Regulation of Phospholipase C-epsilon by Rho and Ras Family Proteins

Jason Paul Seifert

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology.

Chapel Hill
2007

Approved by:

Advisor: Professor T. Kendall Harden

Reader: Professor John E. Sondek

Reader: Professor Keith Burridge

Reader: Professor Lee M. Graves

Reader: Professor Robert A. Nicholas

©2007
Jason Paul Seifert
ALL RIGHTS RESERVED

ABSTRACT

Jason Paul Seifert
Regulation of Phospholipase C-epsilon by Rho and Ras Family Proteins
(Under the direction of Dr. T. Kendall Harden)

Inositol phospholipids are a unique collection of signaling molecules involved in an extensive variety of signaling events including actin remodeling, membrane trafficking, and calcium mobilization. Phosphoinositides regulate signaling responses by binding specific effector proteins, or through the generation of second messenger molecules. The most well characterized phosphoinositide signaling pathway is the hydrolysis of phosphatidylinositol (4,5)-bisphosphate by the phospholipase C (PLC) family of enzymes. PLCs cleaves the inositol headgroup from the glycerol backbone of PtdIns(4,5)P_2, generating the second messenger molecules inositol (1,4,5)-trisphosphate and diacylglycerol. An extensive array of extracellular signaling molecules elicit their physiological effects through receptor-mediated stimulation of PLC isozymes. All PLC enzymes contain a conserved catalytic region; however, elaboration of the catalytic core with various protein binding and/or regulatory domains gives rise to multiple modes of regulation and different PLC subfamilies. Recent work has implicated Ras superfamily GTPases in the regulation of several PLC isoforms adding a new level of complexity to phosphoinositide metabolism.

Phospholipase C-epsilon (PLC-ε) is an elaborate PLC isoform that is regulated by several signaling pathways, including downstream of both heterotrimeric and Ras superfamily GTPases. The work described herein examines the regulation of PLC-ε

enzyme activity through direct interactions with Ras superfamily GTPases. We describe the purification and functional characterization of a catalytically active fragment of PLC-ε with the goal of providing incontrovertible evidence regarding direct Rho and Ras family regulation of enzyme activity. Reconstitution of purified proteins in model phospholipid vesicles demonstrates RhoA and K-Ras individually are sufficient to increase the activity of PLC-ε in both a concentration- and GTPγS-dependent manner. Moreover, RhoA and K-Ras required mutually exclusive sites of interaction to confer increased PLC-ε activity. Finally, when maximally activated by RhoA or K-Ras, PLC-ε activity is increased further by the addition of the complementary GTPase, and the enzyme displayed increased sensitivity to K-Ras in the presence of a maximally activating concentration of RhoA. These results identify PLC-ε as a novel enzyme that is regulated by Rho and Ras family GTPases through distinct domains, leading to individual and dual regulation of enzyme activity.

To my parents, Greg and Marcia, who have given me every opportunity to succeed, have supported every decision I have made, and for whom without, I would have never made it this far. And, to my brother, Joe, who continues to be a positive influence and source of inspiration in everything I have undertaken throughout my life.

ACKNOWLEDGEMENTS

First, and foremost, I would like to thank my dissertation advisor, Dr. T. Kendall Harden. I have learned so much throughout my five years in Ken's lab, most of which extends well beyond the lab bench. I believe the most successful scientists have a unique knowledge regarding the ability to ask the right questions, and in the end, do so regardless of notoriety or applause. These are characteristics exemplified by Ken which I will hopefully utilize throughout my professional career. Additionally, I would like to thank members of my dissertation committee, Dr. John Sondek, Dr. Rob Nicholas, Dr. Lee Graves, and Dr. Keith Burridge for their guidance throughout my graduate student career.

I would also like to thank past and present members of the Harden lab who have made contributions to my education. I would especially like to thank Dr. Erik Bodor for his guidance during my rotation project and long-lasting friendship, and Dr. Dayle Houston for always listening and offering advice. There are so many people to thank that won't get the recognition they deserve here, but I am fully aware that most people don't make it too far in life without the support of their friends and family. So to all of those people who are not mentioned here, I extend my deepest thanks. I would also like to thank my family, especially my parents Greg and Marcia, who have given me every opportunity to be where I am today.

Finally, I would like to extend my thanks to Dr. Hannah Carey at the University of Wisconsin, and Mary Doers at Promega, for my first opportunities to work in the scientific community.

Work presented within the body of this dissertation was originally published elsewhere.

Chapter 2:

Seifert JP, Snyder JT, Sondek J, Harden TK. Direct activation of purified phospholipase C epsilon by RhoA studied in reconstituted phospholipid vesicles. *Methods Enzymol.* 2006; **406**:260-271.

Chapter 3:

Seifert JP, Wing MR, Snyder JT, Gershburg S, Sondek J, Harden TK. RhoA activates purified phospholipase C-epsilon by a guanine nucleotide-dependent mechanism. *J.Biol.Chem.* 2004; **279**:47992-47997.

TABLE OF CONTENTS

LIST OF FIGURES……..xii

LIST OF TABLES…… ..xiii

LIST OF ABBREVIATIONS……..xiv

Chapter

1. INTRODUCTION..... 1

1.1 The Basis of Cell Signaling..... 1

1.2 Inositol Phospholipids..... 1

1.2.1 Structure and Metabolism of Inositol Phospholipids 2

1.3 Phosphoinositide Binding Domains..... 3

1.3.1 PtdIns(4,5)P_2 Binding Domains 4

PH Domains 4

ENTH and ANTH Domains 4

1.3.2 Monophosphate PtdIns Binding Domains..... 5

1.3.3 Other Phosphoinositide Binding Domains 6

1.3.4 PtdIns(4,5)P_2 and Disease..... 6

1.4 Phosphoinositide-Specific Phospholipase C 7

1.4.1 Catalytic Function and Structure of Conserved PLC Domains 9

Catalytic X and Y Box..... 9

X-Y Linker Region 11

PH Domains 12

EF-Hand Repeats 13

C2 Domains 13

1.4.2 Phospholipase C Subfamilies and Regulation 14

Phospholipase C-δ 14

Phospholipase C-β 16

Phospholipase C-γ 18

Phospholipase C-ζ 20

Phospholipase C-η 21

Phospholipase C-ε 22

1.5 Phospholipase C-ε 22

1.5.1 Identification and Cloning 22

1.5.2 Unique Structural and Regulatory Domains 23

RA Domains 23

CDC25 Domain 26

1.5.3 Regulation 27

Ras Family GTPases 27

Heterotrimeric G Proteins 28

Rho Family GTPases 29

Receptor Regulated Activation 30

1.5.4 Physiological Function of PLC-ε 32

1.6 Guanine nucleotide binding proteins 35

1.6.1 Ras Superfamily GTPases 36

Ras GTPases 37

Rho GTPases 39

Rab, Ran, and Arf GTPases 42

1.6.2 Heterotrimeric G proteins 42

1.7 Research Goals 44

2. DIRECT ACTIVATION OF PURIFIED PHOPHOLIPASE C EPSILON BY RHOA STUDIED IN RECONSTITUTED PHOSPHOLIPID VESICLES 51

2.1 Abstract 51

2.2 Introduction 51

2.3 Materials and Methods 53

2.4 Acknowledgements 62

3. RHOA ACTIVATES PURIFED PHOSPHOLIPASE C-EPSILON BY A GUANINE NUCLEOTIDE-DEPENDENT MECHANISM 64

3.1 Abstract 64

3.2 Introduction 65

3.3 Materials and Methods 66

3.4 Results 72

3.5 Discussion 76

3.6 Acknowledgements 78

4. DIRECT, INDEPENDENT, AND DUAL REGULATION OF A RHO AND RAS REGULATED PHOSPHOLIPASE EFFECTOR 85

4.1 Abstract 85

4.2 Introduction 86

4.3 Materials and Methods 88

4.4 Results 93

4.5 Discussion 98

5. CONCLUSIONS AND FUTURE DIRECTIONS..... 111

REFERENCES……....119

LIST OF FIGURES

Figure

1.1 Synthesis and metabolism of phosphoinositides....46

1.2 Domain organization of mammalian phospholipase C (PLC) family members....47

1.3 PLC-ε links heterotrimeric and small G protein signaling pathway in the generation of second messengers....48

1.4 Model of regulated nucleotide cycling of G proteins....49

2.1 Purification of PLC-ε EF-RA2 with metal chelate and size exclusion chromatography....63

3.1 Purification and functional activity of PLC-ε EF-RA2....79

3.2 Characterization of the phospholipase activity of PLC-ε EF-RA2....80

3.3 Activation of PLC-ε by RhoA....81

3.4 Stimulation of PLC isozymes by small GTPases....82

3.5 Loss of Rho-dependent regulation in a PLC-ε fragment lacking a conserved insert within the Y box....83

3.6 Association of purified RhoA with sucrose-loaded phospholipids vesicles....84

4.1 RhoA and H-Ras activation of PLC-ε requires individual domains....102

4.2 Dual activation of PLC-ε in cells overexpressing RhoA and H-Ras....103

4.3 Specificity of G protein regulation of PLC isozymes....104

4.4 Direct *in vitro* activation of PLC-ε by RhoA and K-Ras through distinct domains....105

4.5 Time course of PtdIns(4,5)P_2 hydrolysis by purified PLC-ε....106

4.6 Direct, dual and independent regulation of PLC-ε by purified RhoA and K-Ras....107

LIST OF TABLES

Table

1.1 Tissue distribution and function of mammalian PLC isoforms 50

5.1 RhoA and K-Ras promoted activation of PLC-ε 108

LIST OF ABBREVIATIONS AND SYMBOLS

ADP	Adenosine 5'-diphosphate
ANTH	AP180 N-terminal homology
Ala or A	Alanine
Arg or R	Arginine
Aps or D	Aspartic acid
Asp or N	Asparagine
BSA	Bovine serum albumin
C or Cys	Cystine
cAMP	Adenosine 3',5'-cyclic monophosphate
CDC25	Cell division cycle protein 25-like domain
CDP	Cytidine 5'-diphosphate
cDNA	Complementary deoxyribonucleic acid
CRIB	Cdc42/Rac-interactive binding
CV	Column volumn
DAG	Diacylglycerol
DH	Dbl homology
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ENTH	Epsin 1 N-terminal homology

