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. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

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.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

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

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

REGULATION OF PHOSPHOLIPASE D ACTIVITY BY RALA AND PROTEIN KINASE C IN CELL TRANSFORMATION

by

ARMAND HORNIA

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

1999

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

UMI Number: 9917661

UMI Microform 9917661
Copyright 1999, by UMI Company. All rights reserved.

This microform edition is protected against unauthorized copying under Title 17, United States Code.

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

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.

1/28/99
Date

Chair of Examining Committee
Dr. David A. Foster, Hunter College

1/28/99
Date

Executive Officer
Dr. Richard L. Chappell, Hunter College

Dr. Rivka Rudner, Hunter College

Dr. Anne Henderson, Hunter College

Dr. Thomas Haines, City University of New York Medical School

Dr. Ray Birge, Rockefeller University

Supervising Committee

THE CITY UNIVERSITY OF NEW YORK

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

ABSTRACT

REGULATION OF PHOSPHOLIPASE D ACTIVITY BY RALA AND PROTEIN KINASE C IN CELL TRANSFORMATION

by

Armand Hornia

Advisor: Dr. David A. Foster

We extend previous studies on the non-receptor tyrosine kinase c-src to cells overexpressing a receptor class tyrosine kinase, the epidermal growth factor receptor (EGFR), 3Y1 EGFR cells. Unlike c-Src overexpressing cells, downregulation of PKC isoforms with TPA did not transform the EGFR cells. Treatment of EGFR cells with EGF did transform these cells. We examined the effects of PKC α- and PKC δ-specific inhibitors and the expression of dominant negative mutants of PKC α and δ. Both the PKC δ-specific inhibitor rottlerin and a dominant negative PKC δ mutant transformed the EGFR cells in the absence of EGF. In contrast, the PKC α-specific inhibitor Go6976 and expression of a dominant negative PKC α mutant blocked the transformed phenotype

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

induced by both EGF and inhibition of PKC δ. Interestingly, both rottlerin and EGF induced substantial increases in phospholipase D (PLD) activity. The elevation of PLD activity in response to inhibiting PKC δ, like transformation, was dependent upon PKC α and restricted to the 3Y1 EGFR overexpressing cells. These data demonstrate that PKC isoforms α and δ have antagonistic effects upon both transformation and PLD activity.

The small GTPase RalA exists in a complex with PLD and is required for the activation of PLD activity by oncogenic Src and Ras. Upon EGF treatment, RalA is activated and its activation is dependent upon Ras. The activation of PLD by EGF was dependent upon both HaRas and RalA. The transformed phenotype induced by EGF was also dependent upon RalA; Overexpression of wild type RalA or an activated RalA mutant transformed the 3Y1 EGFR cells in the absence of EGF. The involvement of RalA suggested that elevated PLD activity might explain the RalA dependence for transformation of the EGFR cells in response to EGF. To establish a role for PLD in EGF signaling, 3Y1 EGFR cells were stably transfected with a PLD1 expression vector. Not only did EGFR cells tolerate expression of PLD they were even transformed in the absence of EGF. These data indicate that the Ras/RalA/PLD signaling pathway is an essential component of the growth and transforming properties of EGF.

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

ACKNOWLEDGEMENTS

I would like to express my gratitude and my appreciation to those who have, throughout these many years, provided me with wise advice, kindly support, and friendship.

I offer my thanks to Dr. David A. Foster for his guidance, insight, and monetary support.

I wish to thank all the other members in my thesis committee: Dr. Rivka Rudner, who gave me the opportunity of doing my first lab tutorial with her and in whose lab I learned fundamental molecular biological techniques; Dr. Anne Henderson, Dr. Thomas Haines, and Dr. Ray Birge for their precious time and kindness.

I offer my warmest thanks to my colleagues in Dr. Foster's lab. Above all, to my best friend Dr. Zhimin Lu whose intelligence, sense of humor, high scientific aptitude, and thoughtfulness have made my stay here a truly pleasant one; Taiko Sukezane whose diligence, patience, dedication, and cheerful spirit made for a truly pleasant collaboration; Sergei Bychenok, Paul Frankel, Troy Joseph, Angela Shen, Henghe Tian, Lizhong Xu, Minghao Zhong, Nancy Barile, Albert Carrero, and Thuy Nguyen for making the laboratory an interesting place to work. Dr. Marcello Curto, Dr. Jingqing Luo, Dr. Qun Zang, Dr. Hong Jiang, Dr. Jian-guo Song, and Dr. Youwei Jiang for their technical assistance and friendship; Dr. Larry Feig (Tufts University); Dr. Renato Baserga (Thomas Jefferson University); Dr. Andrew Morris (Stonybrook), and Dr. Shigeo Ohno (Yokohama City University Medical School, Japan) for very generously providing our lab with the RalA, EGFR, PLD1, and PKC plasmids, respectively.

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

Dedication

In memory of my beloved mother, Elga, who passed away in 1989 and to the rest of my family, especially my brother Osmany, who have been the main source of encouragement and the providers of unconditional support in all of my endeavors.

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.

LIST OF FIGURES

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

TABLES

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

LIST OF ABBREVIATIONS

aPKC	atypical PKC
cPKC	conventional PKC
DG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DTT	dithiothreitol
EDTA	ethylenediaminotetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-bis tetracetic acid
nPKC	novel PKC
PA	phosphatidic acid
PBS	phosphate buffered saline
PBu	phosphatidylbutanol
PC	phosphatidylcholine
PI	phosphatidylinositol
PKC	protein Kinase C
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
TPA	12-O-tetradecanoylphorbol-13-acetate

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

CHAPTER I

Introduction

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

The focus of this work elucidates biological roles of protein kinase C isoforms and RalA on the transformation of cells and on phospholipase D activation in EGF receptor overexpressing cells.

1. Tumor promotion

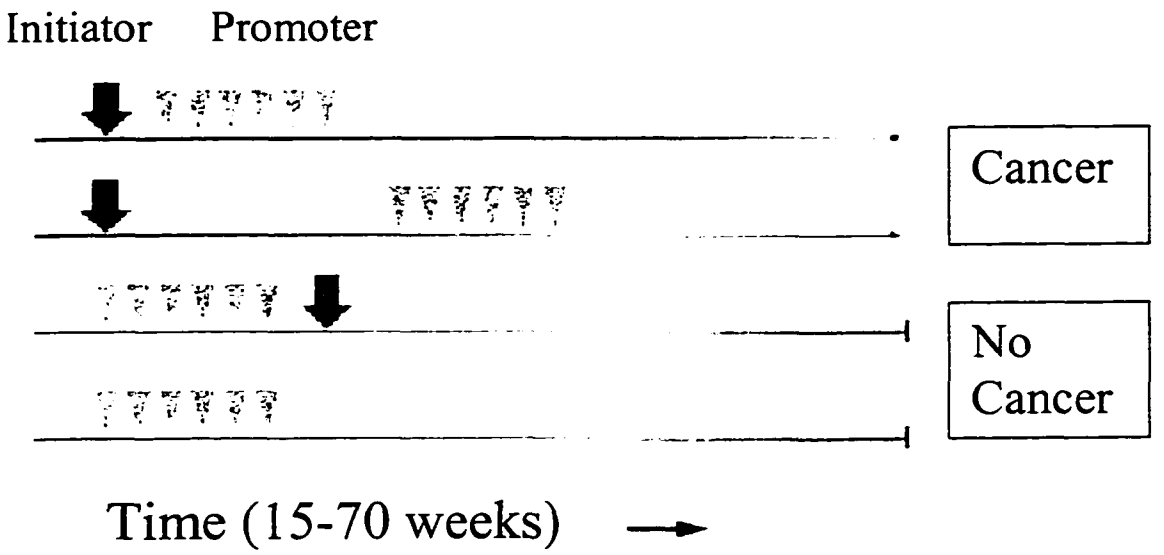
In an effort to further understand cancer formation and eventually develop a cost-effective treatment or cure, research into the mechanism of tumorigenesis has been carried out for decades. In the highly-defined two-stage model of initiation/promotion of skin carcinogenesis (Armuth & Berenblum,1974; Darnell,1990) in the mouse (**Fig.1**), developed in the early 1940's, a low dose of a well-studied chemical carcinogen (dimethylbenzanthracine) is applied to the skin on the back of a mouse. This exposure alone will not cause any statistically significant increase in the number of skin tumors (papillomas) over the lifetime of the animal. A number of environmental chemicals have been determined to be "promoters" of tumor formation in this and other models. A promoting agent is capable of increasing significantly the incidence of papillomas following a single exposure of initiating agent where application of the promoting agent itself causes no increase in cancer.

The latency between exposure to a carcinogen and the onset of cancer can be explained in part by the results of animal experiments that demonstrate that skin carcinogenesis in the rabbit and mouse can be divided into two stages, tumor initiation and promotion. Following single exposure to a subcarcinogenic dose of a carcinogen ("initiation"), the latent period can be shortened and the tumor number or yield increased with certain "promoting agents" (croton oil, benzene, gasoline, UV treatment, phorbol

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

Tumor promoters:
Substances that are not in themselves carcinogenic but increase the potency of known carcinogens.

Tumor initiators:
Substances that cause DNA damage.



Initiator treatment alone does not give rise to tumors.

Fig. 1 Two stage model of carcinogenesis

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

esters, pesticides, and others). Promoters are not carcinogenic in themselves, or only weakly so, but cause significantly elevated cell proliferation in target tissue. These cells are transformed. Transformed cells display many properties of tumor cells and some actually form tumors when injected back into animals. The properties of transformed cells include immortalization, decreased anchorage dependence, decreased dependence on exogenous growth factors, and loss of contact inhibition of growth.

Initiation and promotion are two stages in the development of tumors. Chemical, physical, or biological agents that irreversibly and heritably alter the cell genome typically cause initiation. However, the mechanism of promotion is not well understood. There are many kinds of promoting agents with diverse molecular structures: phorbol esters, estrogen, prolactin, other endogenous hormones, and drugs. Some of the promotors exhibit specific interaction with cell receptors. For example, phorbol esters bind with the intracellular receptor protein kinase C, a serine-threonine kinase, and activate it. The structure of a phorbol ester, TPA, which is a potent activator of PKC is provided (**Fig. 2**). Short-term treatment with TPA leads to the activation of PKC while prolonged treatment down regulates this protein. Consequently, this substance has been used extensively throughout many years in an effort to assess the involvement of PKC in signaling pathways.

The regulation of PKC has been reported to be important in both colon and breast cancer. In colon cancer it appears to function as a tumor suppressor because the activity of PKC is reduced. However, in breast cancer it appears to have an oncogenic role whereby PKC activity is elevated. Interestingly, PKC has also been implicated in

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

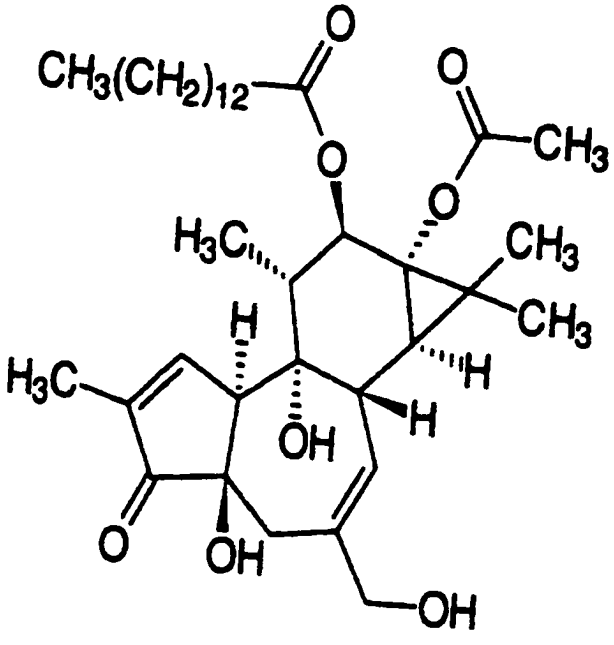


Fig. 2 Tetradecanoyl phorbol acetate (TPA).

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

multidrug resistance (MDR) and metastasis. Elevated levels of PKC correlate with increased drug resistance and metastatic potential (Blobe et al.,1994).

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

2. Protein Kinase C Family (PKC)

The molecular hetereogeneity of the PKC superfamily, involvement in transformation, and their functional divergence make them attractive targets for anticancer drug development in the future.

The protein kinase C superfamily of lipid dependent and diacylglycerol-activated serine-threonine kinases is important as cytosolic intracellular signal tranducers involved in numerous signaling pathways. PKCs are 80 kDa phosphoproteins that play key roles in cellular processes like proliferation and differentiation (Nishizuka,1984) as well as being implicated in receptor desensitization, neurotransmitter release, regulation of gene expression, hormone release, ion channels, mediating immune response, modulating membrane structure, development, tumirogenesis, apoptosis, and neural plasticity (Mellor,1998). PKC has been implicated recently in cell cycle control at two sites, G1/S progression and G2/M transition (Fishman et al.,1998). PKC functions as the transducer of the second messenger, diacylglycerol (DG), and is considered the major receptor for the tumor promoting phorbol esters. DG and phorbol esters recruit PKC to the membrane, a process referred to as translocation, by acting as hydrophobic anchors at the membrane.

There exist multiple PKC isozymes within cells mediating isozyme-specific functions. There are at least 11 known mammalian PKC isoforms (Baron-Delage & Cherqui,1997) to date that have been categorized by structure and cofactor requirements into three distinct groups: Conventional [alpha (α), beta I, II (β1,β2), and gamma (γ)] are calcium/DG/phosphatidylserine-dependent; Novel [delta (δ), epsilon (ε), theta (θ), mu (μ), and eta (η)] are calcium independent and DG/phosphatidylserine-dependent;

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

Atypical [zeta (ζ), lambda (λ), and iota (ι)] are calcium/DG-independent and phospholipid responsive (Nishizuka,1995).

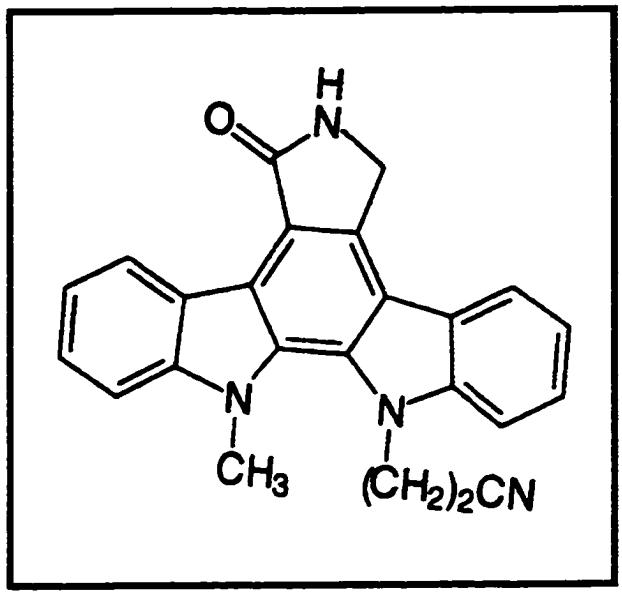
Most cells express more than one type of PKC with each potentially possessing different subcellular localization and cofactor requirements (Ohno & Akita,1991) as well as different levels of expression and availability of target substrates that can vary by cell type. As a consequence, it has been difficult to ascribe a specific role to individual PKCs in cells. However, the recent advent of specific PKC isoform chemical inhibitors (**Fig. 3**) and the availability of PKC constructs that code for isozyme-specific dominant negative inhibitors for the first time provide a means of resolving this critical issue in cell culture models.

Structure:

Comparing their primary structures as inferred from cDNA sequences revealed the structure of the PKC family (Ohno & Akita,1991). PKC has five variable regions and four conserved domains (**Fig. 4**). Two major functional stretches exist coding for the regulatory domain in the amino terminus and a catalytic domain in the carboxyl terminus separated by a hinge region that is cleaved after PKC is membrane bound releasing the short-lived constitutively active kinase domain (PKM) (**Fig. 5**). The regulatory domain (V1-C2) possesses a pseudosubstrate, a C1 phorbol-ester/DG-binding-site, and a C2 phosphatidylserine-calcium-binding site. Within the C1 domain there are two cysteine-rich zinc-finger-like-motif regions. The catalytic domain possesses one ATP and one substrate-binding site.

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

Go6976- PKC α inhibitor



Rottlerin- PKC δ inhibitor

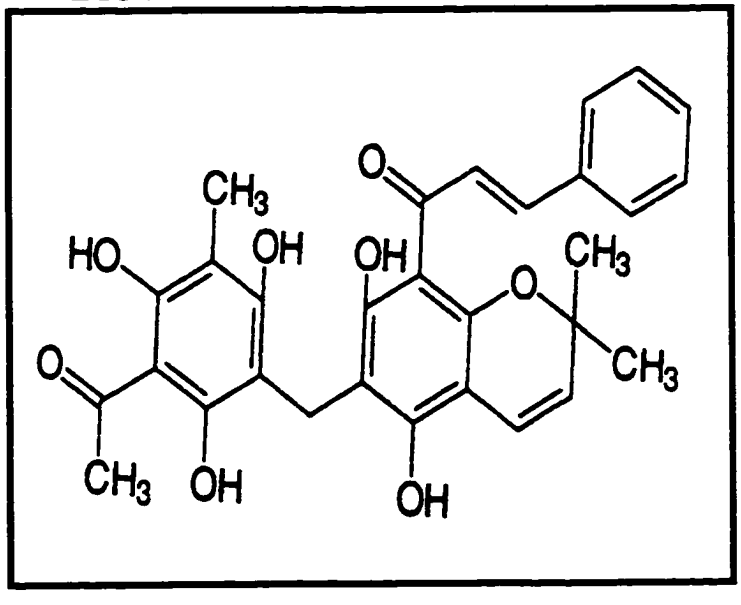


Fig. 3 PKC inhibitors

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

Schematic structure of PKC Isoforms

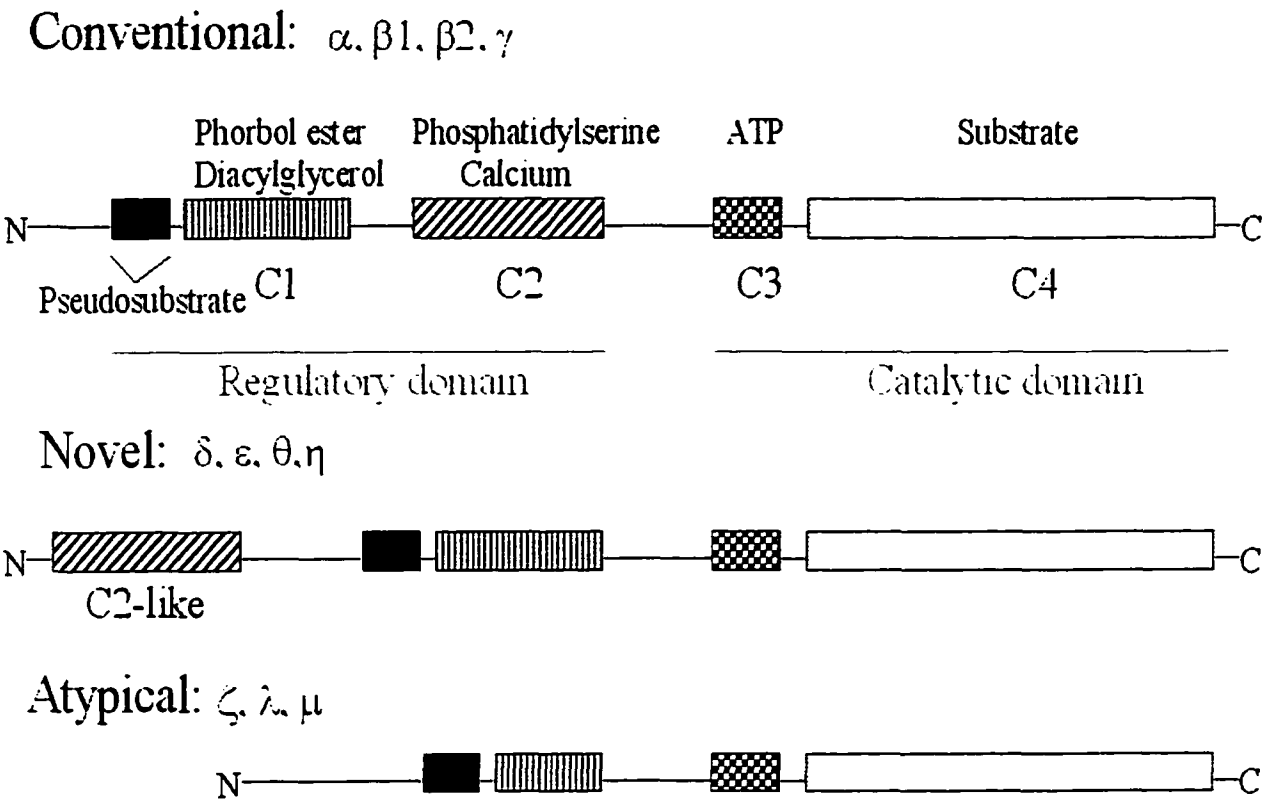
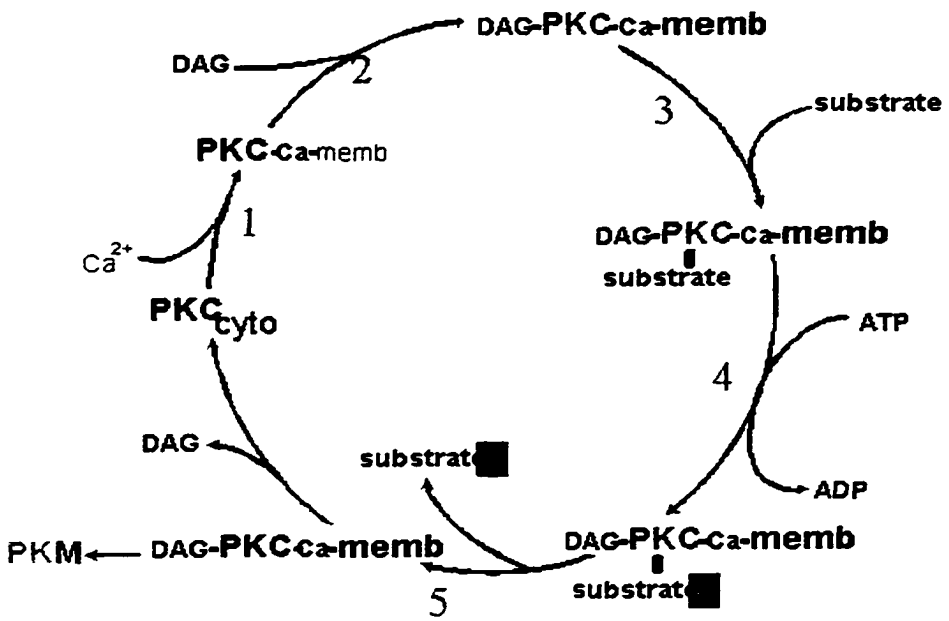


Fig. 4 The schematic structure of PKC isoforms.

