (1999) *Biochem Soc Symp* **65**, 147-72

213. Fuchs, E., and Karakesisoglou, I. (2001) *Genes Dev* **15**(1), 1-14.

214. Horwitz, A. R., and Parsons, J. T. (1999) *Science* **286**(5442), 1102-3.

215. Nabi, I. R. (1999) *J Cell Sci* **112**(Pt 12), 1803-11.

216. Waterman-Storer, C. M., and Salmon, E. (1999) *Curr Opin Cell Biol* **11**(1), 61-7.

217. Lauffenburger, D. A., and Horwitz, A. F. (1996) *Cell* **84**(3), 359-69.

218. Wells, A. (2000) *Adv Cancer Res* **78**, 31-101

219. Mareel, M. M., and De Mets, M. (1984) *Int Rev Cytol* **90**, 125-68

220. Mareel, M. M., Storme, G. A., Dragonetti, C. H., De Bruyne, G. K., Hartley-Asp, B., Segers, J. L., and Rabaey, M. L. (1988) *Cancer Res* **48**(7), 1842-9.

221. Makiyama, N., Matsui, H., Tsuji, H., and Ichimura, K. (1991) *Clin Exp Metastasis* **9**(4), 411-25.

222. Geuens, G. M., Nuydens, R. M., Willebrords, R. E., Van de Veire, R. M., Goossens, F., Dragonetti, C. H., Mareel, M. M., and De Brabander, M. J. (1985) *Cancer Res* **45**(2), 733-42.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

223. Yoshida, D., Piepmeier, J. M., Bergenheim, T., Henriksson, R., and Teramoto, A. (1998) *Br J Cancer* **77**(1), 21-5

224. Prange, E., Schroyens, W., and Pralle, H. (1988) *Clin Exp Metastasis* **6**(2), 107-13.

225. Van Ginckel, R., Vanherck, W., Willebrords, R., Distelmans, W., and De Brabander, M. (1986) *Anticancer Res* **6**(4), 705-8.

226. Irby, R. B., and Yeatman, T. J. (2000) *Oncogene* **19**(49), 5636-42.

227. Nadav, L., and Katz, B. Z. (2001) *Int J Oncol* **19**(2), 237-46.

228. Ha, K. S., Yeo, E. J., and Exton, J. H. (1994) *Biochem J* **303**(Pt 1), 55-9.

229. Cross, M. J., Roberts, S., Ridley, A. J., Hodgkin, M. N., Stewart, A., Claesson-Welsh, L., and Wakelam, M. J. (1996) *Curr Biol* **6**(5), 588-97.

230. Aguirre Ghiso, J. A., Farias, E. F., Alonso, D. F., Arregui, C., and Bal de Kier Joffe, E. (1997) *Int J Cancer* **71**(5), 881-90.

231. Kam, Y., and Exton, J. H. (2001) *Mol Cell Biol* **21**(12), 4055-66.

232. Santy, L. C., and Casanova, J. E. (2001) *J Cell Biol* **154**(3), 599-610.

233. Biscardi, J. S., Belsches, A. P., and Parsons, S. J. (1998) *Mol Carcinog* **21**(4), 261-72.

234. Korah, R. M., Sysounthone, V., Golowa, Y., and Wieder, R. (2000) *Cancer Res* **60**(3), 733-40.

235. Ward, Y., Wang, W., Woodhouse, E., Linnoila, I., Liotta, L., and Kelly, K. (2001) *Mol Cell Biol* **21**(17), 5958-69.

236. McAlpine, R. J. (1991) *Fam Pract Res J* **11**(4), 363-70.

237. Ladeda, V., Aguirre Ghiso, J. A., and Bal de Kier Joffe, E. (1998) *Exp Cell Res* **242**(2), 515-27.

238. Ladeda, V., Frankel, P., Feig, L. A., Foster, D. A., Bal de Kier Joffe, E., and Aguirre-Ghiso, J. A. (2001) *Biochem Biophys Res Commun* **283**(4), 854-61.

239. Reich, R., Blumenthal, M., and Liscovitch, M. (1995) *Clin Exp Metastasis* **13**(2), 134-40.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

240. Williger, B. T., Reich, R., Neeman, M., Bercovici, T., and Liscovitch, M. (1995) *J Biol Chem* **270**(50), 29656-9.

241. Aguirre-Ghiso, J. A., Frankel, P., Farias, E. F., Lu, Z., Jiang, H., Olsen, A., Feig, L. A., de Kier Joffe, E. B., and Foster, D. A. (1999) *Oncogene* **18**(33), 4718-25.

242. Aguirre Ghiso, J. A., Farias, E. F., Alonso, D. F., and Bal de Kier Joffe, E. (1998) *Int J Cancer* **76**(3), 362-7.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.