EPAC	Exchange protein activated by cAMP
FBS	Fetal bovine serium
FERM	Ezrin/radixin/moesin
FYVE	Fab1p, YOTP, Vac1p, and EEA1
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine 5'-diphosphate
GEF	Guanine nucleotide exchange factor
Glu or E	Glutamic Acid
GPCR	G protein-coupled receptor
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
HA	Hemagglutinin
His or H	Histidine
Ins(1,4,5)P3	Inositol (1,4,5)trisphosphate
IP	Inositol phosphate
kDa	Kilodaltons
LARG	Leukemia-associated RhoGEF
Leu or L	Leucine
LPA	Lysophophatidic acid
Lys or K	Lysine
MAPK	Mitogen activated protein kinase

MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
Ni-NTA	Nickel-nitriloacetic acid
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PES	Polyethersulphone
PH	Pleckstrin homology
Phe or F	Phenylalanine
PI or PtdIns	Phosphatidylinositol
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PtdIns(4)P	Phosphatidylinositol (4)phosphate
PtdIns(4,5)P2	Phosphatidylinositol (4,5)bisphosphate
PTEN	Phosphatase and TENsin homologue deleted on chromosome TEN
PTX	Pertussis toxin

PX	Phox homology
RA	Ras-associtating/-association
RBD	Rho-binding domain
RGS	Regulator of G protein signaling
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser or S	Serine
SH2	Src homology 2
SH3	Src homology 3
SHIP	SH2-containing inositol 5'-phosphatase
SPR	Surface plasmon resonance
TEV	Tobacco etch protease
Thr or T	Threonine
TIM	Triose.phosphate isomerase
Tyr or Y	Tyrosine
β-ME	Beta-mercaptoethanol

CHAPTER 1

INTRODUCTION

1.1 The Basis of Cell Signaling

The essential function of any cell, whether a single-celled organism or a member of a multicellular organism, is to increase the chances of overall survival by adapting to environmental changes. Throughout evolution, innumerable mechanisms have arisen whereby cells recognize extracellular chemicals via cell surface "detectors", which transduce information across the membrane and initiate intracellular signaling cascades. In mammals, the most extensively characterized signaling pathways involve the release of hormones, neurotransmitters, growth factors, and other signaling molecules, leading to the activation of transmembrane receptors and subsequent downstream alterations in cellular processes. Whereas signal transduction pathways were once believed to be relatively simple and linear in nature, the discovery of signaling networks has altered this classical view of cell signaling. Crosstalk between virtually all signaling pathways results in complex mechanisms regulating the homeostasis of nearly every cell within an organism.

1.2 Inositol Phospholipids

A family of inositol-containing glycerophospholipids, the inositol phospholipids (PIs), make up a diverse subset of signaling molecules involved in a wide variety of signaling events. Eight naturally occurring inositol phospholipids have been identified, seven of which are phosphorylated in varying combinations at one or more of the free hydroxyl groups of the

inositol ring. Inositol phospholipids elicit signaling responses through the generation of second messenger molecules or by binding specific effector proteins, which in turn control several signaling events including actin remodeling, membrane trafficking, and ion channel function. Inositol phospholipid metabolism is controlled by a family of PI-kinases and PI-phosphatases, as well as hydrolysis of phosphoinositides by phospholipase C enzymes.

1.2.1 Structure and Metabolism of Inositol Phospholipids

Inositol phospholipids are comprised of a diacylglycerol (DAG) lipid moiety and an exposed inositol headgroup (Figure 1.1). The fatty acid chains of the DAG lipid moiety mediate the localization of PIs to various cellular membranes, whereas the polar headgroup is exposed and recognized by distinct phosphatidylinositol-specific binding proteins or phospholipase enzymes. Phosphatidylinositol synthase attaches D-*myo*-inositol to CDP-DAG through a di-ester phosphate bond at the D-1 position of the inositol ring [1]. The end product of the synthesis reaction is phosphatidylinositol (PtdIns), the building block of all inositol phospholipids. Attachment of DAG at the 6-position of the D-*myo*-inositol ring leaves five free hydroxyl groups, three of which are progressively phosphorylated at the 3-, 4-, and/or 5-positions, giving rise to seven naturally occurring phosphoinositides.

Lipid kinases recognize and phosphorylate PtdIns by catalyzing the transfer of a γ-phosphate from ATP to specific locations on the inositol headgroup of PtdIns. The nomenclature of phosphatidylinositol kinases and phosphatidylinositol phosphate kinases is derived from the position on the inositol ring phosphorylated by a specific kinase. For example, PI 3-K phosphorylates the inositol ring at the 3-position of PtdIns to form PtdIns(3)P, whereas PI(4)P 5-K recognizes PtdIns(4)P and phosphorylates the 5-hydroxyl group to form PtdIns(4,5)P_2 [2]. The action of lipid kinases is antagonized by two major

classes of inositol lipid phosphatases, which remove phosphate groups from specific positions on the inositol ring. PTEN (Phosphatase and TENsin homologue deleted on chromosome TEN) and SHIP (SH2-containing Inositol Phophatase) are inositol lipid phosphatases with 3- and 5-position phosphatase activity, respectively [3;4].

Through various combinations of PtdIns phosphorylation and dephosphorylation, lipid kinases and phosphatases create seven species of phosphoinositides. PtdIns is the most abundant inositol lipid in mammalian cells, with levels 10-20 times greater than PtdIns(4)P or PtdIns(4,5)P_2. PtdIns(4)P represents approximately 95% of the total singly phosphorylated PtdIns in cells, with PtdIns(3)P and PtdIns(5)P representing the additional 5% in roughly equal proportions. PtdIns(4,5)P_2 constitutes more than 99% of doubly phosphorylated PtdIns in cells, and represents about 1% of total phospholipids in resting cells. The other two PtdInsP_2 species, PtdIns(3,5)P_2 and PtdIns(3,4)P_2, each constitute about 0.2% of doubly phosphorylated lipids. The only trisphosphoinositide, PtdIns(3,4,5)P_3, exists in the cells at varying concentrations; however, levels are typically low, in a similar range of PtdIns(3,4)P_2 and PtdIns(3,5)P_2 (lipid levels reviewed [4-6]).

1.3 Phosphoinositide Binding Domains

A diverse set of protein domains bind phosphoinositides promoting directed translocation to the plasma membrane and/or regulation of protein function. Proteins with phosphoinositide binding domains are implicated in various cell signaling events, including intracellular trafficking, cytoskeletal remodeling, and receptor promoted signal transduction [7]. Phosphoinositides also regulate several ion channels and transporters, but knowledge regarding the mechanisms governing phosphoinositide regulation of these proteins is limited [8]. Continuous changes in the relative concentrations of different phospholipids, and

variable organelle membrane compositions regulate a myriad of signaling pathways through localized recruitment of phospholipid-binding proteins.

1.3.1 PtdIns(4,5)P_2 Binding Domains

PH Domains

The human genome encodes over 250 PH (pleckstrin homology) domains, the eleventh most common domain in the human proteome. Several proteins involved in cellular signaling, cytoskeletal organization, membrane trafficking and/or phospholipid modification contain PH domains [9-11]. Sequence conservation among PH domains is only 7-30%; however, PH domains are defined by conserved secondary structural elements [9;12]. Approximately 120 amino acids in length, PH domains fold into a small seven-stranded β-sandwich core, capped at one end by a carboxy-terminal α-helix and by three interstranded loops at the other end [12]. These three interstranded loops are highly variable among PH domains and are implicated in the potential binding of phosphoinositides. Indeed, many PH domains bind phosphoinositides, albeit with poor specificity and low affinity [13]. Alternatively, certain PH domains, such as those that bind PtdIns(3,4,5)P_3, and the PH domain of PLC-δ1, which binds PtdIns(4,5)P_2, display high affinity for phosphoinositides by forming specific contacts with lipid headgroups [14;15]. More recently, PH domains have been shown to serve as protein-protein interaction motifs, with binding of the PH domain of β-adrenergic kinase to heterotrimeric Gβγ-subunits, and various PH domains binding to Rho-family GTPases [16-18].

ENTH and ANTH Domains

ENTH (epsin 1 N-terminal homology) and ANTH (AP180 N-terminal homology) domains are α-helical superhelical domains that preferentially bind PtdIns(4,5)P_2 [19;20].

Proteins containing ENTH or ANTH domains are typically involved in clathrin and receptor mediated endocytosis, and because of additional protein recognition motifs are thought to serve as scaffold proteins and assemble protein complexes [21]. Structural studies detail specific interactions made between PtdIns(4,5)P_2 and the ENTH domain of epsin, and between PtdIns(4,5)P_2 and the ANTH domain of CALM [22;23]. Binding of PtdIns(4,5)P_2 to solvent exposed residues within the ANTH domain of CALM contributes to the relatively weak observed affinity (K_d of ~5 μM) [22]. Alternatively, the ENTH domain of epsin forms a binding pocket for the inositol headgroup, and makes additional contacts with the glycerol backbone of PtdIns(4,5)P_2, leading to a 10-fold higher affinity for PtdIns(4,5)P_2 compared to ANTH domains [23]. Additionally, ENTH domains likely penetrate the lipid bilayer to stabilize the association of ENTH domain-containing proteins with membranes [24;25].

1.3.2 Monophosphate PtdIns Binding Domains

Proteins containing PX (Phox homology) domains are typically involved in vesicular trafficking, protein sorting, and lipid modifications [26;27]. Although several phosphatidylinositol lipids (*i.e.* PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(4,5)P_2) interact with PX domains, PtdIns(3)P represents the major lipid binding partner for PX domains [27-29]. Indeed, Yu *et al.* showed that all PX domains identified in *Saccharomyces cerevisiae* preferentially bind PtdIns(3)P [30]. Specificity of PX-domains for PtdIns(3)P is conferred through hydrogen bonding between the PX domain and free hydroxyl groups at the 4- and 5-positions on inositol. The presence of phosphate groups at the 4- and 5-positions would result in steric clashes between the inositol headgroup and the majority of PX domains. The ability of PX domain-containing proteins to anchor to the membrane is aided by a membrane-interaction loop, which penetrates the lipid bilayer.

FYVE domains (Fab1p, YOTP, Vac1p, and EEA1), similar to PX domains, preferentially bind PtdIns(3)P, but with relatively weak affinities [31;32]. Proteins encompassing FYVE domains are involved in endocytosis, regulation of the actin cytoskeleton, and growth factor signaling [33]. Currently, 27 FYVE domain-containing proteins have been identified and typically have zinc finger-like structures, with additional residues coordinating lipid binding [34]. PtdIns(3)P binding is conferred within a shallow hydrophobic pocket, with hydrogen bonds formed between the free hydroxyl groups of the inositol ring and the side-chains of the binding motif [35]. As observed with other low affinity lipid binding domains, FYVE domains partially embed into the membrane to stabilize membrane localization [24].