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



1. Translocation 2. Membrane interaction 3. Activation 4. Phoshorylation 5. Downregulation

Fig. 5 Activation cycle of PKC. (DAG- Diacylglycerol)

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

Common to all members is the presence of the pseudosubstrate in the aminoterminal regulatory domain, which renders the kinase inactive by interacting with the carboxyterminal substrate-binding site of the catalytic domain. Activation of PKC requires the release of this conformational autoinhibitory state leading to its translocation from the cytosol to the membrane. Extracellular stimuli trigger an increase in the level of DG that binds to and activates PKC by changing its conformation so that the pseudosubstrate no longer is bound to the substrate binding site. DG can be produced rapidly by the action of Phospholipase C on phosphatidylinositol or slowly by the action of PLD on phosphatidylcholine producing phosphatidic acid (PA) which is further metabolized to DG by PA phosphohydrolase (Murayama & Ui,1987).

Function:

PKC has a multitude of substrates like MARCKS (myristoylated alanine-rich C kinase substrate), which binds actin, calmodulin, phosphatidylinositol 4,5-bisphosphate (PIP_2), and is implicated in playing a role in phagocytosis, membrane traffic, and cell motility. PKC also has nuclear proteins as substrates, including DNA methyltransferase, CREB, DNA polymerease α, RNA polymerase II, and DNA topoisomerases (Sahyoun et al.,1986).

The exact role of each isoform is unclear; however, the elucidation of the roles of two PKC isoforms, α and δ, in the activation of PLD and transformation of cells overexpressing a protooncogene will be made evident in this thesis. Furthermore a correlation between PKC/PLD activity and transformation gains greater support from this work.

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

3. Lipase: Phospholipase D

Recently, a link between the major PLD product phosphatidic acid (PA) and PKC has been reported. The downstream target(s) of PA are unknown. The researchers noticed that proteins with molecular weights of 29kDa and 32 kDa were phosphorylated when PA was present. This suggested that there exist a PA regulated kinase (PARK). Using the purified 29kDa protein as the substrate they were able to isolate and characterize the PARK. It was found to be PKC α. So, phosphatidic acid produced from the hydolysis of phosphatidylcholine by PLD can in some way activate PKC α (Yokozeki et al., 1998). The importance of PLD in different signal transduction pathways is becoming clearer.

Phospholipase D (PLD) is one of five distinct types of lipases. Lipases are enzymes that hydrolyse ester bonds in phospholipids. These enzymes are involved in the breakdown of phospholipids in cell membranes. All cells have membranes composed of phospholipids and proteins. These enzymes can be subcategorized into two main groups. The aliphatic esterases (phospholipases A1, A2, and B) which liberate fatty acids and the phosphodiesterases (phospholipases C and D) that liberate DG (diacylglycerol) or PA (phosphatidate), respectively. One main type of phospholipid is composed of a glycerol backbone with two fatty acid attachments and a phosphorylated alcohol. Each enzyme catalyses a specific reaction forming unique products. Phospholipases cleave phospholipids at specific sites (**Fig. 6**). Phospholipase B is poorly characterized, but it is known to act on monoacyl phospholipids. The functional significance of PLD remains unclear. Indirect evidence implicates PLD in mitogenesis (Spiegel et al.,1996) since one

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

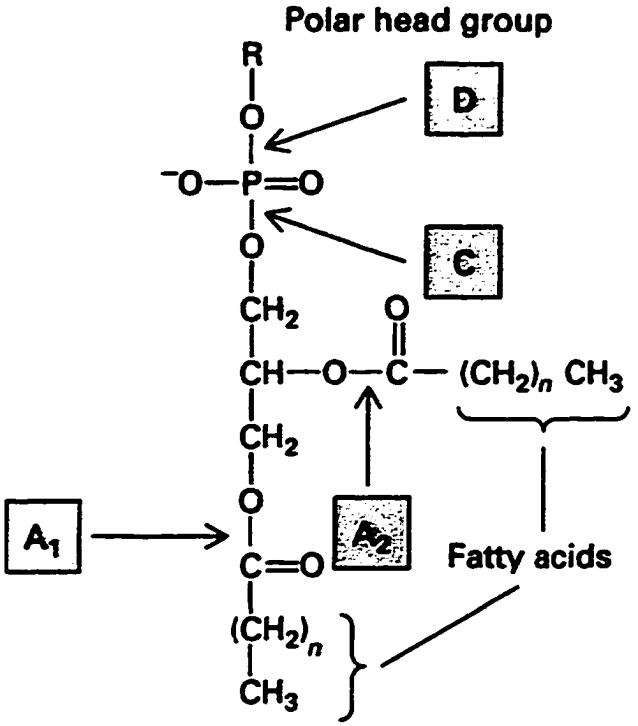


Fig. 6 Phospholipase cleavage sites on phospholipids.

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

of its products, PA, has been linked to mitogenesis and all known growth factors stimulate PLD activity (Fisher et al.,1991;Ben-Av & Liscovitch,1997). Also, the involvement of small GTPases like Ras (Jiang et al.,1995), Rho (Ohguchi et al.,1995), and Arf (Hammond et al.,1995) in the activation of PLD (**Fig. 7**) suggest that PLD activation might play a role in cytoskeleton reorganization and intracellular protein traffic. PLD activity has been detected in the membrane fraction of cells and in the cytoplasmic or soluble fraction from mammalian cells (Chalifa et al.,1990;Taki & Kanfer,1979;Wang et al.,1991).

Phospholipases are responsible for the production of some second messengers like the phospholipid metabolite diacylglycerol (Allan et al.,1978) (DG) which can activate protein kinase C (Nishizuka,1995) (PKC). PLD specifically catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. Furthermore, PA can be converted to DG by the action of PA phosphohydrolase. An example of a kinase that activates PLD to produce DG from PC is the oncogene v-Src (Song & Foster,1993). Phospholipase D has been shown to be essential for meiosis in yeast (Waksman et al.,1996). The yeast gene SPO14 which is essential for meiosis was shown to have the enzymatic properties of the mammalian PIP2-regulated PLD.

The primary metabolite of PLD is PA. PA has been shown to be involved in numerous signaling pathways like mitogenesis and hydrolysis of PIP2 (Murayama & Ui,1987;Ohsako & Deguchi,1981;Kawase & Suzuki,1988;Knauss et al.,1990). It was demonstrated recently that PA generated by interleukin 2-induced DG kinase activity is essential in IL-2 mediated lymphocyte proliferation (Cano et al.,1992). PA can be

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

CHARACTERISTICS	PLD1	PLD2
PKC/ARF/Rho Dependence	Yes	No
PIP2 Dependence	Yes	Yes
Molecular Weight kDa	120	120
Basal activity	Low	High
Substrate Specificity	PC	PC
Transphosphatidylation	Yes	Yes
Subcellular localization	Perinuclear	Plasma membrane

(Mammals possess two distinct members of the same gene family)

Fig. 7 Different Phospholipase Ds

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

converted to Lyso-PA, which has been reported to be mitogenic. DG is an activator of some PKC isoforms. So, both direct and indirect mechanisms for the activation of PKC by PLD exist involving PA or DG, respectively, and PKC can mediate the activation of PLD. Clearly, the interactions between these two proteins are complex and probably highly regulated.

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

4. Epidermal Growth Factor Receptor (EGFR)

We recently published a report in which the depletion or inactivation of PKC δ was shown to be transforming in cells overexpressing the protooncogene c-Src, a non-receptor tyrosine kinase (Lu et al.,1997). The report demonstrated that it is the downregulation and not the activation of PKC by the tumor promoter TPA that is responsible for its transforming ability. c-Src overexpressing cells exhibited elevated PLD activity . DNA synthesis, and increased colony formation in soft agar when treated with TPA, unlike the parental cells lacking the overexpression of c-Src. This finding suggests a possible correlation between transformation and PLD activity. The use of Bryostatin 1, an activator of PKC that has been shown to inhibit tumor promotion on the skin of mice treated with TPA, blocked both the increase in PLD activity and colony formation in c-Src overexpressing cells treated with TPA. An experiment maintaining a constant TPA transforming dose while increasing the concentration of Bryostatin 1 led to the reversion of the transformed phenotype in c-Src cells and correlated well with the inhibition of downregulation of the PKC δ isoform.

In an effort to further implicate PKC δ as a possible tumor suppressor, a different signaling pathway was chosen. The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase. This receptor is the best known member of a group of receptors that form a family. These receptors are designated as HER1 (EGFR), HER2 (erbB2), HER3, and HER4. These receptors are activated when a specific ligand binds the receptor. EGF and TGF α are ligands that bind EGFR. TGF α was first discovered in the supernatant fluid of tumor cells in culture. For some tumor cells, the factor acts in an autocrine fashion, maintaining growth. When appropriate cells are

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

treated with the factor, they not only divide but also temporarily acquire properties of transformed cells (hence the factor's name). Actually, TGF is speculated to be a fetal form of EGF.

Ligand binding by the receptor triggers receptor dimerization, autophosphorylation, and recruitment of SH2-containing proteins. SH2-containing proteins are proteins that have a src-homology-like domain, which can bind tyrosine phosphorylated sites (Sasaoka et al.,1994). EGF receptors can appear on the surface of cells as either high or low affinity receptors. The high affinity receptor is the result of receptor dimerization following ligand binding (**Fig. 8**) and activation while the low affinity receptors are due to the receptor monomers (Collins et al.,1997). EGFR has five autophosphorylation tyrosine sites on its intracellular carboxyl terminal domain (Tyrosines 992,1068,1086,1148, and 1173) (Logan et al.,1997). These sites once phosphorylated can bind different SH2 domain-containing proteins like PLC-γ, and GAP. The EGFR receptor is known to phosphorylate numerous proteins like PLC-γ, GAP-Ras (GTPase activating protein of Ras), and MAP (Mitogen-Activated Protein) kinase after ligand binding and recruitment.

The over-expression of HER1 (EGFR) and HER2 (c-erbB2) have been strongly correlated with a poor prognosis in breast cancer (Jardines et al.,1993). Squamous carcinoma cells have a greatly increased number of EGF receptors on their surfaces as compared with normal keratinocytes. The different members of this family of receptors can heterodimerize. The formation of heterodimers can provide a mechanism for increasing the complexity of a signal triggered by a single ligand. For example, it could

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

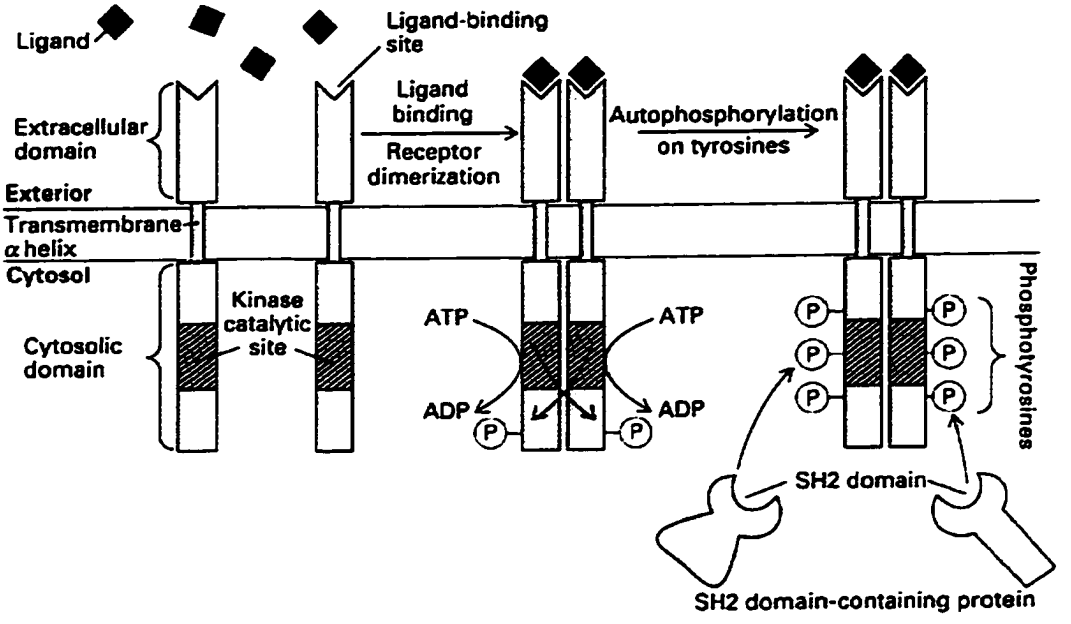


Fig. 8 EGF Receptor activation.

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

greatly increase the influence on different substrates and the activation of different downstream pathways. It could also bring about the juxtapositioning of different substrates that would not normally come together in the homodimers. Heterodimerization can vary the extent of time a receptor signals by altering receptor internalization rates and by varying the rates of the dephosphorylation of the receptor and substrate.

EGFR regulates the activation of the small GTPase Ras through a Grb2-SOS complex (Sasaoka et al.,1994). Grb2 is an adaptor protein with two SH2 (src-homology) domains. Grb2 is found complexed with SOS (Son of Sevenless), a guanine nucleotide exchange factor, in the cytoplasm of cells. After the EGFR binds EGF or TGF α it dimerizes, the Grb2-SOS complex associates with the autophorylated receptors, and the exchange factor then activates the GTPase activity of Ras (**Fig. 9**).

The binding of EGF to the EGF receptor recruits, binds directly to, and phosphorylates PLC γ. In turn, this phospholipase hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DG), the physiologic activator of PKC, as well as producing 1,4,5-trisphosphate, which releases calcium from the endoplasmic reticulum. (Berridge et al., 1987). Alternatively, EGF can activate PKC by the action of phospholipase D- mediated hyrolysis of phosphatidylcholine, generating PA and choline. PA is further metabolized by another enzyme PA phosphatase to DG. The dependence on PKC by EGF in the activation PLD is unclear. We have published that the activation of PLD by EGF is independent of PKC in human cancer cells, A431 (Song et al.,1994). However, reports have been published previously that both support and attack this position (Yeo & Exton,1990; Zhang & Aktar,1998). The resolution of these confusing

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

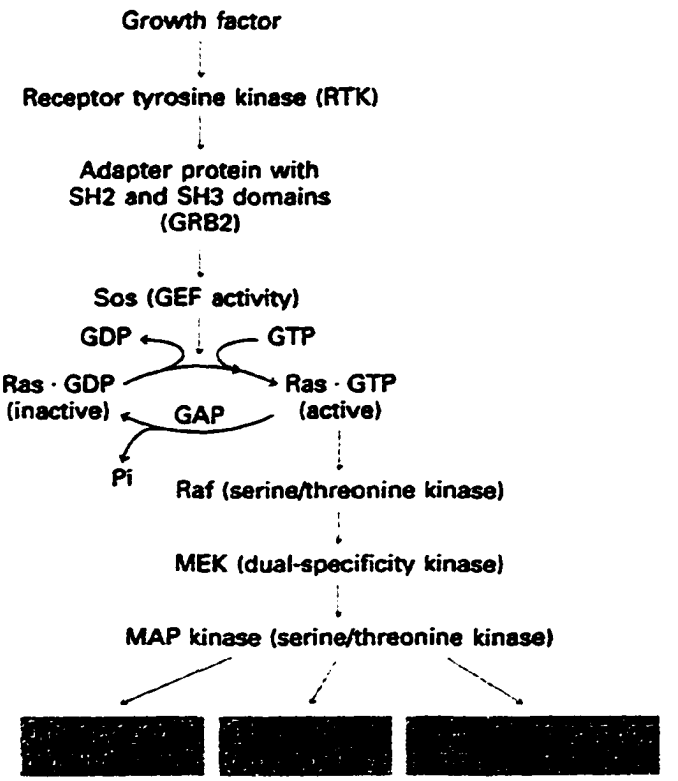


Fig. 9 EGFR signaling cascade.

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

and contradictory reports could be explained by the understanding of the differential roles of specific PKC isoforms and in this thesis this is demonstrated.

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

5. Small GTPase: Ral

Oncogenic forms of Src, a cytosolic tyrosine kinase, and the small GTPase Ras activate PLD. This activation is dependent on the presence of a small GTPase, RalA, which is found to be physically associated with PLD (Luo et al.,1997). EGF when bound to the EGF receptor activates PLD activity. It is unclear if the EGF receptor, a transmembrane tyrosine kinase, shares this dependence for RalA.

Guanine nucleotide binding proteins (G proteins) play many critical roles in many cellular processes. GTPases control numerous processes in cells like growth, apoptosis, translation, cytoskeletal reorganization, and nuclear. They mediate the transduction of signals from the plasma membrane, the sorting of proteins, and translocation of incipient polypeptides to the endoplasmic reticulum (Weismuller & Wittinghofer,1994). There are two types of GTPases. The heterotrimeric G proteins and monomeric GTPases. Heterotrimeric G proteins consist of three subunits α, β, and γ. The GTPase activity of heterotrimeric G proteins is constituted by the α subunit which is around 45 kDa. The small monomeric GTPases are around 20 kDa. The prototypical GTPase is Ras which has been implicated in a variety of cancers (Yuspa & Poirier,1994;Zachos & Spandidos,1997). There are many small GTPases. The recently demonstrated involvement of the small GTPases Ras, Ral, Rho, and Arf (ADP-ribosylating factor) in the activation of PLD are discoveries that strongly suggest the importance of PLD in the small GTPase mediated signaling pathways (Hammond et al.,1995;Jiang et al.,1995;Ohguchi et al.,1995). All small GTPases exist in one of two possible states. They are either in the GDP-bound state when inactive or the GTP-bound state when active (**Fig. 10**). These states are modulated by the action of guanine nucleotide exchange

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

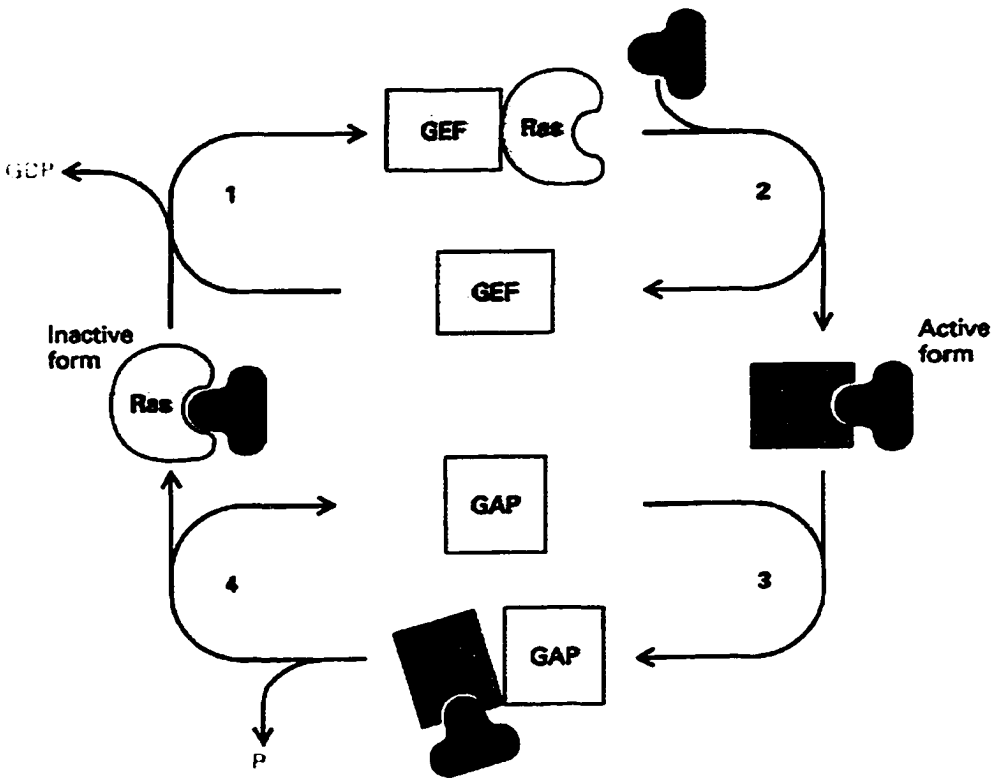


Fig. 10 Small GTPase Cycle.