1.3.3 Other Phosphoinositide Binding Domains

Several other phosphoinositide binding domains have been identified, the majority of which are found in proteins involved in the regulation of the actin cytoskeleton. For instance, PtdIns(4,5)P_2 binding to WASP (Wiskott-Aldrich syndrome protein) enhances WASP-induced Arp2/3-mediated actin polymerization [36]. Additionally, the interaction of PtdIns(4,5)P_2 with profilin or cofilin, two actin binding proteins, inhibits the ability of profilin and cofilin to bind actin monomers [37;38]. FERM domains (ezrin/radixin/moesin), similar in structure to PH domains, bind PtdIns(4,5)P_2 and release a carboxy-terminal inhibitory domain allowing FERM-domain containing proteins to link actin filaments to the plasma membrane [39]. Finally, binding of PtdIns(4,5)P_2 to the carboxy-terminal region of tubby sequesters the protein away from downstream nuclear effectors. Upon hydrolysis of PtdIns(4,5)P_2, tubby translocates to the nucleus, where it serves as transcription factor [40].

1.3.4 PtdIns(4,5)P_2 and Disease

Because of the considerable number of proteins involved in lipid signaling, phosphoinositide metabolism is potentially involved in the aetiology of several diseases. Lithium has historically been used to treat patients with bipolar disorder; however, the precise mechanism of lithium action is unknown. One proposed mechanism entails the ability of lithium to inhibit inositol monophosphatases, thereby reducing the levels of inositol available for the *de novo* synthesis of phosphoinositides [41;42]. Additionally, several PI kinases map to chromosomal regions linked to bipolar disorder [43;44]. An increase in phospholipase activity has also been linked to cardiomyocyte apoptosis, possibly a consequence of decreased PtdIns(3,4,5)P_3 formation, which alters the PKB (protein kinase b) survival pathway [45]. There has also been a correlation found between Lowe's syndrome, which is characterize by severe mental retardation, and elevated PtdIns(4,5)P_2 levels [46]. Several other diseases have been linked to PtdIns(4,5)P_2 metabolism; however, the importance of lipid levels and signaling pathways is not immediately available. Because of the ability to regulate many signaling pathways at multiple different localizations within the cell, it would not be surprising to uncover significant correlations between phosphoinositide metabolism and human diseases.

1.4 Phosphoinositide-Specific Phospholipase C

The cellular action of a variety of extracellular signaling molecules, including hormones, neurotransmitters, and growth factors, is elicited through receptor-mediated stimulation of phosphoinositide-specific phospholipase C (PLC) enzymes. In response to receptor activation, PLC enzymes recognize and hydrolyze the inositol headgroup of PtdIns(4,5)P_2, generating two intracellular second messengers: diacylglycerol, which in turn activates PKC (protein kinase C), and inositol 1,4,5-trisphosphate, which mobilizes

intracellular calcium [47;48]. In addition to generating second messengers, activation of PLC enzymes terminates or promotes downstream signaling of proteins that employ phosphoinositide binding for membrane association and/or regulation of activity.

Hormonal regulation of phosphoinositide metabolism was first observed in the 1950s by Hokin and Hokin, who believed they were studying the incorporation of ^{32}P into RNA. Instead, addition of carbamylcholine or acetylcholine to pigeon pancreatic slices resulted in the incorporation of ^{32}P into the phospholipid fraction, a phenomenon later termed the "PI effect" [49]. A wealth of experiments, spearheaded by Michell, Berridge, and others, further implicated extracellular stimulation of receptors with phospholipid metabolism [50-52]. The Nishizuka group discovered the link between DAG formation and the activation of PKC in the late 1970s and early 1980s [53]. Experiments by Streb *et al.* showed an Ins(1,4,5)P_3-dependent link between receptor activation and increases in intracellular calcium [54]. These experiments, performed over the course of 40 years, laid the groundwork for researchers to dissect intricate signaling pathways integrated through PLC-mediated phosphoinositide metabolism.

By the early 1990s, Rhee and colleagues identified and cloned three subfamilies of PLC enzymes: PLC-β, PLC-γ, and PLC-δ [55;56]. Over the next decade, three additional PLC family members, PLC-ε, PLC-ζ, and PLC-η, were identified. Several PLC subfamilies include multiple members, bringing the total number of active isoforms to fourteen. PLC-L, a PLC-like protein, lacks critical conserved amino acids in the X-region rendering the protein catalytically inactive [57]. PLC enzymes are multidomain, soluble proteins ranging in molecular size from ~70 kDa for the smallest PLC-ζ isozyme, to 125-150 kDa for the PLC-β isozymes, and upwards of ~250 kDa for the largest isozyme, PLC-ε. PLC enzymes are

conserved throughout evolution, with simple PLC-δ-like isoforms expressed in yeasts, slime molds, filamentous fungi, and plants [58]. As cellular signaling components began to diversify, so to did PLC enzymes, leading to the appearance of the more complex PLC subfamilies. Although PLC subfamilies arise from varying sequence conservation and elaborate regulatory domains, all PLC isoforms share a conserved catalytic region for the recognition and hydrolysis the inositol headgroup from phosphoinositide lipids.

1.4.1 Catalytic Function and Structure of Conserved PLC Domains

Collectively, PLC enzymes are calcium-dependent phosphodiesterases that hydrolyze PtdIns(4,5)P_2 to form DAG and Ins(1,4,5)P_3. Although the six subfamilies of PLC enzymes are highly variable, several domains comprising the catalytic region are conserved in all PLC isoforms. Moving sequentially from the amino- to the carboxy-terminal regions of the enzymes, all PLC isozymes contain a PH domain (excluding PLC-ζ), EF-hand repeats, catalytic X- and Y-boxes, and a C2 domain (Figure 1.2). Individual PLC subfamilies arise from elaboration of the conserved regions with additional protein binding and/or modular domains.

Catalytic X and Y Box

The X- and Y-boxes are the most highly conserved regions, with 60-70% amino acid identity among families, with higher conservation found within subfamilies. The X- and Y-boxes fold together with alternating α-helices and β-strands to form a catalytic triose phosphate isomerase-like (TIM) barrel [59]. A three-dimensional crystal structure of the catalytic core (EF-hand through C2 domains) of PLC-δ1 bound to Ins(1,4,5)P_3 revealed the organization of the catalytic regions of PLC-δ as well as the potential mechanism of PtdIns(4,5)P_2 catalysis [60]. The crystal structure of PLC-δ also revealed a hydrophobic

ridge surrounding the active site believed to penetrate the lipid bilayer [61], increasing catalytic activity by allowing maximum access of the catalytic site to membrane-associated phosphoinositide substrate [62;63].

The reaction performed by PLC enzymes results in the cleavage of the oxygen-phosphorus bond connecting DAG to the 1-position of the inositol ring (Figure 1.1). The initial step in hydrolysis is the recognition of phosphoinositide substrate by the catalytic TIM barrel. Although eukaryotic PLC enzymes hydrolyze several phosphoinositide substrates, the rank order of specificity is PtdIns(4,5)P_2 > PtdIns(4)P > PtdIns [64;65]. Structure determination of PLC-δ suggests several amino acids within the catalytic region of PLC are involved in forming hydrogen bonds and salt-bridges between lipid substrate and PLC enzymes. Interactions between Lys-438, Lys-440, Ser-522, and Arg-549 of PLC-δ with the 4- and 5-phosphorylated positions of the inositol ring form phosphomonoesters [66]. The inositol ring also participates in catalysis, as the 2-hydroxyl group is necessary for the nucleophilic attack on the di-ester phosphate bond, thereby generating the cyclic intermediate.

Several amino acids involved in hydrolysis of PtdIns(4,5)P_2 are conserved between all PLC subfamilies, including amino acids His-311, His-356, Glu-341, Asp-343, and Glu-390 of PLC-δ [67]. The coordination of a calcium ion within the active site of PLC enzymes is also required for catalytic activity. In the structure of PLC-δ, a calcium ion is coordinated by the sidechains of Asn-312, Glu-341, Aps-343, and Glu-390, and also forms hydrogen bonds with the 2-hydroxyl group of inositol [66]. Utilizing structural information provided by PLC-δ in complex with various phosphorylated species of inositol headgroups, Ellis *et al.* proposed a three step catalytic mechanism for the hydrolysis of PtdIns(4,5)P_2 [60]. The first

step involves the formation of a 1,2-cyclic inositol phosphate intermediate resulting from the protonation of the DAG leaving group and the in-line nucleophilic attack of the 2-hydroxyl group of inositol. During this step, the calcium ion participates in the formation of the cyclic intermediate by lowering the p*Ka* of the attacking hydroxyl and neutralizing the negative charge generated during the transition state. Following formation of the cyclic intermediate, His-356 utilizes a proton from a water molecule to attack the cyclic intermediate and complete the hydrolysis of PtdIns(4,5)P_2. The final step in PtdIns(4,5)P_2 hydrolysis is the release of soluble inositol phosphate products. Mutation of His-311 and His-356, as well as the amino acids involved in the coordination of the calcium ion, confirmed the requirement of these residues as the catalytic activity of PLC-δ was reduced or destroyed upon mutation [68;69].

X-Y Linker Region

The amino acid sequence linking the X- and Y-catalytic regions is highly variable in both length and sequence among PLC subfamilies. Several studies suggest the linker regions of PLC isozymes provide a mechanism of autoinhibition through occlusion of the active site. Limited proteolysis of the X-Y linker region of PLC-β2 increased basal activity of the enzyme 30-fold relative to uncleaved enzyme [70]. Structural analysis confirmed occlusion of the PLC-β2 active site by the X-Y linker region [71]. Similar proteolysis experiments with PLC-δ1 also resulted in increase basal activity, suggesting the X-Y linker provides a general mechanism of autoinhibition for other PLC isozymes [60]. Finally, replacement of the X-Y linker region of PLC isozymes with a short flexible linker sequence results in a dramatic increase in basal enzyme activity (S. Hicks, T.K. Harden, J. Sondek, unpublished data). Enzyme activity was quantified after transient expression in COS-7 cells of PLC-β2,

PLC-ε, and PLC-δ1 lacking the X-Y linker regions compared or wild type enzymes. Furthermore, when reconstituted in phospholipid vesicles, purified PLC-β2 lacking the X-Y linker region also exhibits 5-10 fold higher activity than PLC-β2. Transfection experiments suggested that PLC-β and PLC-ε lacking the full X-Y linker retained regulation by Rac1 or Gβγ, or RhoA or Ras, respectively. The mechanism by which the X-Y linker region is released from the active site is unknown. However, proteins shown to regulate PLC isozymes, such as Gβγ regulation of PLC-β, or RhoA regulation of PLC-ε, may interact with the X-Y linker region causing release of the X-Y linker from the active site. Alternatively, interfacial activation by repelling the negatively charged linker from the negatively charged membrane is more likely in the activation of PLC-β2 by Rac, which results in no conformational change within the catalytic regions of PLC-β2.