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

factors (GEFs) and the action of GTPase-activating proteins (GAPs) which replace the bound GDP with GTP and turn on the intrinsic GTPase activity of specific GTPases, respectively.

The small GTPase Ras has many downstream targets like Raf (serine/threonine kinase), RalGDS, and PI3Kinase (Weismuller & Wittinghofer,1994;Urano et al.,1996). The Ras pathway has been bridged to the Ral GTPase pathway which in turn connects with the Rho GTPase pathway. Ral GTPase can modulate the Rho pathway since it has been linked to Rho pathways by the discovery of RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. So, the Ras signaling pathway can be depicted as Ras --> RalGDS --> Ral --> RLIP76 --> CDC42/Rac1/Rho where CDC42 and Rac are other small GTPases. RalGDS (GDS-guanine nucleotide dissociation stimulator) associates directly with Ras. Ral proteins make up a distinct group of GTPases that share 58% identity in sequence with Ras. Ral is activated by a nucleotide exchange factor (GEF) and inactivated by a GTPase-activaing protein (GAP) unique to this GTPase (Feig et al.,1996). There are at least three different kinds of Ral proteins: A, B, and C. Ral and Ras are found exclusively in the membrane fraction of cells unlike Rho which can be found in both membrane and cytosol fractions. Although both Ral and Ras are plasma membrane proteins Ral is found, for the most part, in intracellular vesicles. Constitutively activated Ral does not transform cells like the constitutively activated form of Ras.

Recently, in collaboration with our lab, Julio A. Aguirre-Ghiso, from the University of Buenos Aires in Argentina, has discovered that RalA, specifically the Ral28N dominant negative mutant, can reduce the levels of urokinase-type plasminogen

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

activator (uPA) and metalloproteinases (MMPs) in NIH3T3 cells transformed by v-Src and v-Ras (Aguirre Ghiso, unpublished). High levels of these two proteins have been correlated with tumorigenicity, invasiveness, and metastasis in human tumors. Since the activation of PLD in v-Src and v-Ras transformed cells, which possess PLD-mediated high levels of uPA and MMP, is dependent on Ral, its role in regulating the levels of these proteins was investigated. Dominant negative Ral blocked levels of MMP in v-Src transformed cells, but not in v-Ras cells. A most impressive result was that when v-Src or v-Ras transformed NIH3T3 cells expressing Ral 28N were injected subcutaneously into mice these oncogene expressing cells no longer formed tumors. Hence, PLD and Ral are strongly implicated in transformation.

We recently reported that rat fibroblasts overexpressing the non-receptor tyrosine kinase c-Src become transformed upon treatment with the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (Lu et al.,1997). In this cell culture model, TPA was able to induce the amplification of cells containing an initiating mutation (c-Src overexpression) and was therefore functioning very much like a tumor promoter *in vitro*. The tumor promoting effect of TPA was determined to be due to the depletion of the δ isoform of protein kinase C (PKC) (Lu et al., 1997).

Other studies have suggested that PKC δ has negative effects on cell division (Watanabe et al., 1992; Mischak et al., 1993; Goode et al., 1994; Hirai et al., 1994; Borner et al., 1995; Griffiths et al., 1996; Fukumoto et al., 1997; Acs et al., 1997). Phospholipase D (PLD) activity, which is elevated in response to mitogenic signals, was stimulated by downregulation of PKC δ in cells overexpressing c-Src (Lu et al., 1997), suggesting the possibility that PKC δ may also negatively regulate PLD activity.

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

However, the activation of PLD by the mitogenic signals induced by both platelet-derived growth factor and epidermal growth factor (EGF) has been reported to be dependent on PKC (Plevin et al.,1991;Yeo & Exton,1995).

Like the tumor promoting effect of overexpressing c-Src in rat fibroblast, the overexpression of the EGF receptor also primes cells for transformation. 3Y1 rat fibroblasts overexpressing the epidermal growth factor receptor become transformed upon treatment with EGF. A common response to oncogenic and mitogenic stimuli is the activation of phospholipase D (PLD). Overexpression of a tyrosine kinase is a common genetic defect in a variety of human tumors (Dickson et al.,1992;Ottenholf-Kalff et al.,1992) The epidermal growth factor (EGF) receptor, which has an intrinsic tyrosine kinase that is activated in response to EGF, is frequently elevated in human breast and ovarian cancer (Reese & Slamon,1997). However, overexpression of a tyrosine kinase such as the EGF receptor is not sufficient for a fully transformed or cancerous phenotype.

PLD converts phosphatidylcholine to phosphatidic acid and it has been speculated that the change in the charge on the inner surface of the plasma membrane may in some way affect the curvature of the membrane and facilitate invagination and vesicle formation. Studies with PLD have proceeded slowly because it is expressed at very low levels and there is an apparent low tolerance for PLD activity, since it has been very difficult to express PLD in cells at levels that allow molecular analysis. PLD has also been implicated in vesicle transport in the Golgi (Bi et al.,1997;Chen et al.,1997), and therefore useful mutants of PLD have been slow in coming because of the essential nature of this biological cellular process of trafficking proteins in vesicles. Similarly, inhibition of PLD activity is likely to be lethal to cells for the same reason.

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

We demonstrated recently (Luo et al.,1997) that PLD1 associates directly with the small GTPase RalA, a downstream target of Ras (Feig et al.,1996). RalA has been implicated in cell transformation (Urano et al.,1996) and may therefore be an important component of mitogenic signaling mediated by Ras. RalA apparently facilitates the assembly of an active PLD complex that includes RalA, PLD1, and the PLD activator Arf, another small GTPase (Luo et al.,1998). Additionally, RalA was shown to be required for PLD activation in response to the mitogenic signals initiated by v-Src and v-Ras (Jiang et al.,1995). Thus the PLD activated in response to Ras-dependent mitogenic signals is likely mediated by the Ras/RalA GTPase cascade (Feig et al.,1996). Therefore, while inhibition of PLD is difficult because of the numerous roles it likely has in membrane topology, RalA mutants can be used as an indirect indicator for PLD involvement in Ras-dependent signal transduction that may be independent of the role that PLD plays in vesicle transport.

The involvement of Protein kinase C isoforms α and δ in the activation of PLD and in tumor formation as well as what role Ral mediates in the activation of PLD and transformation of EGFR overexpressing cells is presented in this work.

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

CHAPTER II

Antagonistic Effects of PKC α and δ on Both Transformation and Phospholipase D Activity Mediated by the EGF receptor and the Role of RalA in This Pathway

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

RESULTS

3Y1 cells overexpressing the EGF receptor display a transformed phenotype upon treatment with EGF, but not TPA. 3Y1 rat fibroblasts were stably transfected with a plasmid (pPEGFr) containing a puromycin-resistance marker gene and the EGF receptor gene under the control of the SV40 promoter (Coppola et al.,1994). EGF receptor-overexpression was verified by Western analysis of several puromycin-resistant clones as shown in **Fig. 11**. Clone 2 expressed the highest levels of the EGF receptor and unless otherwise indicated, was used for all subsequent experiments. Upon establishment of an EGF receptor-overexpressing cell lines (EGFR cells), we first examined the effect of both long term TPA treatment and EGF on the morphology of these cells. In **Fig. 12 a**, it is shown that the EGFR cells had a flat non-transformed morphology like the parental 3Y1 cells. In response to EGF, the EGFR cells took on a refractile morphology characteristic of transformation (**Fig. 12 a**). This morphological change was observed in the other EGFR cell lines as well; indicating that the ability of EGF to induce this phenotype was not restricted to the clonal EGFR cell line shown. However, in contrast with expectations, TPA treatment did not cause a transformed morphology as observed previously with the c-Src-overexpressing cells (Lu et al.,1997).

We next investigated the ability of the EGFR cells to form colonies in soft agar, and as shown in **Fig. 12 b**, EGF, but not TPA, induced anchorage independent growth. The ability of the EGFR cells to form colonies in soft agar correlated well with the level of expression of the EGF receptor (**Fig. 12 c**). The inability of TPA to induce the transformed phenotype was not due to a lack of PKC isoform downregulation, since this treatment resulted in the same rapid degradation of PKC isoforms reported previously

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

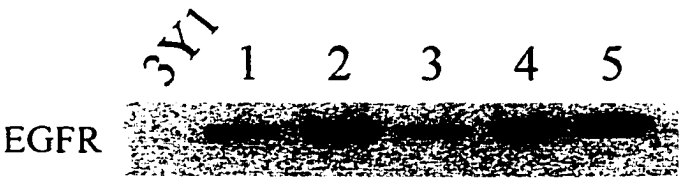


Fig. 11. Establishment of 3Y1 cells overexpressing the EGF receptor. 3Y1 cells were transfected with pPEGFr, which expresses the EGF receptor from the SV40 promoter and contains a puromycin-resistance marker (5). Several puromycin-resistant colonies were picked and analyzed for levels of expression of the EGF receptor. Western blot analysis was performed on lysates from the parental 3Y1 cells (3Y1) and the puromycin-resistant clones using an antibody to the EGF receptor as shown.

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

a.

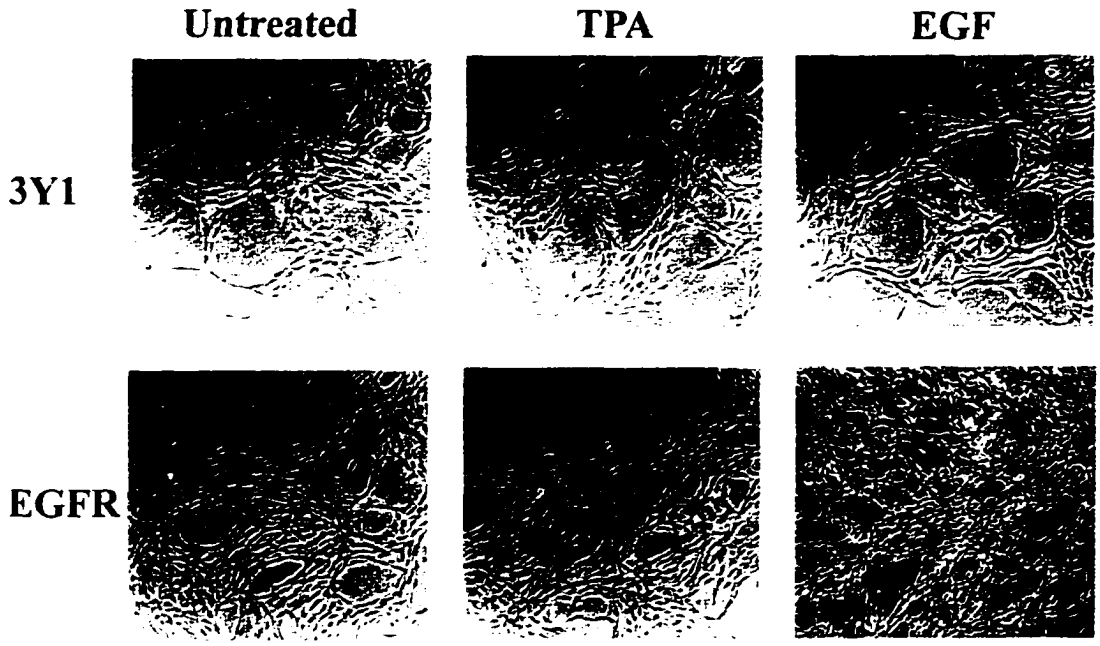
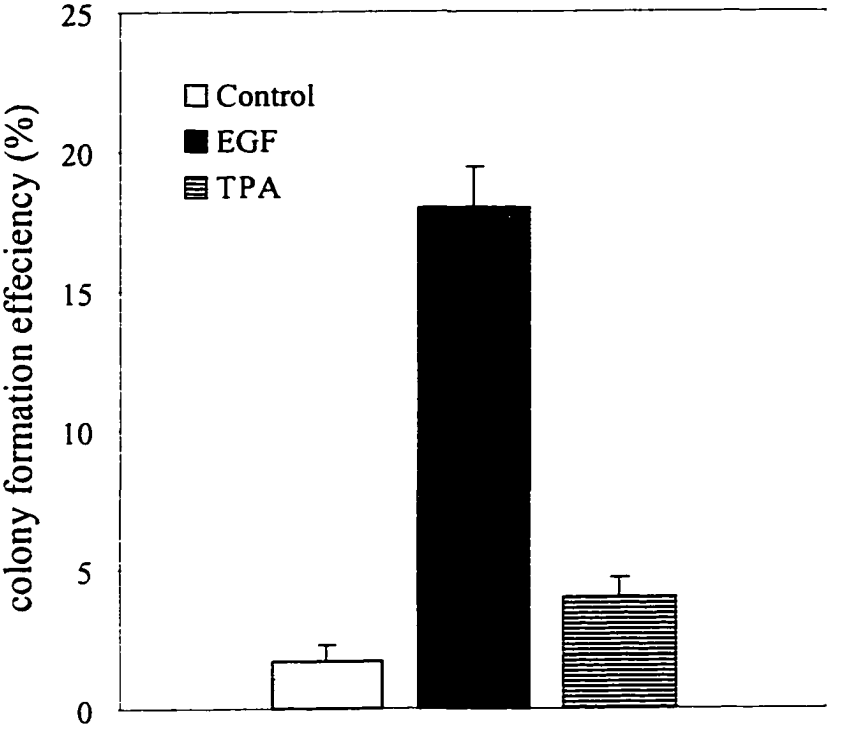
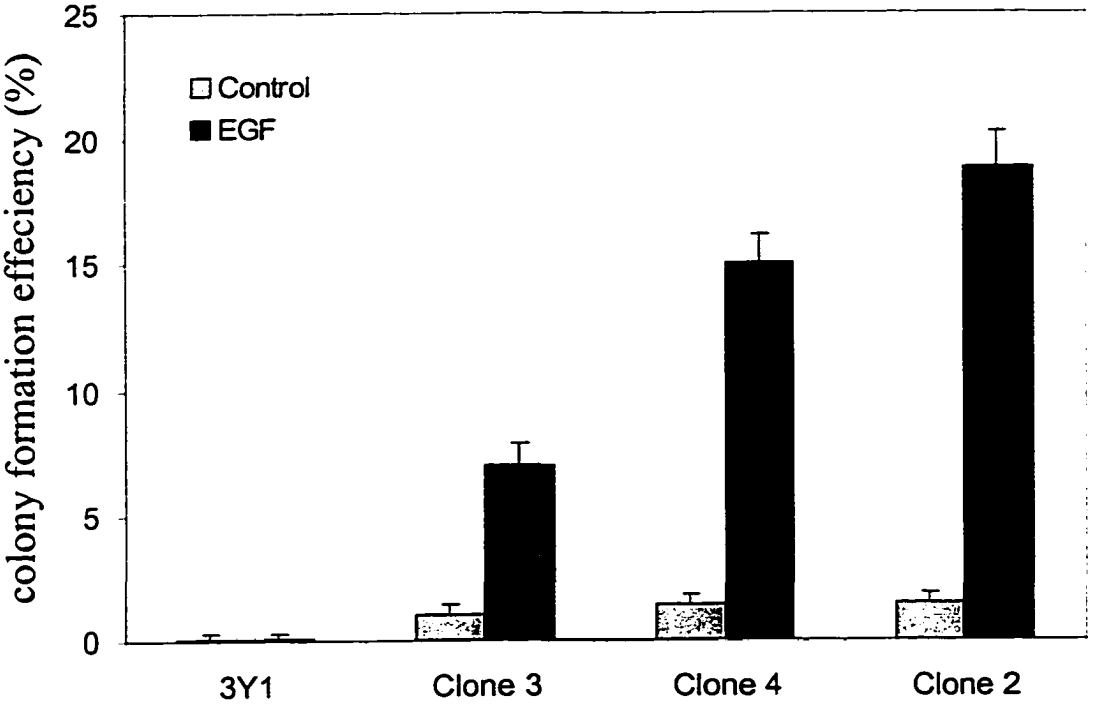


Fig. 12. Cells overexpressing the EGF receptor display a transformed phenotype upon treatment with EGF, but not TPA. (a) 3Y1 or EGFR cells were either untreated or treated with TPA (400 nM) or EGF (100 ng/ml) for 24 h, at which time the morphology of the cells was examined as shown. (b) Anchorage-independent growth of the EGFR cells was examined in the presence or absence of either TPA (400 nM) or EGF (100 ng/ml) as shown. TPA and EGF were replenished every four days. 10^3 cells were suspended in soft agar and the percentage of cells that formed colonies was determined three weeks later. (c) Colony formation in different EGFR clones expressing low (clone 3), midlevel (clone 4), and high (clone 2) expression of EGFR were compared.

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

Fig. 12 b,c





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

(Lu et al.,1997;1998). These data indicate that downregulation of PKC isoforms in response to TPA does not have the same effect in cells overexpressing the EGF receptor as observed previously for cells overexpressing the non-receptor tyrosine kinase c-Src (Lu et al.,1997).

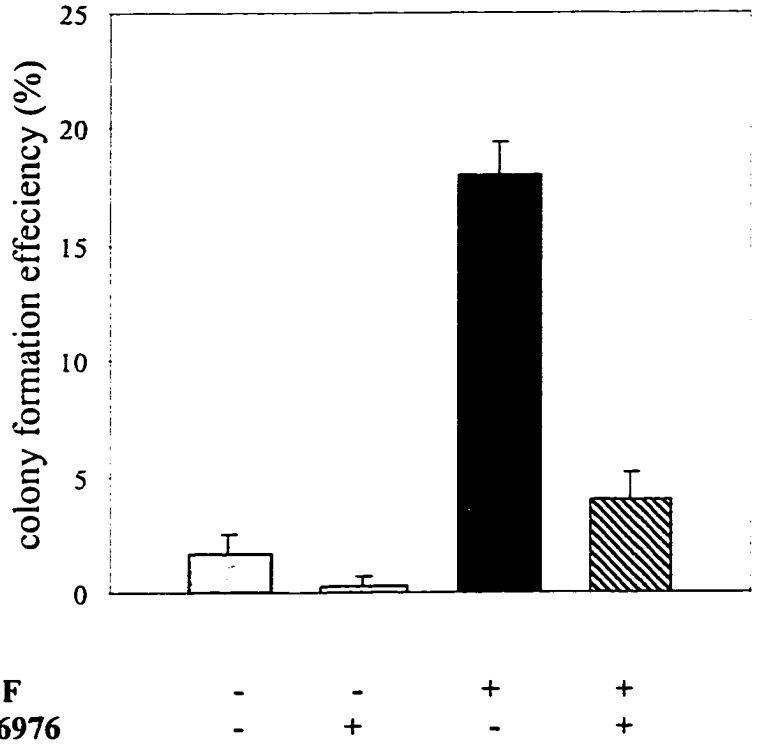
Differential effects of PKC α and δ on EGF receptor-mediated transformation. The inability of prolonged TPA treatment to induce a transformed phenotype in the EGFR cells could be due to a requirement for one of the PKC isoform depleted by the TPA treatment for the EGF receptor to induce a mitogenic signal. We therefore employed PKC inhibitors that were specific for the α and δ isoforms of PKC. We demonstrated previously that activation of PKC isoforms was required for ubiquitination and downregulation in 3Y1 cells and that Go6976 (Martiny-Baron et al.,1993) specifically inhibited PKC α downregulation and rottlerin (Gschwendt et al., 1994) specifically inhibited PKC δ downregulation (Lu et al., 1998). Thus, these two inhibitors were able to distinguish PKC α and δ requirements in these cells. We examined the effect of these two compounds on anchorage independent growth in the EGFR and parental 3Y1 cells. Go6976 strongly inhibited both EGF-induced and background colony formation in the EGFR cells (**Fig. 13 a**), indicating a PKC α requirement for EGF-induced mitogenic signals. Rottlerin, on the other hand, stimulated colony formation of the EGFR cells in the absence of EGF and increased the number of colonies formed in soft agar in the presence of EGF (**Fig. 13 b**). Rottlerin did not induce colony formation in the parental 3Y1 cells. Consistent with rottlerin inducing a transformed phenotype in the EGFR cells, rottlerin also caused a transformed

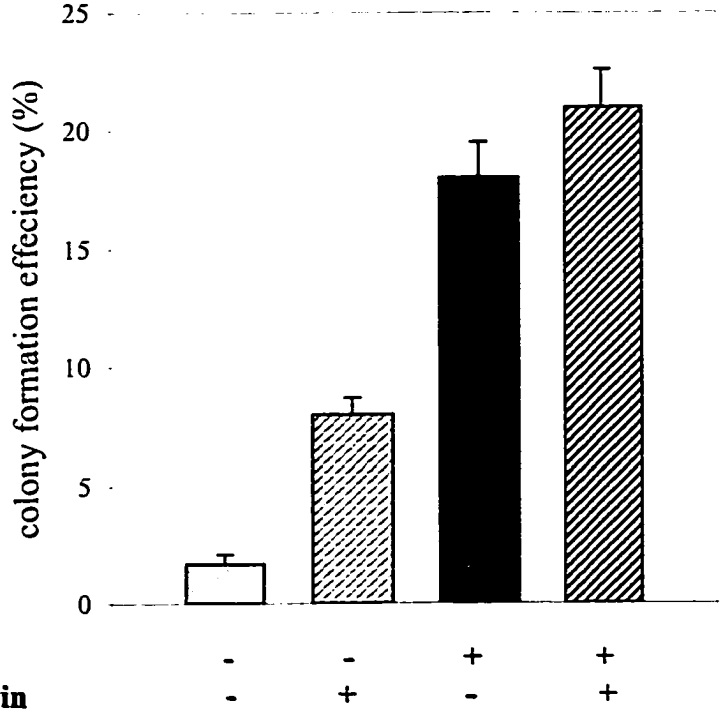
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. 13. The effect of PKC α- and δ-specific inhibitors on EGFR cells. EGFR cells were treated with EGF (100 ng/ml) and either Go6976 (0.5 μM) (a) or rottlerin (15 μM) (b) and then examined for the ability to form colonies in soft agar as in Fig. 2B. (c) The EGFR cells were treated with EGF (100 ng/ml), rottlerin (15 μM), G06976 (0.5 μM), or TPA (400 nM) as shown and the morphology of the cells was examined 24 h later as in Fig. 2A. (d) Colony formation in different EGFR clones expressing low (clone 3), midlevel (clone 4), and high (clone 2) expression of EGFR were compared with or without rottlerin.