PH Domains

With the exception of PLC-ζ, and certain splice variants of PLC-β4, all PLC isozymes have a PH domain preceding the EF-hand repeats and catalytic TIM barrel. As discussed previously, PH domains typically aid in membrane association by recognizing and binding negatively charged lipids. Indeed, the PH domain of PLC-δ1 binds with high affinity to PtdIns(4,5)P_2, which is required for enzyme activity, presumably serving as a tether bringing the catalytic TIM barrel to the membrane associated substrate [14;72;73]. Alternatively, PLC-γ specifically binds PtdIns(3,4,5)P_3 with high affinity, resulting in translocation to the plasma membrane [74]. The PH domains of PLC-β isozymes do not preferentially bind phosphoinositides and are not required for enzyme activity [75]. Interestingly, the PH domains of PLC-β isozymes are involved in protein-protein interactions with heterotrimeric Gβγ-subunits and/or the small GTPase Rac1 (see section 1.4.2). The PH domains of PLC

isozymes, though variable in sequence and binding specificity, appear to serve a common function in assisting membrane association of PLC enzymes.

EF-Hand Repeats

Included within all PLC isoforms, EF-hand repeats are helix-loop-helix structures divided into pairwise lobes [61]. EF-hand repeats, also found in calmodulin and tropinin, typically serve as calcium binding motifs, with each repeat binding to one calcium ion; however, naturally occurring variants have been identified that lack calcium binding [76]. The role of EF-hand repeats in the context of PLC enzymes is unclear. The crystal structure of PLC-δ1 suggests an absence of calcium binding to the EF-hand repeats. However, other studies have shown that calcium binding is necessary for the interaction of the EF-hand domain of PLC-δ1 with PtdIns(4,5)P_2 [77]. Indeed, deletion of this region of PLC-δ1, or mutation of requisite calcium-binding amino acids, results in the loss of enzyme activity [78]. These data suggest an essential role for the EF-hand repeats in modulating PLC-δ1 enzymatic activity. In contrast, although PLC-β and PLC-γ isozymes contain the four helix-loop-helix repeats, the domains lack residues required for metal binding. EF-hand repeats potentially serve as a flexible linker between the PH domains and catalytic regions of PLC isozymes that do not require EF-hand repeats for catalytic activity.

C2 Domains

Typically about 120 amino acids in length, C2 domains are found in a variety of membrane-associated proteins. The general structure of C2 domains is an eight β-stranded antiparallel sandwich, with three loops at one edge of sandwich forming calcium binding sites [79]. Several C2 domains bind calcium to mediate the association of C2 domains with phospholipids. The C2 of PLC-δ1 employs calcium binding to mediate a ternary complex

between the enzyme, a calcium ion, and phosphatidylserine [80]. Under specific *in vitro* conditions, phosphatidylserine binding to the C2 domain of PLC-δ1 increased the functional activity of the enzyme. The calcium-binding regions of PLC-δ1 are well conserved among species, suggesting the C2 domain plays an important regulatory role for PLC-δ isozymes. PLC-β and PLC-γ isozymes lack critical residues required for calcium binding, suggesting an alternate role for the C2 domain not involving calcium-mediated phospholipid interactions. The C2 domain of PLC-β1 has been shown to assist in the association of $G\alpha_q$ to the carboxy-terminal extension of the enzyme [81]. Due to the absence of a PH domain, PLC-ζ may utilize binding of PtdIns(3)P via the C2 domain for membrane association [82]. The regulatory role of C2 domains in the context of the remaining PLC isoforms is unknown, though evidence regarding PLC-δ and PLC-β suggest C2 domains are potential lipid or protein binding regulatory domains.

1.4.2 Phospholipase C Subfamilies and Regulation

Phospholipase C-δ

Mammalian PLC-δ isoforms are archetypical PLC enzymes evolutionary conserved in primitive eukaryotes, including yeast, slime molds, and plant PLC [83-85]. The PLC-δ isoforms are among the simplest PLCs, containing only the conserved PH domain, EF-hand repeats, X- and Y-boxes, and carboxy-terminal C2 domain. There are three mammalian family members, PLC-δ1, δ3, and δ4, sharing approximately 50% homology. PLC-δ2 was originally identified from a bovine brain library; however, further analysis revealed PLC-δ2 was the homologue of human/mouse PLC-δ4 [86]. PLC-δ1 is the most widely expressed PLC-δ isoform, with highest expression in brain, heart, lung, skeletal muscle, and testis (Table 1.1) [55]. Expression of PLC-δ1 is elevated in the brains of patients with Alzheimer's

disease compared to non-affected individuals but the role of PLC-δ1 in the brain and/or disease is unknown [87]. PLC-δ3 and PLC-δ4 have similar expression patterns, with both isotypes found in brain, skeletal muscle, and heart [88-90].

The solved crystal structures of the PH domain of PLC-δ1 in complex with Ins(1,4,5)P_3, and the catalytic EF-C2 region of PLC-δ1, provided the first structural information for the conserved regions found in all PLC isozymes [61;91]. As discussed above, these structures provided insight into the potential regulation of PLC-δ1 through the binding of PtdIns(4,5)P_2 to the PH domain, and the calcium-dependent binding of PS to the C2 domain. In response to these observations, Mark Lemmon proposed a "tether and fix" mode of PLC-δ regulation [92]. The proposed mechanism involves "tethering" of PLC-δ to the membrane via association with PtdIns(4,5)P_2, with subsequent PS binding to the C2 domain "fixing" the enzyme in a proper orientation to expose the active site to PtdIns(4,5)P_2 substrate. Accumulation of soluble Ins(1,4,5)P_3 would serve as a negative regulator or PLC-δ activity by competing with PtdIns(4,5)P_2 for binding to the PH domain. Another theory of PLC regulation, similar to the "tether and fix" model, is the concept of "membrane scooting" originally described by Hershberg and de Hass in 1976 in context of phospholipase A2 regulation [93]. Membrane scooting is a process that involves two successive equilibria, the first of which is the interfacial association of a water-souble enzyme to the membrane, and the subsequent interaction between a single enzyme molecule and a single substrate molecule. In the context of PLC-δ isozymes, the preferential binding of PtdIns(4,5)P_2 to the PH domain would anchor the enzyme to the lipid bilayer allowing the processive hydrolysis of substrate.

PLC-δ is one of the more calcium-sensitive PLC isoforms, potentially the result of calcium-dependent binding of both PtdIns(4,5)P_2 to the PH domain, and PS binding to the C2 domain [77;80]. One hypothesis suggests PLC-δ isoforms are activated as a secondary response to receptor-regulated PLC isoforms, such as PLC-β or PLC-γ [94]. The increase in intracellular calcium resulting from PLC-β or PLC-γ activation would promote the calcium-dependent translocation of PLC-δ isoforms to the plasma membrane, resulting in a prolonged PLC-dependent calcium signal. Finally, evidence suggests PLC-δ is potentially regulated by a novel class of G-protein, Gh. Association of PLC-δ with GDP-bound Gh has been proposed to negatively regulate PLC-δ activity, whereby receptor activation and GTP binding to Gh, PLC-δ is liberated, allowing the enzyme to actively hydrolyze substrate [95].

Phospholipase C-β

PLC-β isozymes contain a unique carboxy-terminal extension of approximately 400 amino acids following the C2 domain (Figure 1.2). The four PLC-β isozymes, PLC-β1-4, have unique tissue distributions and phenotypic traits among knockout mice. PLC-β1 is most widely expressed in the brain, and PLC-β1$^{-/-}$ mice develop epileptic seizures, often leading to sudden death [65]. In mice lacking the hematopoietic cell specific PLC-β2, chemokine signaling is disrupted. However, chemotaxis of hematopoeitic cells from PLC-β2 knockout mice is not altered [96]. PLC-β3 is expressed in several tissues including the liver, parotid gland, and low levels in the brain, and mice lacking PLC-β3 display decreased sensitivity to μ-opioid signaling [97]. Finally, mice lacking PLC-β4, found specifically in the cerebellum and retina, display ataxia, caused by loss of metabotropic glutamate-receptor signaling, and defective visual responses [65;98]. The presence of individual PLC-β isoforms in distinct tissues suggests a non-redundant role for the individual PLC-β isoforms.

Prior to the known involvement of heterotrimeric G proteins in PLC-dependent calcium signaling, several experiments suggested a pertussis toxin (PTX)-dependent and PTX-independent component of phosphoinositide signaling [99;100]. The PTX-independent regulation of PLC involves binding of the carboxy-terminal extension of PLC-β to the GTP-bound heterotrimeric Gα-subunit, $G\alpha_q$ [101-103]. Although all PLC-β isoforms are activated by direct association with $G\alpha_q$, PLC-β1 and PLC-β3 are the most sensitive to $G\alpha_q$ regulation [101;102]. PTX-sensitive regulation of PLC-β isoforms is mediated through association with heterotrimeric Gβγ subunits originating from $G\alpha_i$-dependent signals [104-106]. Deletion of the carboxy-terminal extension of PLC-β does not inhibit Gβγ-dependent activation of PLC-β, suggesting $G\alpha_q$ and Gβγ utilize independent regions of interaction with PLC-β to regulate enzyme activity [107]. Indeed, the region of Gβγ interaction appears to be the PH domain of PLC-β, and possibly another site within the Y-region of the catalytic domain [81]. PLC-β2 and PLC-β3 are the most sensitive to Gβγ regulation, while PLC-β1 is weakly activated, and PLC-β4 is insensitive to Gβγ activation [104;105;108]. This classical view of PLC-β regulation via interactions with various heterotrimeric G proteins positions PLC-β downstream of innumerable hepthahelical receptors coupled to various G proteins.