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

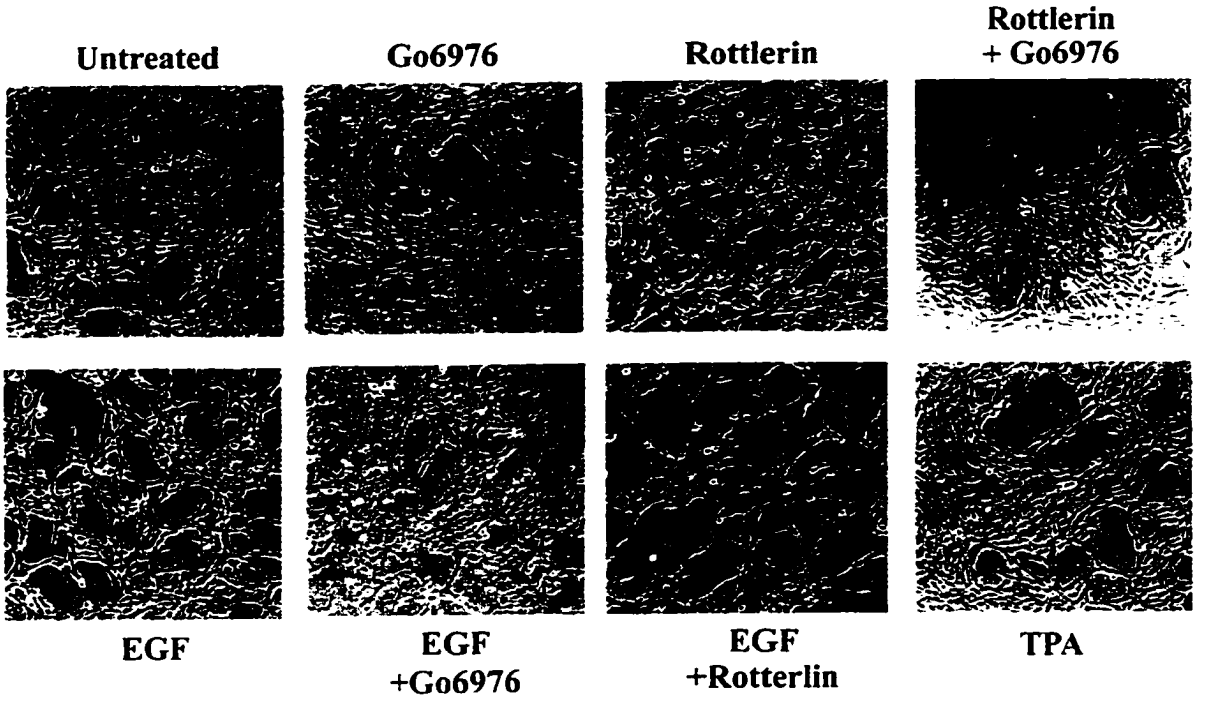
Fig. 13 a,b

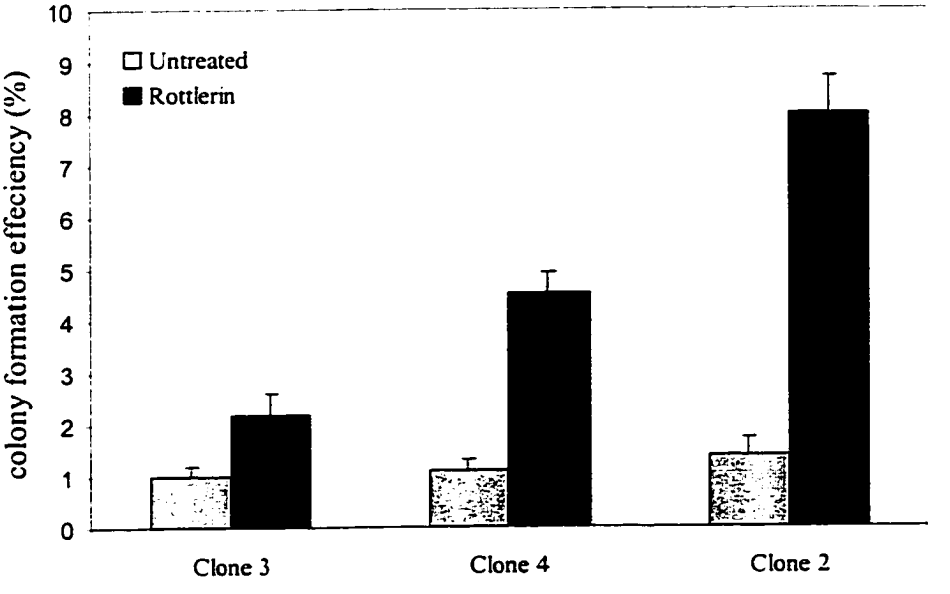




Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. 13 c,d





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

morphology in the EGFR cells in the absence of EGF (**Fig. 13 c**). The effect of rottlerin on colony formation was seen in all of the EGFR clones (**Fig. 13 d**) and therefore, was not due to any clonal variants.

Consistent with the inability of prolonged TPA treatment to induce a transformed phenotype in the EGFR cells, Go6976 inhibited the rottlerin-induced transformed morphology (**Fig. 13 c**). These data indicate that inhibition of PKC δ specifically has a tumor promoting effect similar to that reported previously for cells overexpressing c-Src (Lu et al.,1997). However, in contrast to the case with c-Src, the EGF receptor has a PKC α requirement for mitogenesis that explains why TPA treatment, which down regulates both PKC α and δ, does not cause transformation of the EGFR cells.

To further establish that the effect of the PKC-specific inhibitors Go6976 and rottlerin were due to effects upon PKC α and PKC δ respectively, we introduced dominant negative mutants of PKC α and δ into the EGFR cells. These mutants both have a conserved Lys in the ATP binding site converted to Ala and have been shown previously to act as dominant negative mutants for PKC α and δ (Hirai et al., 1994; Lu et al., 1997; Li, 1995). Expression of the dominant negative PKC α and δ mutants was verified by taking advantage of our previous demonstration that PKC downregulation in response to TPA is dependent upon an active kinase (Lu et al.,1998). Two cell lines transfected with either the kinase inactive PKC α or δ were treated with TPA for 24 h and the levels of PKC α and δ were then determined by Western blot analysis. As shown in **Fig. 14 a**, both PKC α and δ were degraded by TPA treatment to below the level of detection in the parental EGFR cells, whereas PKC α and δ levels in the two cell lines

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

a.

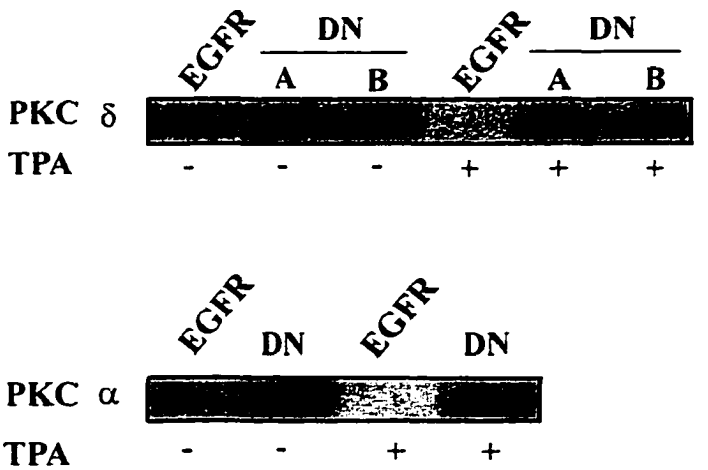
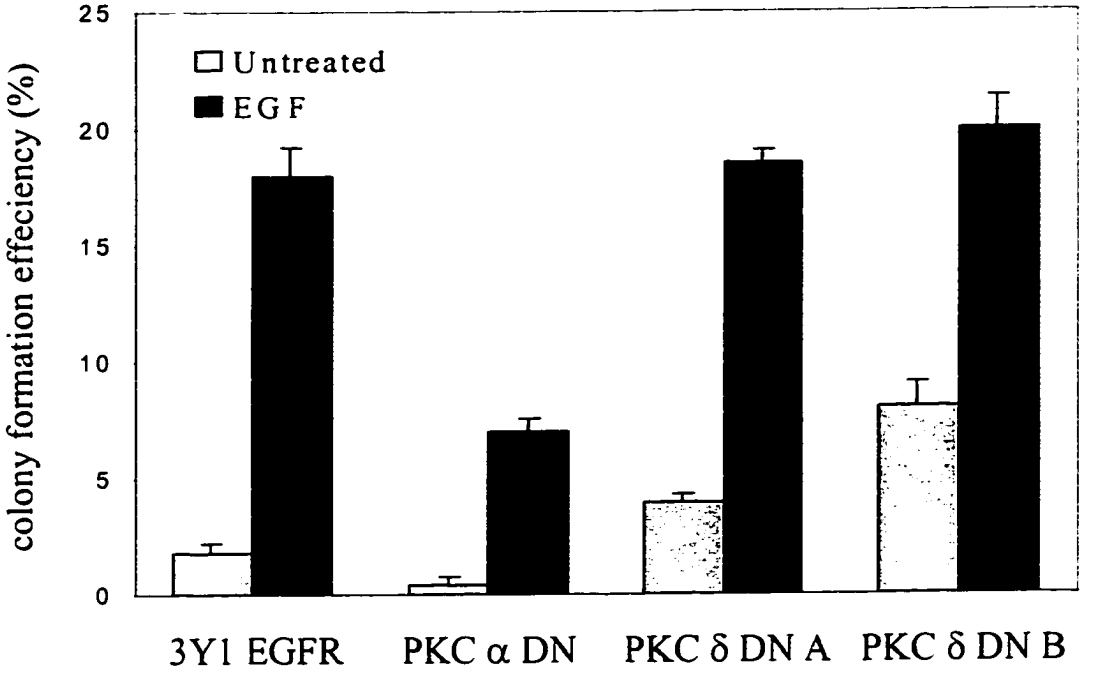


Fig. 14. The effect of dominant negative mutants for PKC α and δ upon the ability of EGFR cells to form colonies in soft agar. (a) EGFR cells were cotransfected with a plasmids expressing either a dominant negative (DN) PKC α or δ mutant and pCEP4 (Invitrogen), which expresses a hygromycin-resistance marker gene. One clone expressing the PKC α mutant and two clones expressing different levels of the PKC δ mutant were analyzed for expression of the mutants by Western blot analysis before and after treatment with TPA (400 nM, 24 h) which downregulates the endogenous wild type PKC α and δ, but not the kinase inactive mutants (24). (b) The ability to form colonies in soft agar of the EGFR cells and the EGFR cell lines expressing the dominant negative PKC α and δ mutants was performed as in Fig. 13 in the presence and absence of EGF (100 ng/ml) as shown.

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

Fig 14. b



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

expressing the dominant negative PKC δ were only reduced by about half, indicating that the kinase inactive dominant negative PKC mutants were being expressed.

Having established that the dominant negative PKC δ was expressed, we next examined the ability of cells to form colonies in soft agar in the presence and absence of EGF. The dominant negative PKC α inhibited EGF-induced colony formation and the cells expressing the dominant negative PKC δ now formed colonies in soft agar in the absence of EGF (**Fig. 14 b**). There was also a correlation between colony number and the level of expression of the dominant negative PKC δ with clone B expressing higher levels of the PKC δ mutant (**Fig. 14 a**) and also forming more colonies. Expression of the dominant negative PKC δ mutant did not increase the number of colonies induced by EGF treatment (**Fig. 14 b**), indicating that activation of the EGF receptor is able to overcome the inhibitory effects of PKC δ (see discussion).

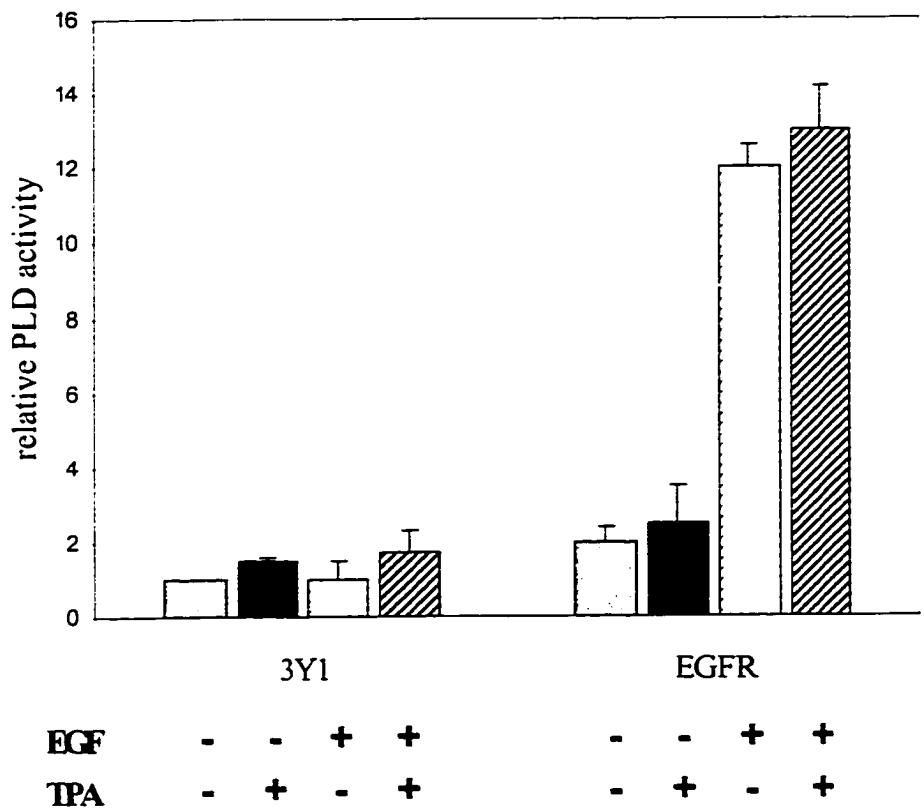
Differential effects of PKC α and δ on PLD activity in cells overexpressing the EGF receptor. We reported previously that in cells overexpressing c-Src, downregulation of PKC isoforms with prolonged treatment with TPA resulted in an increase in PLD activity (Lu et al.,1997). We therefore examined the effect of PKC downregulation by TPA on PLD activity in the EGFR and parental 3Y1 cells in the presence and absence of EGF. Activation of PLD by EGF has been reported previously (Yeo and Exton, 1995; Song et al., 1994) and consistent with these reports EGF strongly elevated PLD activity in the EGFR cells several fold (**Fig. 15 a**). Downregulation of PKC isoforms with prolonged TPA treatment had no dramatic affect upon the PLD activity in either EGF-treated or untreated 3Y1 or EGFR cells (**Fig. 15 a**),

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

Fig. 15. Downregulation of PKC δ elevates PLD activity in cells overexpressing the EGF receptor. (a) 3Y1 and EGFR cells were treated with EGF (100 ng/ml, 5 min) and/or TPA (400 nM, 24 h) as shown in the presence of 1% butanol and PLD activity was determined by examining the levels of the PLD-generated transphosphatidylation product phosphatidylbutanol as described in Materials and Methods. The relative PLD activity was determined by normalizing to the PLD activity in the untreated 3Y1 cells. Error bars represent the standard deviation for two independent experiments performed in duplicate. (b) The effect of the PKC α- and δ-specific inhibitors Go6976 (0.5 μM) and rottlerin (15 μM) upon EGF-induced PLD activity was determined as in A. The relative PLD activity was normalized to the PLD activity in the untreated EGFR cells. (c) EGFR cells expressing dominant negative (DN) mutants of PKC α and δ were examined for their effect upon PLD activity in the presence and absence of EGF as shown. The relative PLD activity was normalized to the PLD activity in the untreated EGFR cells. Error bars for all experiments represent the standard deviation for two independent experiments performed in duplicate, where duplicates varied by less that 10%.

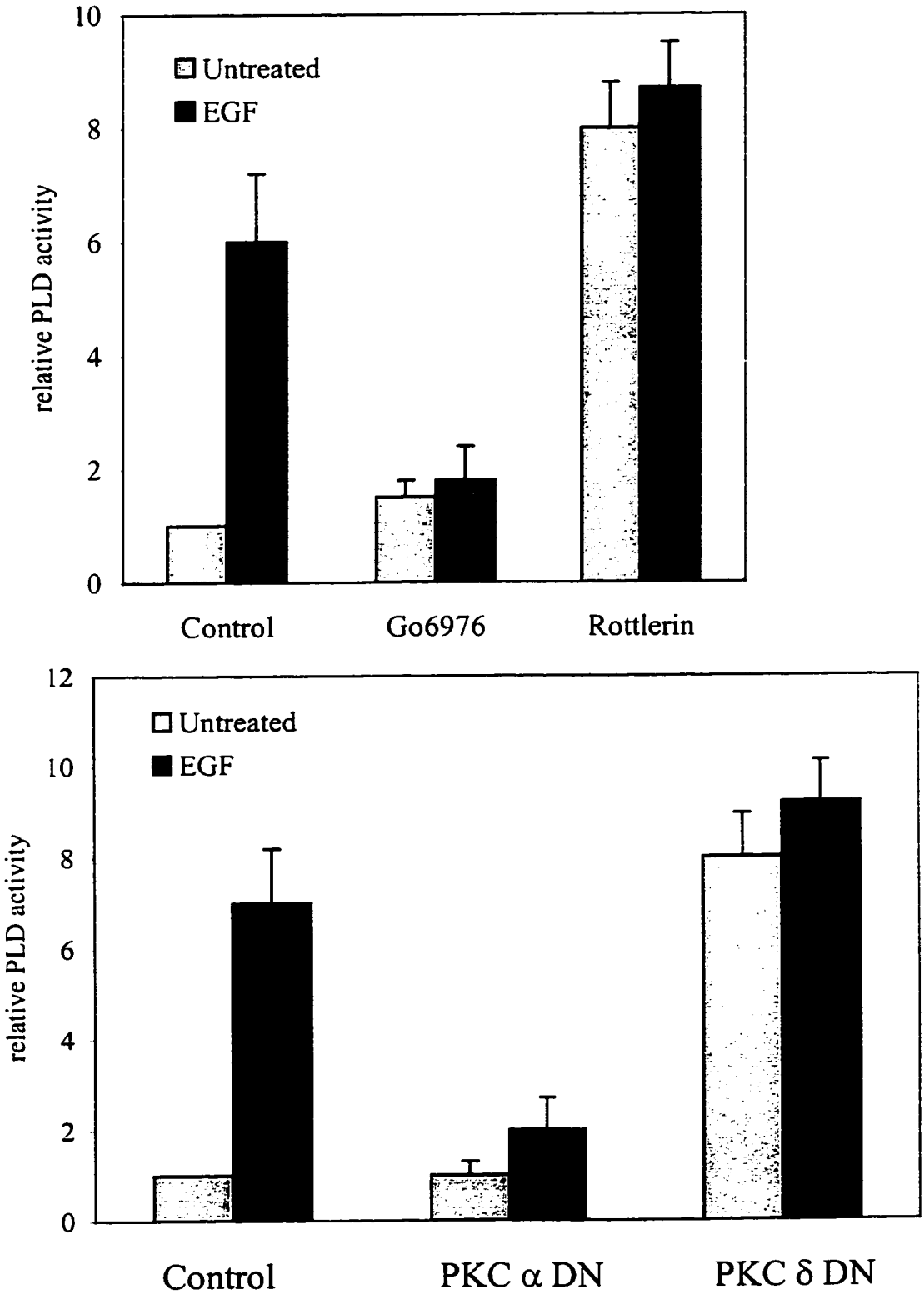
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. 15 a



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

Fig. 15 b,c



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

suggesting that the EGF-induced PLD activity in these cells was independent of PKC. The role of PKC in the activation of PLD in response to EGF has been controversial. A dependence upon PKC was found in fibroblasts (Yeo and Exton,1995), but not in A431 human epidermoid carcinoma cells (Song et al., 1994). Since EGF-induced transformation was inhibited by the PKC α inhibitor Go6976 and enhanced by the PKC δ inhibitor rottlerin, it is possible that downregulation of all PKC isoforms by TPA treatment could have both inhibitory and stimulatory effects that would neutralize each other. We therefore examined the effects of the PKC isoform-specific inhibitors Go6976 and rottlerin upon the PLD activity in the EGFR cells. The PKC α-specific inhibitor Go6976 reduced the EGF-induced increase in PLD activity to approximately the level seen with the inhibitor alone (**Fig 15 b**). The PKC δ inhibitor rottlerin stimulated PLD activity in the EGFR cells to even a higher level than that observed in response to EGF (**Fig. 15 b**). Rottlerin did not substantially elevate PLD activity in the parental 3Y1 cells (data not shown), indicating that the effect may be restricted to the partially transformed EGFR cells. Nor did EGF substantially further elevate PLD activity in the rottlerin treated cells (**Fig. 15 b**), indicating that the effect may be on the same population of PLD molecules activated in response to EGF. These data suggest that PKC α is a positive regulator of EGF-induced PLD activity and that PKC δ is a negative regulator of PLD activity.

We next examined the PLD activity in the EGFR cell lines expressing the dominant negative PKC mutants described in **Fig. 14 a**. If PKC α is required for EGF induced PLD activity as suggested by the data in **Fig. 15 b**, then EGF-induced PLD activity should be reduced in the EGFR cells expressing the dominant negative PKC α,

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

and as shown in **Fig. 15 c**, this was observed. EGF-induced PLD activity was reduced. It would also be anticipated that in the EGFR cells expressing the dominant negative PKC δ would have an elevated basal level of PLD activity that could not be substantially elevated by EGF. And as shown in **Fig. 15 c**, the basal PLD activity was elevated several fold relative to the parental EGFR cells and EGF did not significantly elevate the PLD activity further. These data are consistent with the results obtained with the PKC isoform-specific inhibitors and further indicate a PKC α requirement for EGF-induced PLD activity and an inhibitory effect for PKC δ on PLD activity in the EGFR cells that is lost upon downregulation or inhibition. The effect of the PKC α and δ isoforms upon PLD activity in the EGFR cells mirrors exactly the effect of these isoforms on transformation of these cells.

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

EGF-induced PLD activity is dependent upon the Ras/RalA GTPase cascade.

EGF induces an increase in PLD activity (Song et al.,1994;Yeo and Exton,1995). PLD1 (Hammond et al.,1995) associates directly with RalA (Luo et al.,1997) and we therefore wished to determine whether activation of the Ras/RalA GTPase cascade was required for the EGF-induced increase in PLD activity. To investigate this we established EGFR cell lines that stably expressed various RalA mutant plasmids (**TABLE I**). We transfected into the EGFR cells plasmid vectors expressing wild type RalA, an activated RalA (Q72L), and three inactivating RalA mutants: S28N, which is homologous to the S17N mutation in Ras; D49N, a RalA effector domain mutant that is defective in associating with Ral-BP1 (Cantor et al., 1995); and ΔN11, which has an amino terminal deletion of 11 amino acids unique to Ral GTPases and is defective in recruiting the PLD activator Arf into an active PLD complex (Luo et al.,1998). Expression of these RalA genes was verified by Western blot analysis as shown in **Fig. 16 a**.

TABLE I

RalA expression vectors

NAME	CHARACTERISTIC
WT	NORMAL AND COMPLETE VERSION OF RAL
Q72L	Constitutively active RalA
S28N	GDP/GTP exchange dominant negative mutant of RalA
D49N	Effector domain mutant lacking the binding site for the RalA effector RalBP1
ΔN11	Deletion mutant defective in binding small GTPase ARF

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

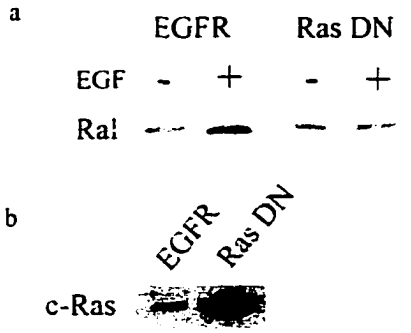


Fig. 16. EGF activates RalA in a Ras-dependent manner. (a) EGFR cells and EGFR cells expressing the S17N dominant negative Ras mutant (Ras DN) were treated with EGF (100 ng/ml) for 10 min. The cells were then lysed and treated with immobilized GST-Ral-BD as described Materials and Methods. The GST-Ral-BD was recovered by centrifugation and the precipitate was subjected to Western blot analysis using an antibody raised against RalA. (b) The parental EGFR cells and the EGFR cells stably transfected with the S17N dominant negative Ras mutant were examined for expression of Ras proteins by Western blot analysis as shown.

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

We then examined the effect of these RalA gene products upon PLD activity in the EGFR cells in the presence and absence of EGF. The effect of the RalA gene products on PLD activity in the EGFR cells is shown in **Fig. 16 b**. Overexpression of wild type RalA and the activated Q72L RalA mutant induced small, but reproducible, increases in both basal and EGF-stimulated PLD activity. Overexpression of all three defective RalA mutants inhibited EGF-induced PLD activity, with the S28N mutant having the largest effect. The mutants had little or no effect upon the basal PLD activity. These data indicate that RalA mediates the PLD activity induced by EGF. And consistent with a Ras/RalA GTPase cascade involvement, the activation of PLD activity by EGF was also inhibited by the dominant negative S17N Ras mutant (**Fig. 16 b**).

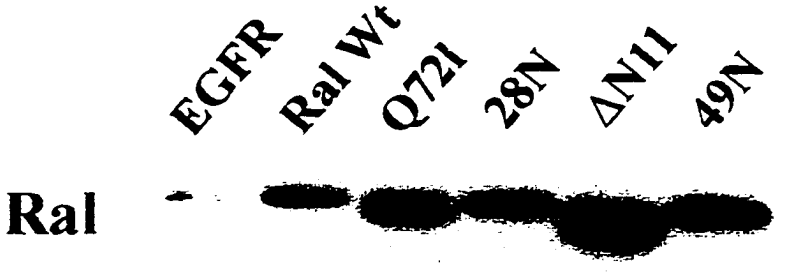
EGF-induced transformation is dependent upon Ral. As shown previously, in response to EGF, the EGFR cells form colonies in soft agar. Moreover, the EGFR cells become transformed upon downregulation of PKC δ. Interestingly the downregulation of PKC δ resulted in the elevation of PLD activity. We therefore examined the role of the Ras/RalA pathway on transformation in the EGFR cells. We investigated the ability of the EGFR cells expressing the various RalA and Ras gene products to form colonies in soft agar in the absence and presence of EGF. The ability of EGFR cells to form colonies in soft agar was inhibited by expression of the dominant negative S17N Ras gene (**Fig. 17**). Overexpression of wild type RalA or the activated Q72L RalA resulted in a substantial increase in colony forming efficiency in the absence of EGF, while not significantly increasing the colony forming efficiency in the presence of EGF (**Fig. 17**). Thus, RalA overexpression or activation can apparently result in at least partial

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

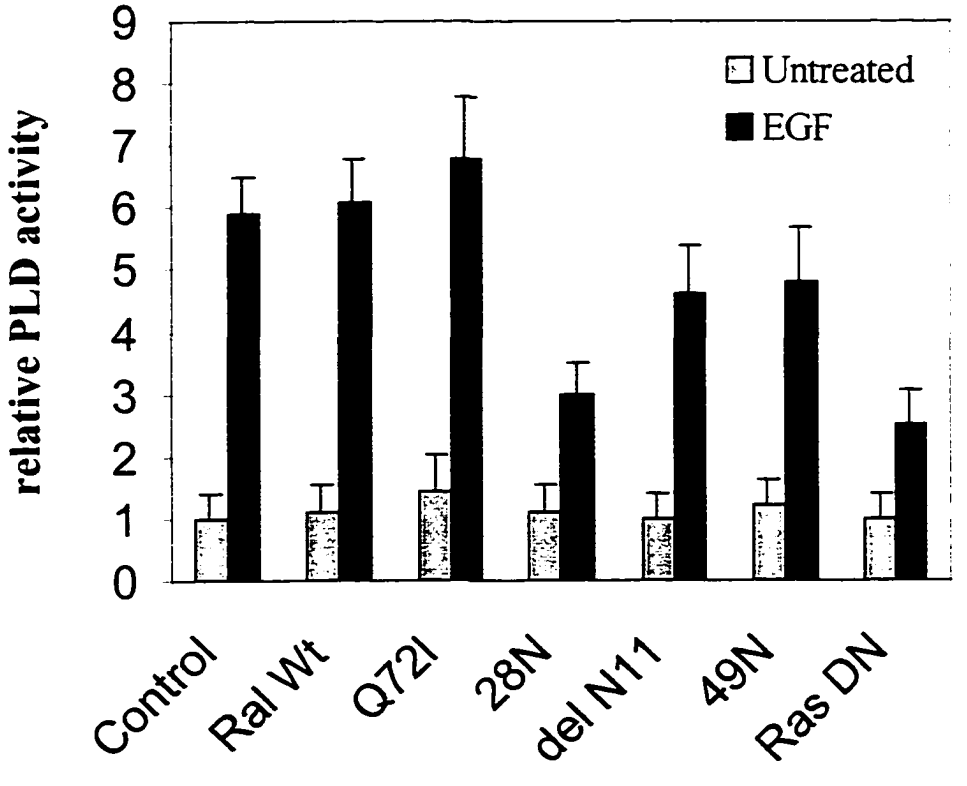
Fig. 17. EGF-induced PLD activity is dependent upon the Ras/RalA GTPase cascade. EGFR cells were stably transfected with plasmid vectors expressing wild type RalA; Q72L, an activated RalA mutant; S28N, an inactivating mutant that is homologous to the S17N mutation in Ras; D49N, a RalA effector domain mutant; and ΔN11, which has an amino terminal deletion of 11 amino acids. Expression of these RalA genes was verified by Western blot analysis as shown in (a). (b) The EGFR cells and these cells expressing the RalA genes were treated with EGF (100ng/ml, 10 min) as shown in the presence of 1% butanol and PLD activity was determined by examining the levels of the PLD-generated transphosphatidylation product phosphatidylbutanol as described in Materials and Methods. The relative PLD activity was determined by normalizing to the PLD activity in the untreated 3Y1 cells. Error bars represent the standard deviation for three independent experiments performed in duplicate.

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

a.



b.



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

transformation of cells overexpressing the EGF receptor. This is similar to the effect of downregulating PKC δ had upon the EGFR cells, which also transformed these cells.

The effect of the dominant negative RalA mutants upon the EGF-induced transformed phenotype. As with the EGF-induced PLD activity, we examined the effect of three RalA mutants with defects in the effector domain (D49N), GDP/GTP exchange (S28N), and Arf recruitment (ΔN11). And as with the EGF-induced PLD activity, all three mutants substantially reduced the ability of EGF to induce colony formation in soft agar (**Fig. 18**). These data indicate that RalA is both required for the EGF-induced transformation of the EGFR cells and that activated RalA is at least able to partially compensate for EGF, possibly through its interaction with PLD1.

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

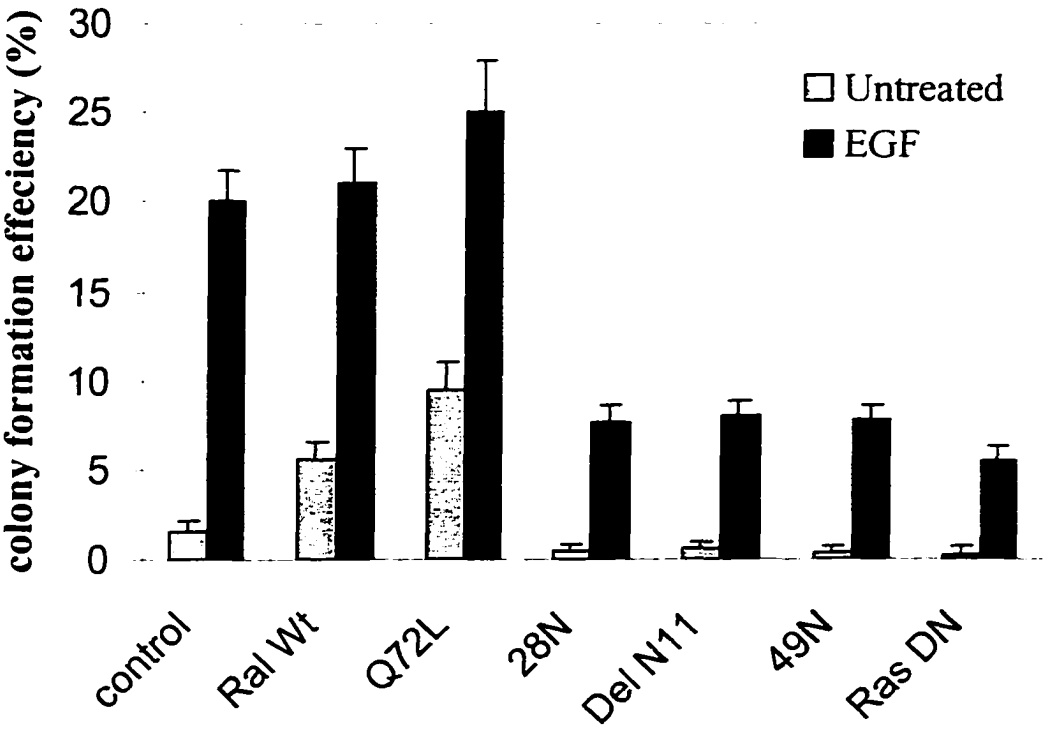


Fig. 18. EGF-induced transformation is dependent upon Ral. Anchorage-independent growth of the EGFR cells and the EGFR cells expressing the dominant negative S17N Ras (Ras DN) and the various RalA genes described in Fig. 17 was examined in the presence and absence of EGF (100 ng/ml) was determined as shown. EGF was replenished every four days. 10^3 cells were suspended in soft agar and the percentage of cells that formed colonies was determined three weeks later. Error bars represent the standard deviation for three independent experiments performed in duplicate.

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

Expression of PLD1 in EGFR cells increases colony forming efficiency. As discussed in the introduction, cells are very intolerant of PLD expression. We nevertheless attempted to express PLD1 in the EGFR cells and in the parental 3Y1 cells. The Flu-tagged PLD1 expression vector, pCGN-hPLD1, was cotransfected into the EGFR cells along with pCEP4 (Invitrogen), which expresses a hygromycin-resistance marker gene. Transfection was attempted in both the EGFR and parental 3Y1 cells. Interestingly, hygromycin-resistant colonies were detected only in the EGFR cells; no hygromycin-resistant colonies were detected in the parental 3Y1 cells (**TABLE II**). This was not due to differences in the transfection efficiency between the two cell lines since the pCEP4 gave equal numbers of colonies in both the EGFR and parental 3Y1 cells (**TABLE II**).

TABLE II

Hygromycin-resistant colonies (%)

	3Y1	EGFR
pCEP4	100	100
pPLD1	0	67

The human PLD1 (pCGN-hPLD1) gene was transfected as described in Materials and Methods. pCEP4 expresses a hygromycin-resistance marker gene and was either co-transfected with pCGN-hPLD1 or transfected by itself into the EGFR and parental 3Y1 rat fibroblasts since pCGN-hPLD1 lacks a selectable marker. Hygromycin-resistant colonies were then counted 10 days later. The number of hygromycin-resistant colonies was then determined and normalized to the number of colonies in the parental 3Y1 cells transfected with pCEP4 alone, which was given a value of 100%.

Thus, the expression of PLD1 in the parental 3Y1 cells is apparently toxic, which is consistent with previous reports suggesting that high levels PLD expression are not tolerated by cells (McNamara et al., 1995). The EGFR cells, however, are apparently able to tolerate higher levels of PLD1 expression. Several hygromycin colonies were

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

expanded and screened for PLD1 expression. Expression of human PLD1 (Flu-tagged hPLD1) could be distinguished from the low levels of endogenous rat PLD1 by virtue of a small difference in electrophoretic mobility as shown by Western blot analysis of a representative hPLD1-expressing clone (**Fig 19. Insert**).

We then compared the ability of this PLD1-overexpressing cell line to form colonies in soft agar with the parental EGFR cells. As shown in (**Fig. 19**), it can be seen that in the absence of EGF, there is an increase in colony forming efficiency in the PLD1-overexpressing cells relative to the EGFR cells in the absence of EGF. The increase was comparable to that seen with overexpression of RalA and the activated Q72L RalA in the absence of EGF. In the presence of EGF, the PLD1 cells had a reduced colony forming efficiency (**Fig. 19**). This was likely due to inhibitory effects of elevated PLD activity

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

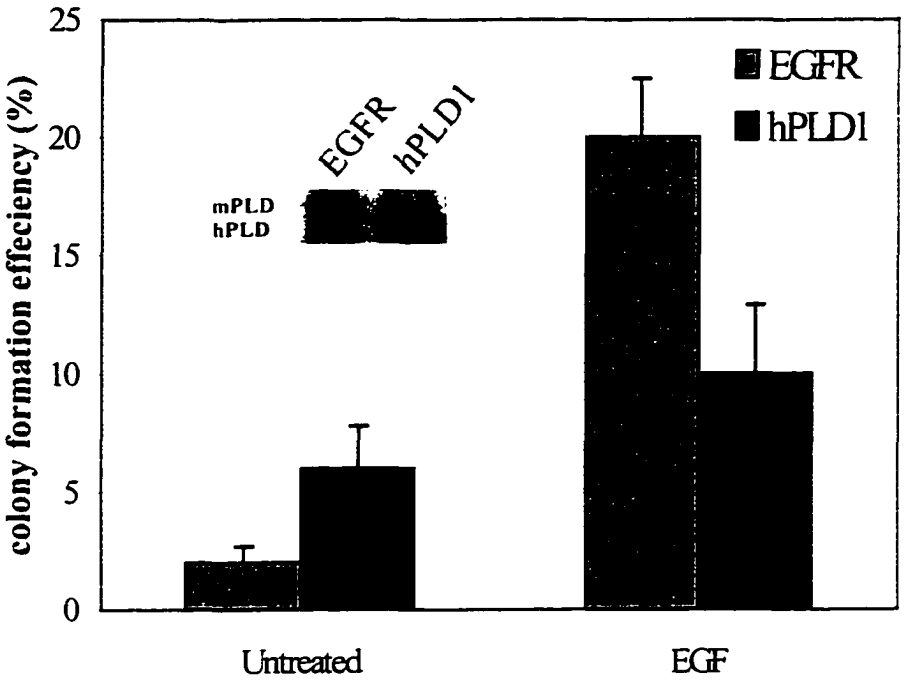


Fig. 19. Expression of PLD1 in EGFR cells increases colony forming efficiency. Several colonies transfected with pCGN-hPLD1 expression vector were picked and screened for hPLD1 expression that can be distinguished from endogenous PLD1 by a difference in electrophoretic mobility. Western blot analysis with an anti-PLD1 antibody of a representative PLD1-expressing clone is shown in the insert. The ability of the hPLD1-expressing and parental 3Y1 cells to form colonies in soft agar was determined in the presence and absence of EGF as described in Fig. 12.

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

in response to EGF and the sensitivity of cells to high levels of PLD activity. We have found that in the presence of EGF, the PLD1-overexpressing cells slow down their growth substantially and many cells die. These data suggest that elevated PLD activity in cells overexpressing the EGF receptor can result in an increase in the ability to form colonies in soft agar and are consistent with the dependence of EGF-induced transformation being dependent upon the Ras/RalA/PLD pathway.