The complexity of PLC-β signaling, and PLC signaling in general, increased dramatically in 1997 when Illenberger and colleagues reported the direct, GTP-dependent activation of PLC-β2 by Rho family GTPases, specifically Rac1 and Cdc42 [109]. PLC-β2 demonstrated the highest sensitivity to Rac activation, while PLC-β3 showed little effect, and PLC-β1 was completely insensitive. Subsequent studies by Snyder *et al.* verified Rac-regulated PLC-β2 activity by coexpressing PLC-β2 with constitutively active mutants of Rac1, Rac2, and Rac3 in COS-7 cells and measuring inositol phosphate accumulation [17].

Additionally, a chimera of PLC-β2 containing the PH domain of PLC-β1 resulted in a loss of Rac-regulated enzyme activity, suggesting Rac interacts specifically with the PH domain of PLC-β2. [110]. Surface plasmon resonance experiments showed the isolated PH domain of PLC-β2 specifically bound activated Rac GTPases, verifying the Rac binding site within PLC-β2 [17].

Jezyk and coworkers ultimately defined the interaction between PLC-β2 and Rac1 by solving the first crystal structure of a PLC enzyme in complex with a protein activator [71]. The structure of a fragment of PLC-β2 spanning the PH to C2 domains in complex with activated Rac1 confirmed the specific interaction of Rac1 with the PH domain of PLC-β2. The switch regions of Rac1 clamp down on a hydrophobic ridge within the PH domain, suggesting the interaction is physiologically relevant as G proteins engage known effectors through interactions with the switch regions. Although binding to Rac1 in cells would result in translocation of PLC-β2 to the plasma membrane, the crystal structure also revealed that binding to Rac1 would align PLC-β2 in a favorable orientation to facilitate access of the catalytic TIM barrel to phosphoinositide substrate. Interestingly, the residues of Rac1 that interact with PLC-β2 share substantial identity with other Rho family GTPases, specifically RhoA and Cdc42. Whereas the electrostatic potential of Rac and the PH domain of PLC-β2 are favorable for complex formation, the electronegative potential of RhoA would repel the like-charged PH domain of PLC-β2. Cdc42 has a similar surface charge potential as Rac1, and although Cdc42 has been shown to increase PLC-β activity *in vitro*, a Phe56 in Cdc42, compared to Trp56 in Rac1, creates an unfavorable situation for complex formation between Cdc42 and the PH domain of PLC-β2.

Phospholipase C-γ

Two PLC-γ isozymes have been identified; the ubiquitously expressed PLC-γ1, and the hematopoietic cell enriched PLC-γ2 (Table 1.1) [55]. The activation of various growth factor receptors, including the receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF) leads to the activation of PLC-γ isozymes [111-113]. PLC-γ isoforms are characterized by unique X-Y linker regions, which contain a split PH domain, two SH2 domains, and an SH3 domain (Figure 1.2). Phosphorylated residues on tyrosine kinase receptors serve as high affinity binding sites for SH2 domains within the X-Y linker of PLC-γ, allowing recruitment of the enzyme to the plasma membrane, and phosphorylation of the enzyme at two residues within the linker region, specifically, Tyr771 and Tyr783 [114]. While phosphorylation of these residues is necessary for PLC-γ activity, neither is sufficient to activate the enzyme. Other studies suggest activation of certain growth factor receptors stimulate PI 3-K to form PtdIns(3,4,5)P_3, which in turn binds the PH domain and carboxy-terminal SH2 domain of PLC-γ, anchoring the enzyme to the membrane. One potential hypothesis is that phosphorylation of PLC-γ releases autoinhibition of the X-Y-linker, while binding to PtdIns(3,4,5)P_3 confers membrane association and enzyme activity.

Several other mechanisms of PLC-γ activation have been identified including phosphorylation by non-receptor tyrosine kinases, activation of immunoglobin receptors, and stimulation of GPCRs [76;115]. Most recently, PLC-γ2, but not PLC-γ1, has been identified as a direct downstream effector of Rac GTPases through a mechanism not involving tyrosine kinase phosphorylation or PtdIns(3,4,5)P_3 generation [116]. While several mechanisms of PLC-γ regulation have been observed, the specific biological function of these isozymes remains unclear. To varying degrees, PLC-γ1 has been associated with several cellular

processes including cell cycle control, transformation, control of the actin cytoskeleton, and development [115]. Indeed, PLC-γ1 deficient mice do not survive past embryonic day nine, suggesting an essential role for PLC-γ1 in development [117]. Knockout of PLC-γ2 is not embryonic lethal; however, these mice exhibit defective immunoglobin receptor signaling and reduced numbers of mature B cells [118].

Phospholipase C-ζ

Following fertilization of mammalian eggs, a series of Ins(1,4,5)P_3-dependent calcium oscillations occur. Several experiments determined that a soluble factor released by sperm is responsible for inducing calcium oscillations in fertilized eggs. Subsequent studies ruled out the involvement of known PLC isoforms, lending support to the hypothesis that an undiscovered PLC isoform released by sperm triggers calcium oscillations. In 2002, Saunders *et al*. identified the novel, sperm specific, PLC-ζ sequence by examining short EST-sequences isolated from mouse and human testis [119]. PLC-ζ is approximately 70 kDa and conserved in at least 8 mammalian species, as well as chickens. Sharing the most homology with PLC-δ, PLC-ζ lacks regulatory domains found in other PLC isoforms, and is the only mammalian PLC isoform devoid of a PH domain. The enzyme retains the core catalytic X- and Y-boxes, as well as four EF-hand repeats and a C2 domain (Figure 1.2).

The mechanism of PLC-ζ regulation in a cellular environment has yet to be fully ascertained. Calcium binding to the EF-hand repeats potentially regulates functional PLC activity, as removal of the first two EF-hands results in a rightward shift of the EC_{50} for calcium and decreases enzyme activity [82]. Interestingly, PLC-ζ is sensitive to calcium concentrations found in typical resting cells (~100 nM), suggesting calcium levels in fertilized eggs potentially regulates the activity of PLC-ζ, but not other PLC isoforms present

in fertilized eggs. The C2 domain of PLC-ζ does not appear to bind calcium; however, calcium-independent binding of PtdIns(3)P to the isolated C2 domain has been observed [82]. C2 domain-mediated membrane association of PLC-ζ may be integral to enzyme regulation as PLC-ζ lacks a PH domain, which is utilized by several PLC isozymes for membrane localization. Finally, a nuclear localization signal (NLS) within the X-Y linker region of PLC-ζ may play a vital role in the regulation of enzyme activity [120]. Fluorescently tagged-PLC-ζ was visualized in cells and shown to alternate between a perinuclear region during interphase when calcium oscillations are absent, to a cytoplasmic localization corresponding to increased calcium oscillations and mitosis [120].

***Phospholipase* C-η**

In 2005, four groups independently identified the newest PLC isoform, PLC-η, using data mining of EST databases and nucleotide sequences [121-124]. Two family members were identified, which mapped to different chromosomes in humans and mice: the shorter, 115 kDa PLC-η1, and the longer, 125 kDa PLC-η2. These isozymes share ~50% homology and both contain the canonical PH domain, EF-hand repeats, catalytic TIM barrel, and C2 domain (Figure 1.2). Following the catalytic region is a long carboxy-terminal Ser/Pro-rich sequence terminating in a PDZ-binding motif. PLC-η1 and PLC-η2 are brain specific enzymes, with enriched expression found in hippocampus, cerebral cortex, Purkinje cells, and olfactory bulb [122]. The regulation of PLC-η isozymes has yet to be determined in detail, though evidence suggests higher calcium sensitivity compared to other PLC isoforms, such as PLC-β [121]. Experiments by Zhou *et al.* indicate increased phosphoinositide accumulation in COS-7 cells transiently transfected with heterotrimeric Gβγ subunits and PLC-η, potentially positioning PLC-η downstream of GPCR activation [123]. Unpublished

in vitro experiments confirm direct regulation of PLC-η by $G\beta_1\gamma_2$ as determined by reconstitution of purified proteins in phospholipid vesicles (E. Zhou and T.K. Harden.).

Phospholipase C-ε

The final PLC isozyme subfamily, PLC-ε, will be discussed in detail in the following section.

1.5 Phospholipase C-ε

1.5.1 Identification and Cloning

In 1986, two groups, Cantley and coworkers, and Wakelam *et al*., independently suggested a role for Ras family proteins in the regulation of phosphoinositide metabolism [125;126]. Nearly twelve years later, while searching for proteins that interact with the nematode *Caenorhabditis elegans* homologue of Ras (LET-60^{12V}), Kataoka and coworkers isolated a novel PLC isoform [127]. The 210 kDa sequence possessed the conserved catalytic X- and Y-regions typified by all PLC isoforms and included the residues required for catalytic phosphodiesterase activity. Additionally, PLC210, the largest PLC isoform, contains novel regulatory domains not found in other PLC isoforms. PLC210 was the first PLC isoform identified encompassing known Ras-associating domains, including an amino-terminal CDC25 (cell division cycle protein 25-like domain) RasGEF domain, and tandem carboxy-terminal RA (Ras-associating) domains. The predicted Ras interacting domains suggests PLC210 is a bifunctional enzyme, acting downstream of activated Ras through the RA domains, and as an activator of Ras GTPases, potentially leading to an auto-feedback mechanism.

Following identification of the *C. elegans* PLC210 gene, three groups independently utilized the sequence information to clone PLC-ε, the mammalian homologue of PLC210.

Song *et al.* and Lopez *et al.* identified two human splice variants of PLC-ε differing in the amino-terminal region, yielding sequences of 1994 and 2303 amino acids, respectively [128;129]. Northern blot analysis with human mRNA revealed highest PLC-ε expression in heart, with lower expression levels in lung, kidneys, and brain [129]. Simultaneously, a single, 2281 amino acid rat ortholog of PLC-ε was cloned and shown to have functional phospholipase activity both *in vivo* and *in vitro* [130]. The mammalian homologues contain a similar domain organization as PLC210, including the carboxy-terminal RA domains and CDC25 domain; however, these groups did not identify a PH domain or EF-hand repeats. A more extensive scanning of the mammalian PLC-ε sequence by Wing *et al.* confirmed the presence of a PH domain, as well as EF-hand repeats [131]. Identification of the additional domains provided a complete domain architecture for PLC-ε, which includes, moving from the amino- to the carboxy-terminus, a Cys rich region, CDC25 domain, PH domain, EF-hand repeats, catalytic X- and Y-boxes, C2 domain, and tandem RA domains.