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

DISCUSSION

In this thesis, we have shown that in cells overexpressing the EGF receptor, inhibition of PKC δ results in a transformed phenotype. This suggests that PKC δ has a tumor suppressing effect that is lost upon inhibition. The addition of EGF to cells overexpressing the EGF receptor led to a transformed phenotype that was dependent upon PKC δ. The ability of EGF to induce a transformed phenotype in the absence of PKC δ downregulation suggests that upon EGF treatment, the inhibitory effects of PKC δ can be overcome.

In this regard, it was shown previously that in response to EGF, PKC δ becomes phosphorylated on tyrosine, leading to an inhibition of PKC δ kinase activity (Denning et al.,1996). Thus, the ability to overcome the negative effects of PKC δ by EGF may be due to the ability to stimulate tyrosine phosphorylation of PKC δ. This is very similar to the differential ability of v-Src and c-Src to transform cells. 3Y1 cells overexpressing c-Src are not transformed; however, downregulation of PKC δ results in the transformation of these cells (Lu et al.,1997). The same cells overexpressing v-Src are already transformed, but interestingly, PKC δ associates with v-Src in these cells and becomes phosphorylated on tyrosine and the tyrosine-phosphorylated PKC δ has a reduced kinase activity (Zang et al.,1997). A reduced kinase activity of tyrosine phosphorylated PKC δ was also observed in cells transformed by v-Ras (Denning et al.,1993). Thus, there is apparently a mechanism whereby PKC δ can be downregulated through tyrosine phosphorylation in response to cell division signals that allows cells to overcome the negative regulatory effects of PKC δ.

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

The data presented here and previously (Lu et al.,1997;Zang et al.,1997) suggest a model where overexpression of c-Src or the EGF receptor primes a cell for division, however cell division is blocked by PKC δ. This is similar conceptually to a model proposed several years ago by Stiles and Pledger where resting cells needed both "competence" and "progression" factors to leave the resting stage of the cell cycle (G_0) and traverse G_1 into S-phase (Stiles et al.,1979). Thus, in our model system, overexpression of c-Src or the EGF receptor is postulated to act as a competence factor which facilitates either the exit from G_0 or prevents entering the resting G_0 state, and the progression factor is postulated to be the inhibition of PKC δ which allows the traversing of G_1. In the tumor promotion model, inhibition or depletion of the tumor suppressing PKC δ allows cell cycle progression and therefore, the amplification of the initiated (c-Src or EGF receptor overexpressing) cells.

In these two models for the regulation of cell proliferation, promotion and progression are similar in that they facilitate passage through G_1 into S-phase. Most of the characterized tumor suppressor genes also affect cell cycle progression through a G_1/S cell cycle checkpoint. p53, $p21^{cip}$, p27, $p16^{ink}$ family, and Rb, are the best studied in what is being called a tumor suppressor pathway (Collins et al.,1997;Sherr,1996) It has been suggested that a defect in this tumor-suppressing pathway, which blocks cell cycle progression in late G_1, is essential for all human tumors (Sherr,1996). We have found that in serum-starved cells overexpressing c-Src, inhibition of PKC δ results in an increase in DNA synthesis that can be detected as soon as 3 hours after treatment (Lu et al.,1997). This rapid induction of DNA synthesis by PKC δ inhibition suggests that, in the absence of serum, at least some of the c-Src-overexpressing cells are blocked in late G_1 and that

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

inhibiting PKC δ facilitates passage through this checkpoint into S phase. Consistent with this hypothesis, PKC δ was recently reported to suppress expression of the G1 cyclins D1 and E (Fukumoto et al.,1997) which facilitate passage from G_1 to S. Thus, PKC δ may be another regulator of this tumor-suppressing pathway that allows progression through the G_1/S cell cycle checkpoint. Overexpression of PKC δ in CHO cells led to an accumulation of cells in G_2/M upon TPA treatment (Watanabe et al.,1992), indicating that PKC δ may negatively regulate cell cycle progression at multiple points.

A role for PLD in cell cycle regulation is poorly understood, however PLD activity is elevated in response to most if not all mitogenic stimuli (Yeo & Exton,1994;Foster,1993). Data presented here also indicate that RalA is required for the transformed phenotype induced by EGF in the EGFR cells. The finding here that downregulation or inhibition of PKC δ in the EGFR cells leads to substantial increases in PLD are consistent with a role for PLD in the cell cycle progression stimulated by inhibition of PKC δ. It is not yet clear how the downregulation of PKC δ might elevate PLD activity, but since phosphorylation of the EGF receptor by PKC negatively regulates the receptor function (Decker,1984; Downward et al.,1985; Friedman et al.,1984; McCaffrey et al.,1984), it is possible that the loss of PKC δ-mediated inhibition of the EGF receptor could lead to the observed increase in PLD activity that may in some as yet undetermined way contribute to cell cycle progression.

We demonstrated previously that cells overexpressing c-Src become transformed upon treatment with TPA, which downregulates both PKC α and δ. However; as demonstrated here, cells overexpressing the EGF receptor do not become transformed with the TPA treatment. This is presumably due to the requirement of PKC α for both

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

EGF-induced transformation and PLD activity. Interestingly, the activation of PLD by Src, in contrast with the activation of PLD by EGF, is independent of PKC α (Lu et al.,1997;Song & Foster,1993). Thus, the PKC α requirement for EGF-induced PLD activity may explain the differential effect of long term TPA treatment has upon the transformed phenotype in cells overexpressing c-Src and cells overexpressing the EGF receptor. These data also indicate that the transformed phenotype induced in EGFR cells by down regulation of PKC δ is not mediated by EGF receptor activation of c-Src. If this were the case, then we would not see the additional requirement of PKC α for both EGF-induced transformation and PLD activity that was not observed in the cells overexpressing c-Src.

The differential effect of PKC α and δ upon PLD activity likely explains previously reported discrepancies in the dependence of EGF-induced PLD activity upon PKC (Song et al.,1994;Yeo and Exton,1995). Downregulation of all phorbol ester responsive isoforms by TPA would be expected to have both inhibitory and stimulatory effects upon PLD activity in cells overexpressing the EGF receptor. And as reported here in fibroblasts overexpressing the EGF receptor and previously in human epidermoid carcinoma A431 cells that also overexpress the EGF receptor (Yeo and Exton,1995), downregulation of PKC isoforms with TPA had little or no effect upon PLD activity. However, as shown here, the PKC α-specific inhibitor Go6976 blocked EGF-induced PLD activity very effectively. These data are also consistent with previous reports implicating PKC α as an activator of PLD activity *in vitro* (Singer et al.,1996) and with a requirement for PKC in the activation of PLD by EGF as reported by Exton and colleagues (Yeo and Exton,1995). The apparent lack of a PKC requirement for EGF-

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

induced PLD activity seen in the human cancer cell line A431, which like the cells used in this study, overexpress the EGF receptor, was likely due to both stimulatory and inhibitory effects of downregulating all phorbol ester-responsive PKC isoforms with TPA.

Overexpression of a tyrosine kinase is a common genetic alteration in human cancers (Dickson et al.,1992). This kind of genetic change alone does not result in a tumor. This initiating event does however give the cell a selective growth advantage over other cells in the presence of promoting substances that suppress the cell cycle control proteins that prevent cell cycle progression.

In this thesis, we have presented further evidence that inhibition of PKC δ is sufficient to stimulate anchorage-independent growth of cells overexpressing a receptor class tyrosine kinase, which is common in human tumors. Li et al. recently reported that PKC δ was required for transformation induced by insulin-like growth factor I (IGF-I) receptor (Li,1998). This suggests the possibility that PKC δ may also have positive roles in regulating cell division in response to other stimuli. However, the data presented here and previously (Lu et al.,1997) suggest that PKC δ by substances that either inhibit or downregulate PKC δ can enhance the promotion phase of tumor progression in cells where either c-Src or the EGF receptor is overexpressed. It will be important to determine whether epigenetic events such as diet, hormones, and cigarette smoke are able to either inhibit or downregulate PKC δ such that cells containing mutations that elevate tyrosine kinase activity would be amplified and therefore subject to progression to a more cancerous phenotype.

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

Previous reports have indicated that the PLD activity elevated in response to mitogenic signals is mediated by RalA (Jiang et al.,1995), which interacts directly with PLD1 (Luo et al.,1997). Additionally, RalA is required for v-Src-induced PLD activity (Jiang et al.,1995) and also blocks transformation of fibroblasts in culture (Urano et al.,1996) as well as the formation of tumors in mice (Aguirre Ghiso, unpublished). Interestingly, an essential role of Ral proteins in PKC signaling to PLD in HEK-293 cells was recently reported (Schimdt et al.,1998).

In this thesis, we have demonstrated that the Ras/RalA GTPase cascade mediates the activation of PLD by EGF. The transformed phenotype induced by EGF on the EGFR cells was dependent upon the Ras/RalA GTPase cascade suggesting that an important component of EGF-induced transformation is the activation of PLD. Consistent with this hypothesis, overexpression of PLD1 in the EGFR cells led to the formation of colonies in soft agar by the EGFR cells in the absence of EGF. The correlation between PLD activity and the transformed phenotype was not absolute. Overexpression of wild type RalA or the activated RalA led to a significant increase in colony forming efficiency in the EGFR cells in the absence of EGF, but led to only very small increases in PLD activity in the EGFR cells. Thus, the effect of wild type RalA and activated RalA had a much bigger effect upon transformation than upon PLD activity. Similarly, the effect of the defective RalA mutants was more pronounced on transformation than on PLD activity. We believe that this is likely due to RalA only affecting a subset of cellular PLD activity that is mediated by Ras. Since Ras is largely restricted to caveolae and caveolae-related membrane microdomains (Anderson,1998), the PLD activated in response to EGF may be restricted to this portion of the plasma membrane. It is unlikely that PLD involved in

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

other cellular functions such as vesicle formation in the Golgi is mediated by Ras. The effect of wild type RalA, the activated RalA, and PLD1 expression upon colony forming efficiency was clearly less than that induced by EGF. This could be due to EGF acting more efficiently than overexpressed RalA or PLD1, which may be restricted because of the apparent toxic effects of PLD. Alternatively, it is also possible that activation of other downstream targets of the EGF receptor by EGF leads to more efficient passage of cell cycle checkpoints. The specifics of the more efficient colony formation in response to EGF remain to be established.

Many signaling molecules including Ras, Src, PKC (Oka et al.,1997), and the EGF receptor localize to the sphingolipid- and cholesterol-enriched caveolar membrane microdomains (Anderson,1998). We have found that there is an enrichment of both RalA and PLD activity in caveolar membrane fractions. Thus, PLD may in some way regulate signaling molecules in this plasma membrane microdomain where so many signaling molecules are localized.

In the presence of an overexpressed tyrosine kinase, the effect of PLD can apparently lead to progression through the cell cycle and the amplification of partially mutated cells, or tumor promotion. The mitogenic effects of EGF likely involve multiple downstream effector molecules. The data provided here demonstrate that overexpression of RalA or the overexpression of activation of PLD1 can complement the partial mitogenic signal of EGF receptor overexpression. The dependence of EGF-induced transformation upon RalA indicates that the Ras/RalA/PLD signaling pathway is also an essential component of mitogenic signaling mediated by the EGF receptor.

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

In this thesis, data was presented extending previous work with c-Src to further implicate not only PKC δ but also the small GTPase RalA in the transformation and PLD activation of cells overexpressing the EGF receptor. We described the antagonistic effects of PKC α and δ on both transformation and PLD activity in cells that overexpress the EGF receptor, which like c-Src, is implicated in many human cancers (Biscardi et al.,1998) and have also exploited a variety of RalA mutants to implicate it in PLD activation in cells overexpressing the EGF receptor. Also, both RalA and, indirectly, PLD are implicated in the transformation of these cells. Finally, the overexpression of PLD1 in the EGFR cells for the first time directly implicates PLD with transformation.

The involvement of PKC, Ral, and PLD in transformation and their co-localization in caveolae with Src and EGF receptor is intriguing. The major transmembrane scaffolding protien of caveolae is caveolin 1 (Galbiati et al.,1998). Reduced levels of this protein have already been correlated with transformation and tumorigenesis (Galbiati et al.,1998). Importantly, reintroduction of a recombinant caveolin 1 in transformed cells inhibited the colony formation characteristic of these cells (Lee et al.,1998). It would be very interesting to investigate the effect of PKC and Ral mutants as well as the effect of overexpression of PLD on the level of caveolin 1 in the cell lines used in this work in the future.

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

CHAPTER III

Materials and Methods

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

Cells and Cell Culture Conditions

Rat 3Y1 cells obtained from American Type Culture Collection (ATCC) or rat 3Y1 cells expressing the EGF receptor were maintained in Dulbecco's modified Eagle medium (DMEM, Life Technologies) supplemented with 10% bovine calf serum (HyClone), 100 U/ml penicillin G, 100 ug/ml streptomycin, in a humidified, 10 % CO_2 atmosphere, and grown at 37 °C. Cell cultures were made quiescent by growing to confluence and then replaced with fresh media containing 0.5% bovine calf serum for one day. For growth of cells in soft agar, 1 x 10^3 cells were suspended in top agar (DMEM, 20% calf serum, 0.38% agar) and overlaid onto hardened bottom agar (DMEM, 20% calf serum, 0.7% agar) (Qureshi et al.,1993). Old media was replaced with fresh DMEM with or without EGF added every four days for two to three weeks. Colonies were counted and the average from triplicates of each sample calculated.

Transfection

Cells were plated at a density of 10^5 cells/100 mm dish 18 h in 5% calf serum DMEM prior to transfection. Transfections were performed by using lipofectamine reagent (GIBCO) according to the vendor's instructions. Just prior to being exposed to DNA the media was replaced with plain DMEM lacking any antibiotics or serum. Expression vectors (0.2 ug) were transfected keeping a Lipofectamine to DNA ratio of 10:1. After two hours incubation with the liposome:DNA complexes, cells were placed into medium containing 20% calf serum DMEM for 1 hour and subsequently replaced by 5% calf serum DMEM. After one day, cells were typsinized, replated in two 100 mm plates in 5%

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

calf serum DMEM. The PKC (pSRDPKCα or pSRDPKCδ), PLD1 (pCGN-hPLD1), and RalA (pZIPNeoSV(X)1/RalWT,Q72L,S28N,D49N,ΔN11) expression plasmids lacked selectable markers so they were cotransfected with pCEP4 (PKC & PLD1) or pSV2Neo (RalA and mutants) expressing Hygromycin or Neomycin resistance, respectively. The EGFR cell lines were made by transfecting 3Y1 cells with pPEGFr, which expresses the EGF receptor from the SV40 promoter and contains a Puromycin-resistance marker (Coppola et al., 1994). Transfected cultures were selected with either puromycin (5 ug/ml) for EGFR cell line; puromycin (5 ug/ml) and G418 (Neomycin analog) (400 ug/ml) for the EGFR/RalA cell lines; or puromycin (5 ug/ml) and hygromycin (200 ug/ml) for EGFR/PKC and EGFR/PLD1 cell lines for 10-14 days at 37°C. At that time antibiotic-resistant colonies were picked and expanded for further analysis under selective conditions.

Materials

[^{3}H] myristate was obtained from New England Nuclear (NEN). The PKC inhibitors rottlerin and Go6976 were obtained from Calbiochem. Monoclonal antibodies to the EGF receptor, PKC α, Ras, and RalA were obtained from Transduction Laboratories; a polyclonal antibody for PKC δ was obtained from Santa Cruz Biotechnology; PLD1 antibody was obtained from UBI. pCEP4, which contains the hygromycin resistance gene was obtained from Invitrogen. PLD1 (pCGN-hPLD1) expression vector was a kind gift from Dr. Andrew Morris of Stonybrook. This plasmid was created by taking a fragment of the hPLD1 cDNA encoding the entire open reading frame and was then subcloned in-frame downstream of the CMV (cytomegalovirus) promoter and Flu epitope tag in the

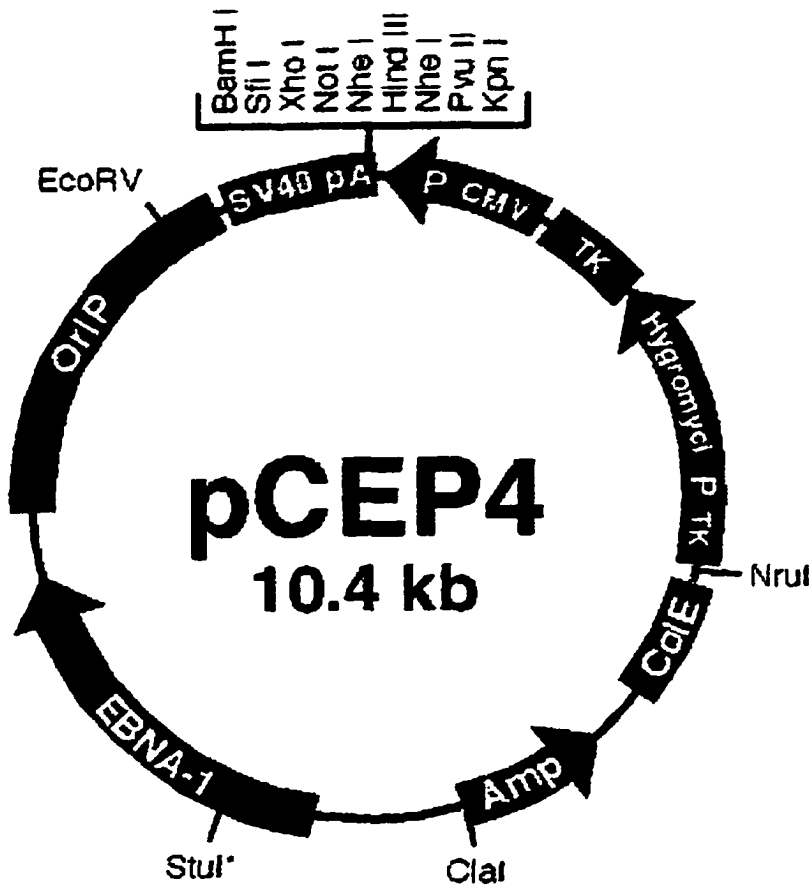
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

mammalian expression vector pCGN. Introduction of this gene into cells was carried out using co-transfection with pCEP4 (Invitrogen), which expresses a hygromycin-resistance marker gene. The EGFR expression plasmid pPEGFr was a kind gift from Dr. Renato Baserga, Thomas Jefferson University. The EGFR plasmid was created from the plasmid pXER, expressing the EGFR cDNA under the control of the SV40 early promoter. After digestion with XhoI and SalI, a fragment containing the SV40 early promoter driving the EGFR cDNA followed by the SV40 splice junction and the SV40 polyadenylation signal was isolated. This DNA fragment was treated with Klenow enzyme in the presence of deoxynucleoside triphosphate to repair the 5' overhangs of XhoI and SalI. Plasmid pBSpacΔp was linearized with PvuII. pBSpacΔp contains the pac (puromycin resistance) cDNA under the control of the early SV40 promoter followed by the SV40 early promoter followed by thte SV40 polyadenylation signal and the ampicillin resistance gene. The two fragments were ligated to form pPEGFr. PKC expression plasmids (pSRDPKCα and pSRDPKCδ) were kind gifts from Dr. Shigeo Ohno, Yokohama City University, Japan. pSRD is a 3.4 Kb plasmid. PKC α was inserted at EcoRI restriction site and PKC δ was inserted at the EcoRI/PstI restriction site downstream of the SV40 promoter.