1.5.2 Unique Structural and Regulatory Domains

RA Domains

RA (RalGDP/AF6 or Ras-associating) domains are approximately 100 amino acid ubiquitin-like protein folds originally defined by Ponting and Benjamin [132]. Although over 100 RA domains have been identified, only a subset of RA domain-containing proteins are authentic Ras-family effectors. Sequence homology among RA domains is generally low and RA domains are characterized by conserved secondary structural elements. Two regions within the carboxy-terminal region of the *C. elegans* protein PLC210 share predicted secondary sequence homology with previously confirmed RA domains, including RalGDS and AF-6. Utilizing purified RA domain constructs of PLC210 (RA1 and RA2), Shibatohge

et al. showed direct association of GTP-bound H-Ras with the RA1 domain. However, binding of H-Ras to the RA2 domain was not observed [127]. Mutations within the switch regions of H-Ras caused a loss of interaction with PLC210 as observed in a two-hybrid system, further implicating the RA1 domain as an authentic Ras-binding domain.

Cloning of mammalian PLC-ε homologs also revealed the presence of tandem carboxy-terminal RA domains. In contrast to the observed interaction between the RA1 domain and Ras GTPases in *C. elegans*, it is the RA2 domain of mammalian PLC-ε isoforms that interact with Ras GTPases [130]. Studies by Song *et al.* show a direct, GTP-dependent association of H-Ras and Rap1a with a fragment of human PLC-ε encompassing both RA1 and RA2 domains [128;133]. Additional two-hybrid analysis suggested RA domain specificity, as other GTPases, such as Rho GTPases, RalA, and R-Ras, did not interact with the RA domains. A sequence alignment of the RA2 domain of PLC-ε with several RA domains from other Ras effectors revealed several critical amino acids required for Ras binding [130]. Two lysine residues (K2150 and K2152) in the second RA domain of rat PLC-ε were mutated to either alanine or glutamic acid. Mutation of Lys2150 to alanine or glutamic acid reduced *in vitro* H-Ras binding by 59% and >99%, respectively. Mutation of Lys2152 was not as significant as Lys2150; however, a charge reversal of both Lys2150 and Lys2152 to glutamic acid reduced H-Ras binding by 97% of wild-type binding. Additionally, mutations within the Ras effector regions were tested for binding to the RA domains of PLC-ε. The RA2 domain of PLC-ε retained the ability to bind H-Ras E37G and H-Ras D38N, with less binding observed with H-Ras T35S and H-Ras Y40C. Taken together, these experiments suggest PLC-ε is an authentic H-Ras effector, utilizing

interactions with switch regions of GTP-bound H-Ras for specific contacts within the RA domains of PLC-ε [130].

Biochemical analysis of a variety of RA domains from various proteins, including those from PLC-ε, confirmed binding of H-Ras and Rap1B to the RA2 domain of PLC-ε. Isothermal titration calorimetry experiments derived affinities of 0.82 and 8.4 μM for H-Ras and Rap1B binding to the RA2 domain of PLC-ε, respectively [134]. Similar experiments with the RA1 domain of PLC-ε showed no significant affinity for H-Ras or Rap1B. Subsequently, NMR structures of the individual RA domains and the crystal structure of RA2 in complex with activated H-Ras were solved [135]. Contacts made between the RA2 domain and H-Ras are unique among Ras effectors, sharing contacts previously observed only between H-Ras and PI 3-Kγ [136]. In particular, while most effectors make contacts only with residues in switch I region of H-Ras, PLC-ε and PI 3-Kγ contact residues within switch I and II regions of H-Ras. Structural comparison between the RA domains of PLC-ε reveals several negatively charged residues within the potential H-Ras binding region of the RA1 domain of PLC-ε. The presence of these negatively charged residues creates an unfavorable surface for H-Ras binding due to the negatively charged surface potential of the switch regions of H-Ras. Finally, the lower affinity of Rap GTPases for the RA2 domain can be partially explained by a charge reversal of Glu31 in H-Ras to a positively charged lysine in Rap GTPases, which would disrupt favorable charge interactions with the RA2 domain [135].

While the understanding of PLC-ε RA domain interactions suggests regulation of the PLC-ε by various Ras family proteins, the function of tandem RA domains remains a mystery. AF-6 is the only other Ras-family effector known to display tandem RA domains,

and similar to PLC-ε, only one of the RA domains serves as a binding partner for Ras (note: several proteins have tandem RBDs) [137]. Structural analysis of the RA domains suggests that they are loosely tethered by a flexible linker and exist as independent entities. The presence of tandem RA domains may be the result of genetic duplication during the evolution of PLC-ε. This is an interesting proposition considering the RA1 domain of PLC210 seems to confer Ras binding, while mammalian PLC-ε binds Ras only through the RA2 domain. The functional significance of the RA1 domain is unclear but could potentially aid in protein stability, serve as a structural spacer sequence allowing PLC-ε to bind Ras proteins independent of enzyme activation, or have some yet undefined binding partners.

CDC25 Domain

The amino-terminal region of PLC-ε shares a region of homology with the catalytic CDC25 domain of various RasGEF proteins [138]. Ras-family GEFs link activated membrane receptors to Ras activation by catalyzing the exchange of GDP for GTP on Ras family proteins, resulting in downstream activation of Ras-dependent signaling pathways. Approximately twenty mammalian Ras-family GEFs have been identified and characterized, and although CDC25 domains are specific for Ras-family proteins, individual RasGEFs confer specificity for particular Ras-family members [138]. Lopez *et al.* observed that overexpression of PLC-ε in TSA201 cells increased GTP-bound H-Ras quantified by co-precipitation with GST-tagged Ras binding domain [129]. Increased levels of activated H-Ras were not due to increased phospholipase activity, as a mutation that inhibits the catalytic activity of PLC-ε (H1144L) retained ability to increase GTP-bound H-Ras. Furthermore, overexpression of full-length PLC-ε, or an amino-terminal construct containing the CDC25 region increased the phosphorylation of MAP kinase, a Ras-dependent pathway.

In contrast to the conclusions of Lopez *et al*., Jin *et al.* suggested that the CDC25 domain of PLC-ε catalyzes nucleotide exchange on Rap1A, but displays no specificity for classical Ras GTPases, such as H-, K-, or N-Ras [139]. Purified PLC-ε constructs containing the CDC25 domain increased the rate of GDP release from Rap1A, but a construct lacking the CDC25 domain showed no GEF activity. Overexpression of the same constructs in COS-7 cells increased the levels of GTP-bound Rap1A and activated B-Raf, a downstream Rap1A-dependent kinase. Additionally, epidermal growth factor stimulation of cells overexpressing Rap1A resulted in a sustained translocation of PLC-ε to perinuclear compartments of the cell, whereas PLC-ε lacking the CDC25 domain only transiently located to the same compartment. The different observations made between the two groups may be attributed to the use of different PLC-ε splice variants and further experiments examining the specificity of the CDC25 domain would potentially resolve conflicting results. Likewise, the physiological consequence of RasGEF activity conferred by PLC-ε is unknown and requires further characterization.

1.5.3 Regulation

Ras Family GTPases

The identification and characterization of the RA domains of PLC-ε, as well as the ability to bind Ras GTPases *in vitro*, suggests the potential regulation of PLC-ε by various Ras family proteins. Several groups have reported increased PLC-ε activity in cells overexpressing PLC-ε with various Ras family GTPases. In addition to classical Ras proteins (H-, K-, and N-Ras), Rap1A, Rap2A, Rap2B, TC21, R-Ras, and Ral have all been reported to increase inositol phosphate accumulation in PLC-ε-dependent manner [140]. Additionally, mutations within the RA2 domain of PLC-ε, or point mutations within the

switch regions of H-Ras, decrease Ras-dependent regulation of PLC-ε similar to the decreased binding observed by Kelley and colleagues (see section 1.5.2). Reconstitution of purified PLC-ε with lipidated Ras GTPases in phospholipid vesicles has produced conflicting results regarding the direct activation of PLC-ε by Ras GTPases. Song *et al.* and Bunney *et al.* observed increased PLC-ε activity in reconstituted lipid vesicles when presented with a single concentration of GTP-bound H-Ras or K-Ras, respectively [128;141]. Alternatively, experiments by Kelley and colleagues suggested H-Ras does not directly regulate PLC-ε activity in a similar assay system [130].

Taken together, *in vitro* binding experiments and *in vivo* transfection data suggests PLC-ε enzyme activity is directly regulated by association with Ras GTPases. However, the mechanism of Ras-dependent PLC-ε activation is unknown. Ras GTPases, similar to other G proteins, are post-translationally modified by covalent attachment of lipid moieties, resulting in membrane localization of Ras proteins. Binding of PLC-ε to activated Ras GTPases could therefore result in the translocation of PLC-ε to various plasma membranes leading to increased activity due to closer proximity to PtdIns(4,5)P_2 substrate. This hypothesis is supported by experiments coexpressing PLC-ε and H-Ras resulting in translocation of PLC-ε from the cytoplasm to the plasma membrane [133]. Another hypothesis involves conformational changes within PLC-ε in response to Ras binding to the RA domains causing a favorable conformation for lipid recognition and hydrolysis. Finally, Bunney and colleagues hypothesize that the RA domains of PLC-ε autoinihibit enzyme activity by forming a complex with the catalytic region of the enzyme, which is disrupted upon binding of Ras and translocation to the plasma membrane [141].

Heterotrimeric G Proteins

Cotransfection of TSA201 cells with mammalian PLC-ε in combination with various heterotrimeric Gα-subunits identified $G\alpha_{12}$ as a regulator of PLC-ε-dependent inositol phosphate accumulation [129]. Although initial experiments by Lopez *et al.* showed no apparent $G\alpha_{13}$-dependent PLC-ε activity, subsequent studies by Wing *et al.* implicated both $G\alpha_{12}$ and $G\alpha_{13}$ as regulators of PLC-ε-dependent inositol phosphate accumulation in COS-7 cells [142]. Activation of PLC-ε by $G\alpha_{12}$ and $G\alpha_{13}$ occurs via a Ras-independent pathway as removal of the RA domains does not inhibit increased PLC-ε activity by $G\alpha_{12}$ or $G\alpha_{13}$. Several well defined downstream effectors of $G\alpha_{12/13}$ are Rho-family GEFs (*i.e.* p115RhoGEF and LARG), which activate Rho-family GTPases, suggesting $G\alpha_{12/13}$ may regulate PLC-ε indirectly through the activation of Rho GTPases [143-146]. Indeed, expression of C3 toxin, which ADP-ribosylates and inactivates Rho GTPases, with $G\alpha_{12}$ or $G\alpha_{13}$ inhibited PLC-ε-dependent inositol phosphate accumulation, but did not inhibit $G\alpha_q$ activation of PLC-β [147]. In addition to Gα-subunits, coexpression of heterotrimeric Gβγ dimers with PLC-ε increases the accumulation of inositol phosphates in COS-7 cells [131]. Heterotrimeric Gβγ subunit regulation of p114RhoGEF potentially stimulates PLC-ε activity through downstream activation of Rho GTPases [148]. Whether the activation of PLC-ε by heterotrimeric $G\alpha_{12}$, $G\alpha_{13}$, Gβγ-subunits is direct has yet to be elucidated.

Rho Family GTPases

Shortly after Gierschik and colleges identified PLC-β2 as a direct downstream effector of activated Rac GTPases, Wing *et al.* provided evidence that Rho-family GTPases also regulate PLC-ε activity [142]. When coexpressed with PLC-ε in COS-7 cells, constitutively active mutants of RhoA, RhoB, and RhoC markedly increased the accumulation of inositol phosphates in a PLC-ε-dependent manner. Other Rho family

GTPases, specifically Rac1, Rac2, Rac3, and Cdc42, failed to increase the activity of PLC-ε in the same experiments. Removal of the PH domain of PLC-ε did not inhibit Rho-mediated inositol phosphate accumluation, suggesting a unique mechanism of regulation compared to Rac-mediated regulation of PLC-β. Additionally, truncation of the RA1 and RA2 domains of PLC-ε results in an enzyme insensitive to Ras GTPases but that retains ability to be regulated by Rho GTPases. Truncation of the amino- and carboxy-terminal regions of PLC-ε narrowed the Rho-mediated effector region to the catalytic core of PLC-ε, specifically a location between the EF-hand repeats and the C2 domain.

To define the region within PLC-ε required for enzyme regulation by Rho family GTPases, multiple sequence alignments were performed against the other PLC isoforms. Sequence comparisons identified a unique insert within the Y-box of the catalytic region of PLC-ε not found in other PLC isoforms. Removal of the unique 70 amino-acid region within the Y-box of PLC-ε results in the loss of Rho-dependent enzyme activiation, but not the loss of H-Ras or Gβγ activation [142]. GST pull-down assays showed direct association of wild-type, and Y-box deleted, PLC-ε with GTP-bound GST-RhoA and GST-H-Ras, but no interaction with GST-Rac, which specifically interacted with PLC-β2 in a GTP-dependent manner. These results suggest the Y-insert region is sufficient for Rho-mediated PLC-ε activation; however, a complete loss of RhoA binding was not observed. The identification of the required regions within PLC-ε for Rho and Ras activation suggests PLC-ε is potentially a direct, independent and dual effector of Rho and Ras GTPases.

Receptor Regulated Activation

Several potential pathways involved in PLC-ε regulation have been identified using a combination of transfection and reconstitution experiments; however, these experiments do

not identify extracellular signals and/or receptors involved in PLC-ε regulation. As $G\alpha_{12/13}$ overexpression increases PLC-ε activity, so does activation of GPCRs (G protein coupled receptors) coupled to $G\alpha_{12/13}$. Studies by Hains *et al.* showed increased PLC-ε activity in cells expressing the LPA (lysophosphatidic acid) or PAR1 (protease activated receptor) treated with LPA or SFLLRN, respectively [147]. Additionally, these experiments confirmed $G\alpha_{12/13}$-mediated activation of PLC-ε occurs through activation of Rho-family GEFs, specifically p115RhoGEF. Expression of the RGS domain of p115RhoGEF, which binds to $G\alpha_{12/13}$, inhibited the receptor-mediated stimulation of PLC-ε by LPA or thrombin receptors. Likewise, expression of C3 toxin inhibited PLC-ε-dependent inositol phosphate formation following LPA or PAR1 receptor stimulation. Similar studies by Kelley and colleagues showed similar receptor mediated PLC-ε activation, with the addition of the S1P (sphingosine 1-phosphate) receptor, another $G\alpha_{12/13}$ coupled receptor [140]. CD44, a hyaluronan receptor coupled to LARG (Leukemia-associated RhoGEF) also increases the activity of PLC-ε, presumably through activation of Rho GTPases [149].

Work by Schmidt and colleagues identified additional GPCRs coupled to $G\alpha_s$-subunits that regulate PLC-ε activity downstream of activated Rap GTPases. Receptors coupled to heterotrimeric $G\alpha_s$ subunits, such as the β2-adrenergic or prostaglandin E1 receptors, stimulate adenylyl cyclase resulting in an increase in cAMP [150]. The accumulation of cAMP stimulates the cAMP-dependent GEF, EPAC (exchange protein activated by cAMP), which specifically activates Rap2B. Increased PLC-ε activity in cells simulated with $G\alpha_s$-coupled receptor agonists mimicked the activity observed with EPAC overexpression, confirming the hypothesized Rap2B-dependent pathway. In contrast,

GPCRs that couple to $G\alpha_i$ pathways, which inhibit adenylyl cyclase, decreased activated Rap2B and inhibited PLC-ε activity [151].

In addition to GPCR-mediated PLC-ε activation, several growth factor receptors have been shown to regulate PLC-ε activity. Following receptor stimulation, RasGEFs are recruited to autophosphorylated residues on the receptors, resulting in nucleotide exchange and activation of Ras GTPases. Indeed, activation of EGF or PDGF receptors in cells expressing PLC-ε leads to increased inositol phosphate accumulation [128;140;152]. Regulated PLC-ε activity is believed to be a direct consequence of Ras and/or Rap activation as removal of the RA domains of PLC-ε abolishes growth factor receptor-mediated activity. Upstream regulation of PLC-ε activity by such a multitude of receptors places PLC-ε at the nexus of multiple signaling pathways involving both heterotrimeric and monomeric GTPases. The physiological consequences of regulated PLC-ε activity become increasingly relevant as more pathways linked to PLC-ε regulation are discovered.

1.5.4 Physiological Function of PLC-ε

The presence of 14 mammalian PLC isoforms performing the same enzymatic reaction would suggest potential redundancy or compensatory action upon genetic manipulation or inactivation of specific PLC isoforms. Unique expression patterns for each PLC isoform may account for regulated function of individual isoforms, and molecular studies of regulation have provided significant advances in understanding the pathways involved in PLC regulation. To provide a more detailed understanding of the physiological role of PLC-ε, several groups have manipulated or knocked-out PLC-ε genes. In the first such study, Kataoka and colleagues deleted the PLC210 gene in *C. elegans*, *plc-1* [153]. Ovulation and fertility in *C. elegans* is regulated by an $Ins(1,4,5)P_3$-dependent signaling

pathway activated upstream by LET-23, a receptor tyrosine kinase. Deletion of *plc-1*, a gene encoding an enzyme that catalyzes the formation of $Ins(1,4,5)P_3$, is potentially involved in ovulation and fertility. Indeed, removal of catalytically required regions of PLC210 resulted in reduced fertility due to ovulation defects. These studies provided the first example of the physiological function of PLC-ε in an intact organism.

Kataoka and colleagues also created mice expressing inactive PLC-ε by removing portions of the catalytic X-box (PLC-$\varepsilon^{\Delta X/\Delta X}$) [154]. Studies exploring the onset of skin carcinomas revealed a crucial role for PLC-ε downstream of Ras-induced *de novo* carcinogenesis. Mice lacking catalytic PLC-ε function exhibited delayed average time of onset and reduced incidence of skin tumors in response to chemical carcinogens. Tumors that did form were generally smaller in size, contained fewer cells, and failed to undergo malignant progression into carcinomas. Additionally, tumors formed in PLC-$\varepsilon^{\Delta X/\Delta X}$ mice failed to undergo basal layer cell proliferation and epidermal hyperplasia in response to chemical carcinogens. Activated H-Ras was found in all cancer cells, regardless of PLC-ε background, suggesting a critical role for PLC-ε in tumor development and progression downstream of Ras signaling.

PLC-$\varepsilon^{\Delta X/\Delta X}$ mice were also used to study the role of PLC-ε enzyme activity in cardiac defects. Although pups were otherwise normal, they displayed enlarged hearts resulting from congenital malformation of the aortic and pulmonary valves [155]. The defects in valve formation caused a mild degree of stenosis and varying degrees of regurgitation resulting in ventricular dilation. Whereas normal mice undergo remodeling of valves during the formation of slender leaflets, mice lacking PLC-ε activity showed increases in valve thickness due to an increased number of cells. These results suggest a role for PLC-ε in

proliferation and/or apoptosis in semilunar valve cells during the late stages of valvulogenesis. This phenotype is shared in mice with attenuated EGF receptor signaling and in mice lacking the heparin-binding EGF-like receptor, suggesting a crucial role for PLC-ε activity during cardiac valve formation downstream of EGF-mediated Ras or Rap activation.

A second mouse model created by Wang and colleagues removed the entire PLC-ε gene to study the role of PLC-ε in cardiac function [156]. PLC-ε expression in cardiac myocytes is upregulated at both the protein and mRNA levels in patients undergoing heart failure, as well as in two mouse models of cardiac hypertropic stress. PLC-$\varepsilon^{-/-}$ mice showed decreased cardiac function, decreased response to isoproterenol, reduced calcium response resulting from β-adrenergic receptor activation in cultured cells, and mice are more likely to develop cardiac hypertrophy in response to chronic stress. These data, combined with the fact that no other PLC isozymes are upregulated in PLC-$\varepsilon^{-/-}$ mice, suggests PLC-ε is required for maintenance of maximum contractile reserve in response to β-adrenergic signaling under conditions of cardiac stress. The increased expression of PLC-ε may be a compensatory mechanism in response to the desensitization of β-adrenergic receptors during heart failure. Previous studies, as addressed above, indicate β-adrenergic stimulation of a $G\alpha_s$ pathway, increasing cAMP, leading to activation of EPAC and Rap2B. It has yet to be determined if this pathway is responsible for the PLC-ε-dependent signaling observed in the PLC-$\varepsilon^{-/-}$ mouse model.

A recent study has identified naturally occurring mutations of PLC-ε in human patients suffering from nephrotic syndrome [157]. Nephrotic syndrome is characterized by proteinuria, hypoalbuminermia, and edema, often leading to end-stage kidney disease,

requiring dialysis or kidney transplantation for survival. A whole-genome search performed to identify causative genes involved in nephrotic syndrome revealed seven different homozygous PLC-ε mutations. Six of the identified mutations were either nonsense or frameshift mutations, with the additional mutation causing a missense mutation of amino acid 1484 from serine to leucine within the catalytic X-box. Of the 12 patients identified with PLC-ε mutations, 9 developed end-stage kidney disease by the age of 5, suggesting an essential role for PLC-ε in preventing severe nephrotic syndrome. Interestingly, two of the identified patients responded to treatment with corticosteroids or cyclosporin A, representing the first case of a molecular cause of nephrotic syndrome that was responsive to therapy.