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

Comments for pCEP4:
10380 base pairs

SV40 Poly A signal. bases 7-405
Multiple Cloning Site. bases 406-463
CMV Promotor. bases 467-1311
TK Poly A signal: bases 1473-1643
Hygromycin gene. bases 1644-2693
TK Promotor: bases 2694-3136
Ampicillin resistance/pUC origin: bases 3635-5526
EBNA-1 gene. bases 5533-6113
Ori P. bases 8142-10078



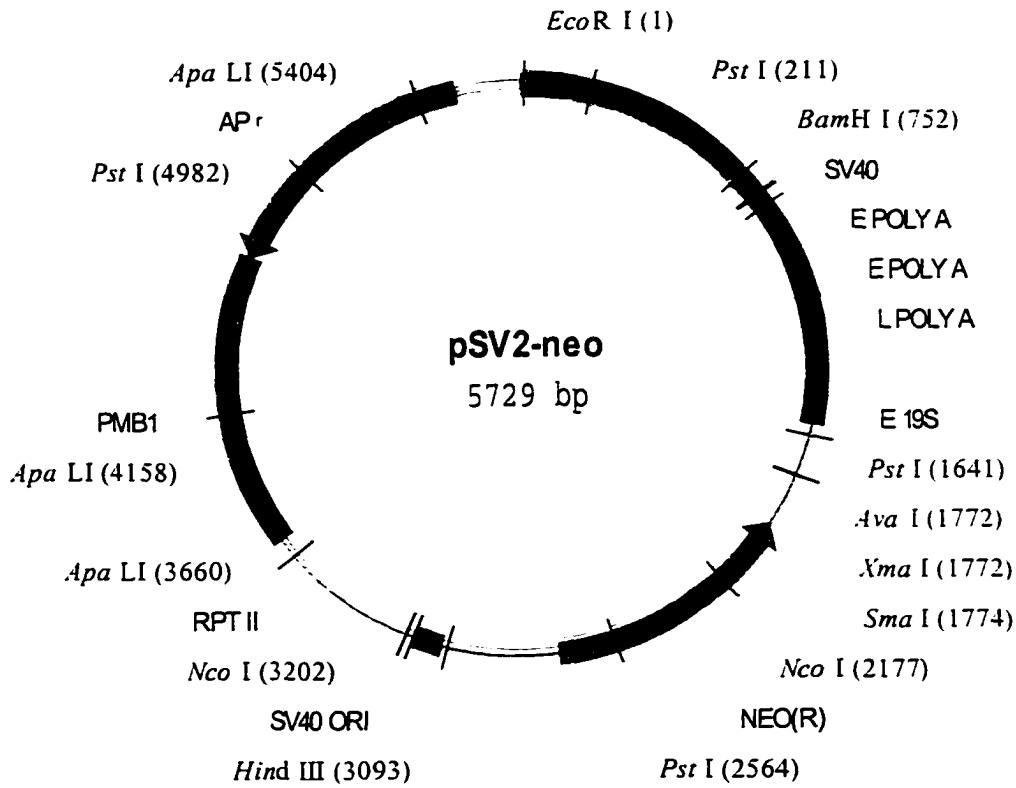
* Stu I is sensitive to dcm methylation. If you intend to digest the plasmid with Stu I, be sure that the cell line you are propagating the plasmid in is dcm-.

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

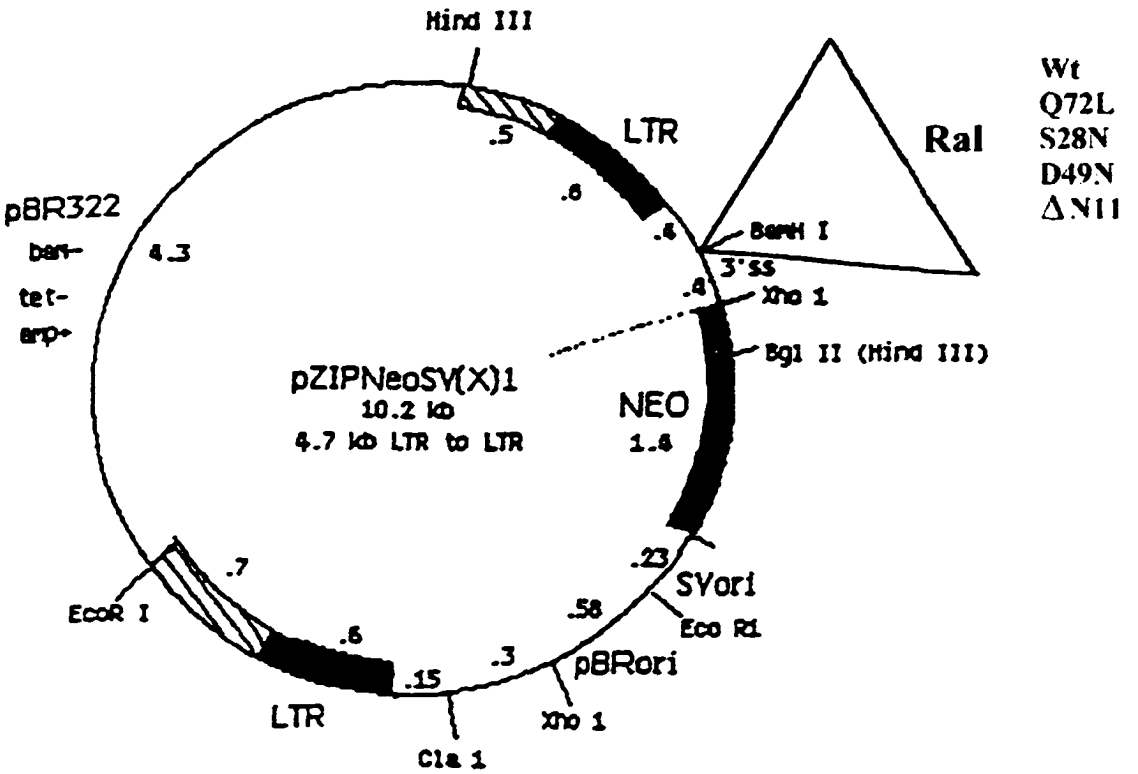
pSV2 neo Vector Information
5.7 kb

Restriction Map of pSV2neo. Unique restriction sites are in bold.

Description:
Selection vector that confers neomycin resistance to eukaryotic cells. The neomycin resistance gene of pSV2 neo can be used as a dominant selectable marker to select for stably transformed mammalian cells using neomycin (G418). pSV2 neo contains a Col E1 origin of replication and ampicillin resistance for propagation and selection, respectively, in E. coli.



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



Ral Transfection plasmid.

Origin of Fragments:

PBR322 – a pBr322 BAM derivative; from Hind III to R1
Left (5') LTR to BAM site – MLV sequences from pZIP clone of Hoffman, Tabin, and Weinberg; The BAM site was the old Pst I site at 1.0 mu of pZIP. S – the 3' splice site of MLV (from pZIP); This was the Bgl II to Xba I fragment from 5.9 6.3 mu.
NEO – from pBR NEO; was the Hind III to Bam fragment of Neo in pBR.
SVori – SV40 origin; RII to Hind III fragment of SV40 (160-5134 nucleotide numbers of SV40)
PBRori – fragment of pBR322 from 2521 to 3102 nucleotides (orientation uncertain) and nucleotides 1415 to 1424 of pBR (next to the Xho site which is drawn 3' to this fragment)
Xho I to the end of the 3' LTR – all original pZIP-MLV;Xho I site was the Hpa I site at 7.75 mu of pZIP
Hatched boxes – flanking mouse cell DNA sequences.

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

Western Analysis

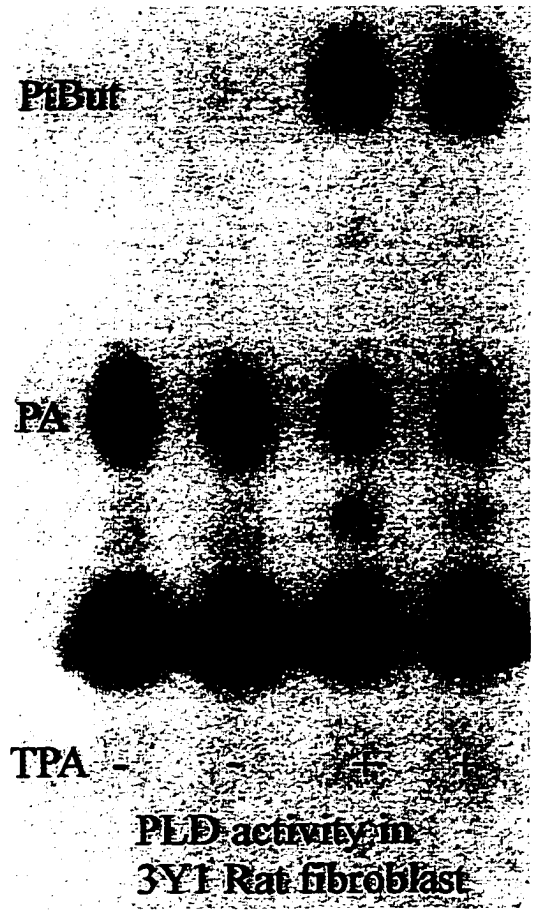
Extraction of proteins from cultured cells was performed as previously described (Lu et al.,1997;1998). Confluent monolayers in 100mm plates were washed three times with ice-cold phosphate buffered saline (PBS:136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4, pH 7.4) and scraped in hypotonic buffer (20 mM Hepes [pH 7.4], 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 5 mM iodoacetic acid, 10 ug/ml each of aprotinin, pepstatin, leupeptin). Protein content was determined by using the Biorad Dc protein assay (Biorad). Equal amounts of protein were subjected to SDS-PAGE using an 8% acrylamide separating gel, transferred to nitrocellulose and blocked overnight at 4°C with 5% non-fat dry milk isotonic phosphate buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4). The nitrocellulose filters were washed three times for five minutes in PBS and then incubated with antibodies. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG was used for detection using the ECL system (Amersham).

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

Phospholipase D Assay

Confluent 35 mm culture dishes were prelabeled for 4 h with [^{3}H]-Myristate, 3 μCi (40 Ci/mmol) in 3 ml of media containing 0.5% newborn calf serum. Phospholipase D (PLD) catalyzed transphosphatidylation in the presence of 1% butanol was performed as described previously (Song et al.,1991;Song & Foster,1993). Extraction and characterization of lipids by thin layer chromatography (TLC: Silica gel 60A, American Scientific Products) was performed as previously described (Song and Foster,1993). Lipids were extracted at 4°C with the addition of 0.65 ml $CHCl_3$. Phase separation was obtained by adding 200ul of 1M NaCl. The organic phase was re-extracted with 0.65 ml of 0.3 M NaCl and 0.2 ml of MeOH:6N HCl (50:1). Lipids were dried under a N_2 stream and redissolved in $CHCl_3$:MeOH (95:5). Samples were then spotted on TLC plates and placed in 100ml of solvent (Upper phase of ethylacetate:trimethylpentane:acetate:water {9:5:2:10} by volume) containing sealed glass tank for 90 minutes. TLC plates were then air dried for 30 minutes in the hood and film (Kodak) placed over the plastic wrapped plates. TLC plates were then exposed at -70°C (Revco) for three to seven days for autoradiography. Relative levels of trasphosphatidylation product phosphatidylbutanol were determined by densitometric analysis of the film using a laser scanning densitometer (Molecular Dynamics). Example of one of my TLC autoradiographs is provided. PtBut, Phosphatidylbutanol; PA, Phosphatidic acid; (T P A) Tetradecanoylphorbol acetate.

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



Sample TLC autoradiograph.

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

RalA Activation Assay.

The detection of activated RalA was performed as described by (Wolthuis et al. 1998a; 1998b). Cells were first lysed with 15% glycerol, 50mM Tris-HCl pH = 7.4, 1% NP40, 200 nM NaCl, 5mM MgCl2, 1mM PMSF, 10ug/ml leupeptin, 10ug/ml aprotinin, 10 ug/ml soybean trypsin inhibitor. The lysates were then treated with glutathione-S-transferase (GST)-Ral-BD fusion protein immobilized with glutathione-agarose beads. Ral-BD is the Ral binding domain of Ral-BP that binds exclusively to activated GTP-bound Ral proteins (Wolthuis et al., 1998a). The activated Ral proteins were then recovered by centrifugation and subjected to Western blot analysis using an antibody raised against RalA (Transduction Laboratories).

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

CHAPTER IV

References

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

Acs, P., Q.J. Wang, K. Bogi, A.M. Marquez, P.S. Lorenzo, T. Biro, Z. Szallasi, J.F. Mushinski, and P.M. Blumberg. 1997. Both the catalytic and regulatory domains of protein kinase C chimeras modulate the proliferative properties of NIH 3T3 cells. *J. Biol. Chem.* **272:**28793-28799.

Aguirre Ghiso, J., P. Frankel, Z. Lu, H. Jiang, E. Farías, A. Olsen, L.A. Feig, E. Bal de Kier Joffé, and D.A. Foster, D.A. 1998. RalA requirement for v-Src- and v-Ras-induced tumorigenicity and overproduction of urokinase-type plasminogen activator and metalloproteases. *Submitted for Publication.*

Allan D, Thomas P, Michell RH. 1978. Rapid transbilayer diffusion of 1,2-diacylglycerol and its relevance to control of membrane curvature. *Nature* Nov 16;276(5685):289-90

Anderson, R.G.W. 1998. The caveolae membrane system. Ann. Rev. Biochem.67:199-225.

Armuth V, Berenblum I. 1974. Promotion of mammary carcinogenesis and leukemogenic action by phorbol in virgin female Wistar rats. *Cancer Res* Oct;34(10):2704-7

Baron-Delage S, Cherqui G. 1997. [Protein kinase C and tumorigenic potential]. *Bull Cancer* Aug;84(8):829-32

Ben-Av P, Liscovitch M. 1989. Phospholipase D activation by the mitogens platelet-derived growth factor and 12-O-tetradecanoylphorbol 13-acetate in NIH-3T3 cells. *FEBS Lett* Dec 18;259(1):64-6

Bi, K., Roth, M.G., and Ktistakis, N.T. 1997. Phosphatidic acid formation by phospholipase D is required for transport from theendoplasmic reticulum to the Golgi complex. *Curr. Biol.* 7:301-307.

Biscardi J.S., A.P. Belsches, and S.J. Parsons. 1998. Characterization of human epidermal growth factor receptor and c-Src interactions in human breast tumor cells. *Mol. Carcinog.* 21:261-272.

Blobe GC, Obeid LM, Hannun YA. 1994. Regulation of protein kinase C and role in cancer biology. These studies highlight the importance and significance of regulation of PKC activity in vivo. *Cancer Metastasis Rev* Dec;13(3-4):411-31

Borner, C., M. Ueffing, S. Jaken, P.J. Parker, and I.B. Weinstein. 1995. Two closely related isoforms of protein kinase C produce reciprocal effects on the growth of rat fibroblasts. *J. Biol. Chem.* **270:**78-86.

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

Brown HA, Gutowski S, Moomaw CR, Slaughter C, Sternweis PC. 1993. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* Dec 17;75(6):1137-44

Cano E, Munoz-Fernandez MA, Fresno M. 1992. Regulation of interleukin-2 responses by phosphatidic acid. *Eur J Immunol* Jul;22(7):1883-9

Cantor, S.B., Urano, T., and Feig, L.A. 1995. Identification of Ral-binding protein 1, a potential downstream target of Ral GTPases. *Mol. Cell. Biol.* 15:4578-4584

Chalifa V, Mohn H, Liscovitch M. 1990. A neutral phospholipase D activity from rat brain synaptic plasma membranes. Identification and partial characterization. *J Biol Chem* Oct 15;265(29):17512-9

Chen, Y.-G., Siddhanta, A., Austin, C.D., Hammond, S.M., Sung, T.-C., Frohman, M.A., Morris, A.J., and Shields, D. 1997. Phospholipase D stimulates release of nascent secretory vesicles from the trans-Golgi network. *J. Cell Biol.* 138:495-504.

Collins, K., T. Jacks, and N.P. Pavletich. 1997. The cell cycle and cancer. *Proc. Natl. Acad. Sci.* USA **94:**2776-2778.

Coppola, D., A. Ferber, M. Miura, C. Sell, C. D'Ambrosio, R. Rubin, R. Baserga. 1994. A functional insulin-like growth factor I receptor is required for the mitogenic and transforming activities of the epidermal growth factor receptor. *Mol. Cell. Biol.* **14:**4588-4595.

Cowley GP, Smith JA, Gusterson BA. 1986. Increased EGF receptors on human squamous carcinoma cell lines. *Br J Cancer* Feb;53(2):223-9

Darnell, J., Lodish, H., and Baltimore, D. 1990. *Molecular Cell Biology.* 1285-1288.

Dingiovanni, J. 1992. Multistage carcinogenesis in mouse skin. *Pharmacol. Ther.* **54:**63-128.

Decker, S.J. 1984. Effects of epidermal growth factor and 12-O-tetradecanoylphorbol-13-acetate on metabolism of the epidermal growth factor receptor in normal human fibroblasts. *Mol. Cell. Biol.* **4:**1718-1724.

Denning, M.F., A.A. Dlugosz, M.K. Howett, and S.H. Yuspa. 1993. Expression of an oncogenic *ras*^{Ha} gene in murine keratinocytes induces tyrosine phosphorylation and reduced activity of protein kinase C δ. *J. Biol. Chem.* **268:**26079-26081.

Denning, M.F., A.A. Dlugosz, D.W. Threadgill, T. Magnuson, and S.H. Yuspa. 1996. Activation of the epidermal growth factor receptor signal transduction pathway stimulates tyrosine phosphorylation of protein kinase C δ. *J. Biol. Chem.* **271:**5325-5331.

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

Dickson, R.B., D.S. Salomon, and M.E. Lippman. 1992. Tyrosine kinase receptor-nuclear protooncogene interactions in breast cancer. *Cancer Treat. Res.* **61**:249-273.

Downward, J., M.D. Waterfield, and P.J. Parker. 1985. Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. Effect on tyrosine kinase activity and ligand binding affinity. *J. Biol. Chem.* **260**:14538-14546.

Engelman JA, Wykoff CC, Yasuhara S, Song KS, Okamoto T, Lisanti MP. 1997. Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *J Biol Chem* Jun 27;272(26):16374-81

Exton JH. 1990. Signaling through phosphatidylcholine breakdown. *J Biol Chem* Jan 5;265(1):1-4

Feig, L.A., and G.M. Cooper. 1988. Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell. Biol.* 8:3235-3243.

Feig, L.A., T. Urano, and S. Cantor. 1996. Evidence for a Ras/Ral signaling cascade. *Trends Biochem. Sci.* 21:438-441.

Fisher GJ, Henderson PA, Voorhees JJ, Baldassare JJ. 1991. Epidermal growth factor-induced hydrolysis of phosphatidylcholine by phospholipase D and phospholipase C in human dermal fibroblasts. *J Cell Physiol* Feb;146(2):309-17

Fishman DD, Segal S, Livneh E. 1998. The role of protein kinase C in G1 and G2/M phases of the cell cycle. *Int J Oncol* Jan;12(1):181-6

Foster, D.A. 1993. Intracellular signalling mediated by protein-tyrosine kinases: networking through phospholipid metabolism. *Cell. Signalling* **5**:389-399.

Friedman, B., A.R. Frackelton, A.H. Ross, J.M. Connors, H. Fujiki, T. Sugimora, and M.R. Rosner. 1984. Tumor promoters block tyrosine-specific phosphorylation of the epidermal growth factor receptor. Proc. Natl. Acad. Sci. USA. **81**:3034-3038.

Fukumoto, S., Y. Nishizawa, M. Hosoi, H. Koyama, K. Yamakawa, S. Ohno, and H. Morii. 1997. Protein kinase C δ inhibits the proliferation of vascular smooth muscle cells by suppressing G1 cyclin expression. *J. Biol. Chem.* **272**:13816-13822.

Galbiati F, Volonte D, Engelman JA, Watanabe G, Burk R, Pestell RG, Lisanti MP. 1998. Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *EMBO J* Nov 16;17(22):6633-6648

Goode, N.T., M.A. Hajibagheri, G. Warren, and P.J. Parker. 1994. Expression of mammalian protein kinase C in *Schizosaccharomyces pombe*: isotype-specific induction of growth arrest, vesicle formation, and endocytosis. *Mol. Biol. Cell.* **5**:907-920

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

Griffiths, G., B. Garrone, E. Deacon, P.Owen, J. Pongracz, G. Mead, A. Bradwell, D. Watters and J. Lord. 1996. The polyether bistratene A activates protein kinase C-δ and induces growth arrest in HL60 cells. *Biochem. Biophys. Res. Comm.* **222:**802-808

Gschwendt, M., H.-J. Muller, K. Kielbassa, R. Zang, W. Kittstein, G. Rincke, and F. Marks. 1994. Rottlerin, a novel protein kinase inhibitor. *Biochem. Biophys. Res. Comm.* **199:**63-98.

Hammond, S.M., Altshuller, Y.M., Sung, T.C., Rudge, S.A., Ross, K., Engebrecht. J., Morris, A.J., and Frohman, M.A. 1995. Human Arf-activated phosphatidylcholine-specific phospholipase D defines a new highly conserved gene family. *J. Biol. Chem.* 270:29640-29643.

Hofer, F., Fields, S., Schneider, C., and Martin, G.S. 1994. Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. *Proc. Natl. Acad. Sci.* USA 91:11089-11093.

Hirai, S., Y. Izumi, K. Higa, K. Kaibuchi, K. Mizuno, S. Osada, K. Suzuki, and S. Ohno. 1994. Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKC δ. *EMBO J.* **13:**2331-2340.

Hofer, F., Berdeaux, R., and Martin, G.S. 1998. Ras-independent activation of Ral by a Ca(2+)-dependent pathway. *Curr. Biol.* 8:839-842.

Howe, L.R., Leevers, S.J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C.J. 1992. Activation of the MAP kinase pathway by the protein kinase raf. *Cell* 71:335-342.

Jardines L, Weiss M, Fowble B, Greene M. 1993. neu(c-erbB-2/HER2) and the epidermal growth factor receptor (EGFR) in breast cancer. *Pathobiology*;61(5-6):268-82

Jiang, H., J.-Q. Luo, T. Urano, Z. Lu, D.A. Foster and L. Feig. 1995. Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature.* **378:**409-412.

Jiang H, Lu Z, Luo JQ, Wolfman A, Foster DA. 1995. Ras mediates the activation of phospholipase D by v-Src. *Biol Chem Mar* 17;270(11):6006-9

Kawase T, Suzuki A. 1988. Phosphatidic acid-induced calcium mobilization in osteoblasts. *J Biochem* Apr;103(4):581-2

Kikuchi, A., Demo, S.D., Ye, Z., Chen, Y., and Williams, L.T. 1994. ralGDS family members interact with the effector loop of ras p21. *Mol. Cell. Biol.* 14:7483-7491

Knauss TC, Jaffer FE, Abboud HE. 1990. Phosphatidic acid modulates DNA synthesis, phospholipase C, and platelet-derived growth factor mRNAs in cultured mesangial cells. Role of protein kinase C. *J Biol Chem* Aug 25;265(24):14457-63

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

Ktistakis, N.T., Brown, H.A., Waters, M.G., Sternweis, P.C., and Roth, M.G. 1996. Evidence that phospholipase D mediates ADP-ribosylation factor-dependent formation of coated vesicles. *J. Cell Biol.* 134:295-306.

Kusaka I. 1975. Degradation of phospholipid and release of diglyceride-rich membrane vesicles during protoplast formation in certain gram-positive bacteria. *J Bacteriol* Mar;121(3):1173-9

Lane MD, Slieker LJ, Olson TS, Martensen TM. 1987. Post-translational acquisition of ligand binding- and tyrosine kinase-domain function by the epidermal growth factor and insulin receptors. *J Recept Res*;7(1-4):321-54

Lange-Carter CA, and Johnson G. 1994. Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science* 265:1458-1461.

Lee SW, Reimer CL, Oh P, Campbell DB, Schnitzer JE. 1998. Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells. *Oncogene* Mar;16(11):1391-7

Li, W., Y.X. Jiang, J. Zhang, L. Soon, L. Flechner, V. Kapoor, J.H. Pierce, and L.H. Wang. 1998. Protein kinase C-delta is an important signaling molecule in insulin-like growth factor I receptor-mediated cell transformation. *Mol. Cell. Biol.* 18:5888- 5898

Logan, S.K., Falasca, M., Hu, P., and Schlessinger, J. 1997. Phosphatidylinositol 3-kinase mediates epidermal growth factor-induced activation of the c-Jun N-terminal kinase signaling pathway. *Mol. Cell. Biol.* 17:5784-5790.

Lu, Z., A. Hornia, Y.-W. Jiang, P. Frankel, Q. Zang, and D.A. Foster. 1997. Tumor-promotion by depleting cells of protein kinase C δ. *Mol. Cell Biol.* **17**:3418-3428.

Lu, Z., D. Liu, A. Hornia, W. Devonish, M. Pagano, and D.A. Foster. 1998. Activation of protein kinase C triggers its ubiquitination and degradation. *Mol. Cell. Biol.* **18**;839-845.

Luo, J.-Q., X. Liu, S.M. Hammond, W.C. Colley, L.A. Feig, M.A. Frohman, A.J. Morris, and D.A. Foster. 1997. Ral interacts directly with the Arf-responsive PIP2-dependent phospholipase D1. *Biochem. Biophys. Res. Comm.* **235**:854-859.

Luo, J.-Q., X. Liu, P. Frankel, T. Rotunda, M. Ramos, J. Flom, H. Jiang, L.A. Feig, A.J. Morris, R.A. Kahn, and D.A. Foster. 1998. Functional association between RalA and Arf in active phospholipase D complexes. *Proc. Natl. Acad. Sci.* USA. **95**:3632-3637.

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

Luttrell, D.K., A. Lee, T.J. Lansing, R.M. Crosby, K.D. Jung, D. Willard, M. Luther, M. Rodriguez, J. Berman, and T.M. Gilmer. 1994. Involvement of pp60$^{c\text{-}src}$ with two major signaling pathways in human breast cancer. *Proc. Natl. Acad. Sci.* USA **91**:83-87.

Mao W, Irby R, Coppola D, Fu L, Wloch M, Turner J, Yu H, Garcia R, Jove R, Yeatman TJ 1997. Activation of c-Src by receptor tyrosine kinases in human colon cancer cells with high metastatic potential. *Oncogene* 1997 Dec 18; 1 5(25):3083-3090

Martiny-Baron, G., M.G. Kazanietz, H. Mischak, P.M. Blumberg, G. Kochs, H. Hug, D. Marme, and C. Schlachtele. 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole G0 6976. *J. Biol. Chem.* **268**:9194-9197.

Marshall, C.J. 1996. Ras effectors. Curr. Opin. Cell. Biol. 8:197-204. Okamoto, T., Schlegel, A., Sherer, P., Lisanti, M.P. 1998. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J. Biol. Chem.* 273:5419-5422.

McCaffrey, P.G., B. Friedman, and M.R. Rosner. 1984. Diacylglycerol modulates binding and phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* **259**:12502-12507.

McNamara P.J. 1995.Toxic phospholipases D of Corynebacterium pseudotuberculosis, C. ulcerans and Arcanobacterium haemolyticum: cloning and sequence homology. *Gene.* Apr 14;156(1):113-8.

Mellor H, Parker PJ. 1998. The extended protein kinase C superfamily. *Biochem J* Jun 1;332 (Pt 2):281-92

Mischak, H., J. Goodnight, W. Kolch, G. Martiny-Baron, C. Schaechtle, M.G. Kazanietz, P.M. Blumberg, J.H. Pierce, and J.F. Mushinski. 1993. Overexpression of protein kinase C-δ and -ε in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. *J. Biol. Chem.*, **268**:6090-6096.

Moolenaar WH, Kruijer W, Tilly BC, Verlaan I, Bierman AJ, de Laat SW. 1986. Growth factor-like action of phosphatidic acid. *Nature* Sep 11-17;323(6084):171-3

Moolenaar WH. 1991. Mitogenic action of lysophosphatidic acid. *Adv Cancer Res*;57:87-102

Murayama T, Ui M. 1987. Phosphatidic acid may stimulate membrane receptors mediating adenylate cyclase inhibition and phospholipid breakdown in 3T3 fibroblasts. *J Biol Chem* 25;262(12):5522-9

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

Nakanishi H, Brewer KA, Exton JH. 1993. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* Jan 5;268(1):13-6

Nishizuka Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* Apr 19-25;308(5961):693-8

Nishizuka Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* Oct 23;258(5082):607-14

Nishizuka Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* Apr;9(7):484-96

Ohguchi K, Banno Y, Nakashima S, Nozawa Y. 1995. Activation of membrane-bound phospholipase D by protein kinase C in HL60 cells: synergistic action of a small GTP-binding protein RhoA. *Biochem Biophys Res Commun* Jun 6;211(1):306-11

Ohmichi M, Decker SJ, Pang L, Saltiel AR. 1991. Nerve growth factor binds to the 140 kd trk proto-oncogene product and stimulates its association with the src homology domain of phospholipase C gamma 1. *Biochem Biophys Res Commun* Aug 30;179(1):217-23

Ohno S, Akita Y. 1991. Structural and functional diversities of a family of signal transducing protein kinases, protein kinase C family;two distinct classes of PKC, conventional cPKC and novel nPKC. *Adv Enzyme Regul*;31:287-303

Ohsako S, Deguchi T. 1981. Stimulation of phosphatidic acid of calcium influx and cyclic GMP synthesis in neuroblastoma cells. *J Biol Chem* Nov 10;256(21):10945-8

Oka N, Yamamoto M, Schwencke C, Kawabe J, Ebina T, Ohno S, Couet J, Lisanti MP, Ishikawa Y. 1997. Caveolin interaction with protein kinase C. Isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide. *J Biol Chem* Dec 26;272(52):33416-21

Osada S, Mizuno K. 1990. A phorbol ester receptor/protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin. *J Biol Chem* Dec 25;265(36):22434-40

Ottenholf-Kalff, A.E., Rijksen, G., van Beurden, E.A.C.M., Hennipman, A., Michels, A., and Stall, G.E.J. 1992. Characterization of protein-tyrosine kinases from human breast cancer: involvement of the c-src oncogene product. *Cancer Res.* 52:4773-4778.

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

Plevin R, Cook SJ, Palmer S, Wakelam MJ. 1991. Multiple sources of sn-1,2-diacylglycerol in platelet-derived-growth-factor-stimulated Swiss 3T3 fibroblasts. Evidence for activation of phosphoinositidase C and phosphatidylcholine-specific phospholipase D. *Biochem J* Oct 15;279 (Pt 2):559-65

Qureshi, S.A., C.K. Joseph, R. Gupta, M. Hendrickson, J. Song, J. Bruder, U.R. Rapp, and D.A. Foster. 1993. A dominant-negative Raf-1 mutant prevents v-Src-induced transformation. *Biochem. Biophys. Res. Comm.* **192:**969-975.

Reese, D.M., and Slamon, D.J. 1997. HER-2/neu signal transduction in human breast and ovarian cancer. *Stem Cells* 15:1-8.

Sahyoun N, Wolf M, Besterman J, Hsieh T, Sander M, LeVine H 3d, Chang KJ, Cuatrecasas P. 1986. Protein kinase C phosphorylates topoisomerase II: topoisomerase activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. *Proc Natl Acad Sci* U S A Mar;83(6):1603-7

Sasaoka T, Langlois WJ, Leitner JW, Draznin B, Olefsky JM. 1994. The signaling pathway coupling epidermal growth factor receptors to activation of p21ras. *J Biol Chem* Dec 23;269(51):32621-5

Schaap, D., van der Wal, J., Howe, L.R., Marshall, C.J., and van Blitterswijk, W.J. 1993. A dominant-negative mutant of raf blocks mitogen-activated protein kinase activation by growth factors and oncogenic p21ras. *J. Biol. Chem.* 268:20232-20236.

Schmidt M, Voss M, Thiel M, Bauer B, Grannass A, Tapp E, Cool RH, de Gunzburg J, von Eichel-Streiber C, Jakobs KH. 1998. Specific inhibition of phorbol ester-stimulated phospholipase D by Clostridium sordellii lethal toxin and Clostridium difficile toxin B-1470 in HEK-293 cells. Restoration by Ral GTPases. *J Biol Chem* Mar 27;273(13):7413-22

Sherr, C.J. 1996. Cancer cell cycles. *Science* **274:**1672-1677.

Singer, W.D., H.A. Brown, X. Jiang, and P.C. Sternweis. 1996. Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase actiivity. *J. Biol. Chem.* **271:**4504-4510.

Song, J., L.M. Pfeffer, and D.A. Foster. 1991. v-Src Increases Diacylglycerol Levels via a Type D Phospholipase-mediated hydrolysis of Phosphatidylcholine. *Mol. Cell. Biol.* **11:**4903-4908.

Song, J., and D.A. Foster. 1993. v-Src activates a phospholipase D activity that is distinguishable from phospholipase D activity activated by protein kinase C. *Biochem. J.* **294:**711-717.

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

Song, J., Y.-W. Jiang, and D.A. Foster. 1994. EGF induces the production of biologically distinguishable diglyceride species from phosphatidylinositol and phosphatidylcholine: evidence for the independent activation of type C and type D phospholipases. *Cell Growth and Differ.* **5**:79-85.

Spaargaren, M., and Bischoff, J.R. 1994. Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras and Rap. *Proc. Natl. Acad. Sci.* USA 91:12609-12613.

Spiegel, S., Foster, D.A., and Kolesnick, R. 1996. Signal transduction through lipid second messengers. *Curr. Op. Cell. Biol.* 8:159-167.

Stamnes, M., G. Schiavo, G. Steinbeck, T.H. Sollner, and J.E. Rothman. 1998. ADPribosylation factor and phosphatidic acid levels in Golgi membranes during budding of cotamer-coated vesicles. *Proc. Natl. Acad. Sci.* USA 95:13676-13680.

Stiles, C.D., G.T. Capone, C.D. Scher, H.N. Antoniades, J.J. Van Wyk, and W.J. Pledger. 1979. Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proc. Natl. Acad. Sci.* U S A **76:**1279-1283.

Taki T, Kanfer JN. 1979. Partial purification and properties of a rat brain phospholipase. *J Biol Chem* Oct 10;254(19):9761-5

Thompson NT, Garland LG, Bonser RW. 1993. Phospholipase D: regulation and functional significance. *Adv Pharmacol*;24:199-238

Ueda, Y., S. Hirai, S. Osada, A. Suzuku, K. Mizuno, and S. Ohno. 1996. Protein kinase C δ activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. *J. Biol. Chem.* **271:**23512-23519.

Urano, T., R. Emkey, and L.A. Feig. 1996. Ral GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. *EMBO J.* **16:**810-816.

Van Corven EJ, van Rijswijk A, Jalink K, van der Bend RL, van Blitterswijk WJ, Moolenaar WH. 1992. Mitogenic action of lysophosphatidic acid and phosphatidic acid on fibroblasts. Dependence on acyl-chain length and inhibition by suramin. *Biochem J* Jan 1;281 (Pt 1):163-9

Waksman M, Eli Y, Liscovitch M, Gerst JE. 1996. Identification and characterization of a gene encoding phospholipase D activity in yeast. *J Biol Chem* Feb 2;271(5):2361-4

Walker, F., A. Kato, L.J. Gonez, M.L. Hibbs, N. Pouliot, A. Levitzki, and A.W. Burgess. 1998. Activation of the Ras/mitogen-activated protein kinase pathway by kinase-defective epidermal growth factor receptors results in cell survuval but not proliferation. *Mol. Cell. Biol.* 18:7192-7204.

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

Wang P, Anthes JC, Siegel MI, Egan RW, Billah MM. 1991. Existence of cytosolic phospholipase D. Identification and comparison with membrane-bound enzyme. *J Biol Chem* Aug 15;266(23):14877-80

Watanabe, T., Y. Ono, Y. Taniyama, K. Hazama, K. Igarashi, K. Ogita, U. Kikkawa, and Y. Nishizuka. 1992. Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C-δ subspecies. *Proc. Natl. Acad. Sci.* USA **89:**10159-10163.

Wiesmuller L, Wittinghofer F. 1994. Signal transduction pathways involving Ras. Mini review. *Cell Signal* Mar;6(3):247-67

Wolthuis, R.M.F, F. Zwartkruis, T.C. Moen, and J. L. Bos. 1998. (a) Ras-dependent activation of the small GTPase Ral. *Curr. Biol.* 8:471-474.

Wolthuis, R.M.F., B. Franke, M. van Triest, B. Bauer, R.H. Cool, J.H. Camonis, J.W. Akkerman, and J. L. Bos. 1998. (b) Activation of the small GTPase Ral in platelets. *Mol. Cell. Biol.* 18: 2486-2491.

Yeo, E.J., and J.H. Exton. 1995. Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells. *J. Biol. Chem.* **270:**3980-3988

Yokozeki T, Homma K, Kuroda S, Kikkawa U, Ohno S, Takahashi M, Imahori K, Kanaho Y. 1998. Phosphatidic acid-dependent phosphorylation of a 29-kDa protein by protein kinase C alpha in bovine brain cytosol. *J Neurochem* Jul;71(1):410-17

Yuspa, S.H., and M.C. Poirier. 1988. Chemical carcinogenesis: from animal models to molecular models in one decade. *Adv. Cancer. Res.* **50:**25-70.

Yuspa, S.H., A.A. Dlugosz, C.K. Cheng, M.F. Denning, T. Tennenbaum, A.B. Glick, and W.C. Weinberg. 1994. Role of oncogenes and tumor suppressor genes in multistage carcinogenesis. *J. Invest. Dermatol.* **103:**90S-95S

Yuspa, S.H. 1998. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. *J. Dermatol. Sci.* **17:**1-7.

Zachos G, Spandidos DA. 1997. Expression of ras proto-oncogenes: regulation and implications in the development of human tumors. *Crit Rev Oncol Hematol* Jul;26(2):65-75

Zang, Q., P. Frankel, and D.A. Foster. 1995. Selective activation of protein kinase C isoforms by v-Src. *Cell Growth Differ.* **6:**1367-1373.

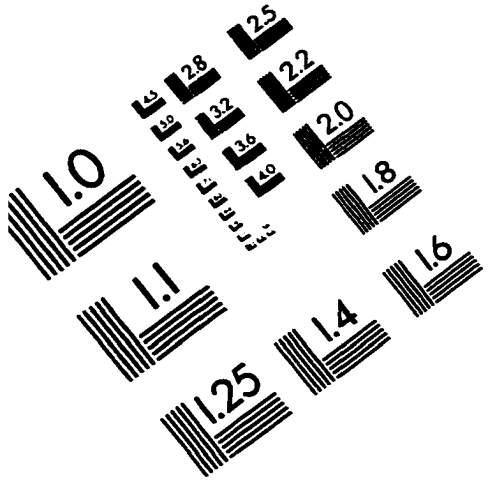
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

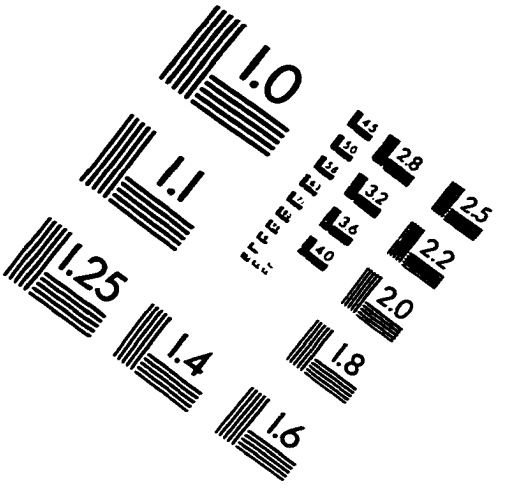
Zang, Q., Z. Lu, M. Curto, N. Barile, D. Shalloway, and D.A. Foster. 1997. Interaction between v-Src and protein kinase C δ in v-Src-transformed fibroblasts. *J. Biol. Chem.* **272:**13275-13280.

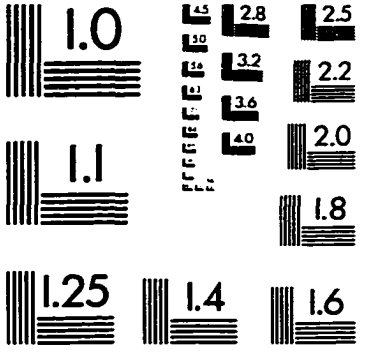
Zhang Y, Akhtar RA. 1998. Epidermal growth factor stimulates phospholipase D independent of phospholipase C, protein kinase C or phosphatidylinositol-3 kinase activation in immortalized rabbit corneal epithelial cells. *Curr Eye Res* Mar;17(3):294-300

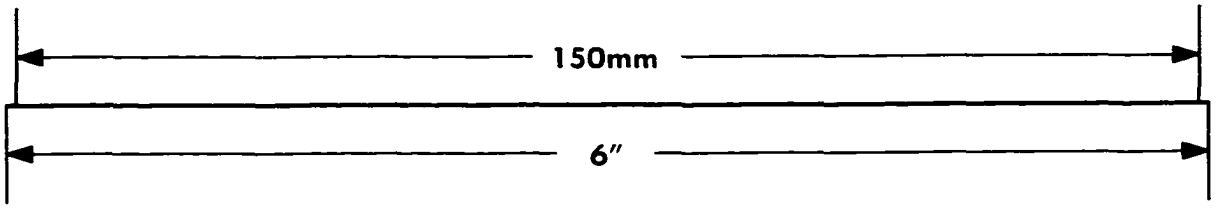
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

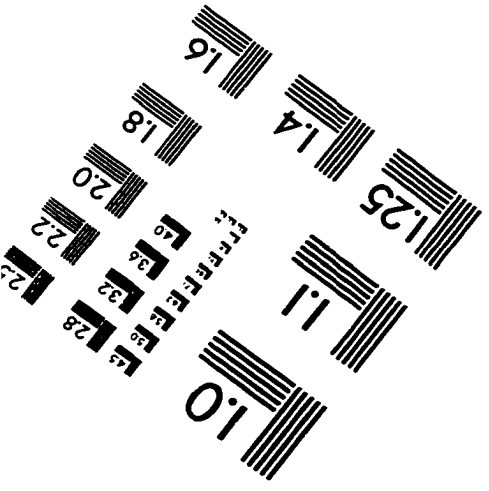
IMAGE EVALUATION
TEST TARGET (QA-3)





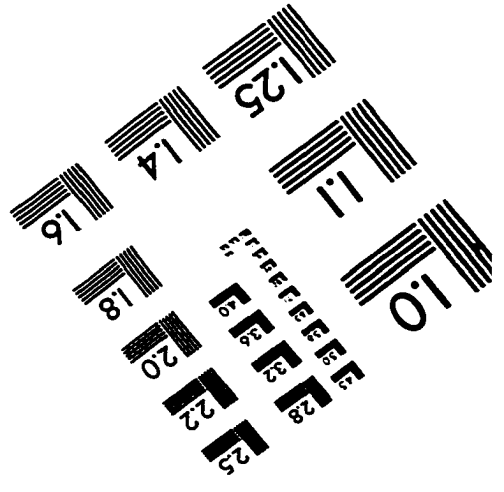






APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved



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