1.6 Guanine nucleotide binding proteins

Guanine nucleotide binding proteins, or G proteins, are a family of GTP hydrolases sharing a common structural core [158]. The >150 eukaryotic GTPases are grouped into two superfamilies; the smaller Ras-like proteins, and the heterotrimeric Gα-subunits. Both families of GTPases act as binary molecular switches that are activated by the tensioned binding of GTP, resulting in conformational changes within the switch regions favorable for association with downstream effector proteins. The hydrolysis of GTP to GDP returns the switch G protein switch regions to the "off" position, relaxing the switch regions and terminating downstream effector interactions. The exchange of GTP and the subsequent hydrolysis to GDP is controlled by a high affinity for guanine nucleotides, the intrinsic activity of the GTPase, and by various regulator proteins such as guanine nucleotide exchange factors (GEFs) and GTPase-accelerating proteins (GAPs) as discussed below (Figure 1.4).

The conserved structural domain shared among all G proteins is an approximately 20 kDa G domain, which is a variation of a classical nucleotide-binding fold [159]. The G domain fold consists of a six-stranded β-sheet surrounded by α-helices. Four structural elements within this region serve a common purpose in all G proteins: a rigid phosphate-binding loop (P-loop), which contacts the α- and β-phosphates on the guanine nucleotide and utilizes a serine or threonine to coordinate a Mg^{2+} ion; the switch I region, which aids in Mg^{2+} coordination; switch II, which is the GTP γ-phosphate sensor and binding site, and contains a catalytic glutamine residue present in most G proteins; and a conserved NKXD sequence for recognition of the guanine ring of the nucleotide [160;161]. Although the mechanism of GTP hydrolysis by GTPases remains a point of conjecture, the reaction is believed to involve the in-line nucleophilic attack of water upon the γ-phosphate group, deprotonation of the attacking water molecule, formation of a transition state complex, and release of Pi [162;163]. Evidence suggests the γ-phosphate group is the catalytic base for the reaction and that Mg^{2+} is required to increase the basicity of the γ-phosphate [164;165].

1.6.1 Ras Superfamily GTPases

Small GTPases are monomeric G proteins with molecular masses ranging from 20 to 40 kDa and that exist in eukaryotes ranging from yeast to human. Like all G proteins, monomeric G proteins have consensus sequence specifying interactions with guanine nucleotides. Over 150 small G proteins are encoded in the human genome, which make up the Ras superfamily of GTPases [166]. Members of the Ras superfamily are structurally classified into five different families: the Ras, Rho, Rab, Arf, and Ran families. Among subfamilies, Ras proteins share approximately 50-55% homology, but are less conserved, approximately 30%, between families [167;168]. Ras superfamily GTPases are implicated in

an extensive array of cell biological processes regulated by many upstream activators, downstream effectors, and negative regulators. All Ras family GTPses are structurally similar and bind effectors through interactions with the switch regions. However, distinct intracellular localizations and interaction with specific downstream effectors accounts for functional specificity of various Ras family GTPases.

Ras GTPases

The most highly studied and founding members of the Ras superfamily are the Ras proteins (rat sarcoma), including the classical Ras proteins (H-, K-, and M-Ras), the Rap proteins (Rap1A, 1B, 2A, 2B), and Ral proteins. H-Ras and K-Ras genes were first identified as oncogenes in sarcoma viruses during the late 1970s and the cellular oncogenes were found in several human carcinomas [169-171]. The carboxy-terminal end of most Ras GTPases contains a CAAX (C=Cys, A=aliphatic, X= any amino acid) region comprising the membrane targeting sequence. Farnesyltransferase or geranylgeranyltransferase I recognize the CAAX motif and catalyze the covalent attachment of a farnesyl or geranylgeranyl isoprenoid, respectively, to the Cys residue of the tetrapeptide motif [172-174]. The specificity of transferases is predicated by sequence within, or surrounding, the CAAX motif, which are classified into two main groups. The first group, including H- and N-Ras, contains an additional Cys residue upstream of the CAAX motif, which is palmitoylated. Other farnesylated Ras GTPases, including K-Ras, have a polybasic stretch of amino acids directly upstream of the CAAX motif. The second type of lipid modification, including the Rap GTPases, recognizes Leu or Phe in the X-position resulting in geranylgerynlation of the Cys CAAX residue. Following attachment of a lipid moiety, the -AAX portion of the CAAX motif is proteolytically removed and the exposed cysteine residue is carboxymethylated.

Attachment of an isoprenoid moiety is not sufficient for proper trafficking of Ras GTPases to lipid membranes, but requires the additional palmitoylation (*i.e.* H-Ras) or polybasic amino acids (*i.e.* K-Ras) [175].

A large number of extracellular signaling molecules activate Ras family guanine nucleotide exchange factors (GEFs) leading to the formation of active, GTP-bound Ras proteins [176;177]. A complex between the CDC25 catalytic domain of the RasGEF SOS (Son of Sevenless) and H-Ras revealed information regarding the catalytic activity of nucleotide exchange [178]. Binding of the CDC25 domain to H-Ras induces conformational changes in the switch regions opening the nucleotide binding pocket, thereby reducing the affinity for Mg^{2+} and the nucleotide phosphate groups, and stabilizing the GTPase in a nucleotide-free conformation. Once the nucleotide binding pocket is opened and GDP is released, the molar excess of GTP over GDP in the cell results in activation of the GTPase through binding GTP.

Termination of Ras signaling is achieved by hydrolysis of GTP to GDP. Ras family GTPases have a notoriously slow intrinsic rate of hydrolysis (k_{cat} ~0.02 min^{-1}), which is aided by various RasGAPs (Ras-family GTPase activating proteins) [179]. The crystal structure of p120GAP in complex with H-ras provided extensive information regarding the mechanism by which RasGAPs aid in GTP hydrolysis [180]. Whereas heterotrimeric Gα-subunits bind aluminum fluoride in the presence of GDP, mimicking the transition state of hydrolysis, Ras GTPases lack a critical arginine residue provided in *trans* by RasGAPs. The structure of p120GAP in complex with GDP-bound Ras and aluminum fluoride suggests the *trans* arginine provides a strong hydrogen bond donor to the nucleotide binding pocket and stabilizes the catalytic conformation of the Gln side-chain in the switch II region.

Genuine Ras effectors are characterized by at least one of three distinct ~100 amino acid folds: two different Ras-binding domains (RBD) or Ras-association (RA) domains. Although sequence homology among Ras-interacting domains varies greatly, all three domains form a similar ubiquitin-like superfold [181]. Activated Ras GTPases regulate a diverse cascade of signaling pathways, including the widely studied activation of MAP kinase pathways after stimulation of growth factor receptors, ultimately leading to gene transcription. Ras GTPases are also involved in lipid metabolism through activation of PI3K, which phosphorylates PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3 [182]. Various other studies have implicated classical Ras proteins in cell proliferation, differentiation, morphology, and apoptosis [183-186]. Other Ras family proteins, such as Rap, Ral, or R-Ras, possess unique functions independent of the function of classical Ras GTPases. Most relevant to this work is that in addition to H-, K-, and N-Ras, Rap GTPases have been shown to regulate the activity of PLC-ε in an RA2 dependent manner [128;187].

Rho GTPases

Rho GTPases are monomeric GTPases characterized by an α-helical "insert region" (amino acids 125-135 in Rac) not present in Ras family GTPases [188;189]. Based on sequence and function, 22 mammalian Rho GTPases have been identified, which are divided into 5 groups: the Rho-like (RhoA, RhoB, and RhoC), the Rac-like (Rac 1, Rac2, Rac3, and RhoG), the Cdc42-like, Rnd, and RhoBTB subfamilies. With the exception of the Rnd proteins, which are constitutively active due to inability to hydrolyze GTP, all Rho family GTPases are regulated by GDP/GTP cycling. The most extensively characterized Rho GTPases are RhoA, Rac1, and Cdc42 which cause the formation of stress fibers, lamellipodia, and filopodia, respectively [190-192]. Like Ras-family GTPases, most Rho

GTPases contain a carboxy-terminal CAAX motif resulting in covalent attachment of farnesyl or geranylgeranyl lipid moieties, cleavage of the -AAX sequence, and carboxymethylation of the cysteine residue. Post-translational modification of Rho family G proteins enhances membrane association and leads to distinct cellular localizations [193;194].

The activation of Rho family GTPases is regulated by a large family of RhoGEFs, which catalyze the exchange GDP for GTP. Unlike the relatively small number of RasGEF proteins, over 60 RhoGEFs have been identified, which are broken down into three families [195]. The Dbl family RhoGEFs represent the largest subfamily of exchangers, containing a catalytic Dbl homology (DH) domain followed by a PH domain, which aids in membrane localization and/or cooperates with the DH domain to assist nucleotide exchange. Some RhoGEFs are fairly promiscuous, exchanging on several Rho family GTPases, whereas other appear to confer specificity of exchange (reviewed:[196;197]). The regulated activity of RhoGEFs ultimately leads to the activation of Rho-family signaling pathways, which has prompted intense characterization of upstream signaling pathways. Growth factor receptor activation, which leads to the activation of Ras family GTPases can lead to the activation of Rho GTPases through the activation of several RhoGEFs, including Tiam1 [198]. Activation of hepthahelical receptors also leads to Rho GTPase activation through RhoGEFs that encompass an RGS (regulator of G protein signaling) domain for heterotrimeric Gα-subunits in addition to a DH/PH exchange region. Specifically, activated $G\alpha_{12/13}$ family members engage the RGS box of p115-RhoGEF or LARG, leading to the termination of heterotrimeric signaling and the activation of Rho family GTPases. The integration of multiple signaling

pathways through RhoGEFs suggests Rho family GTPases mediate a multitude of cellular responses.

Rho family GTPases are negatively regulated by GAP proteins and RhoGDIs (guanine nucleotide dissociation inhibitors). RhoGAP proteins share a ~140 residue GAP domain, which enhances the rate of GTP hydrolysis and share no resemblance to RasGAPs [199]. Several RhoGAPs display substrate specificity for Rho family GTPases, while others are more promiscuous [200]. RhoGDIs bind to GDP-bound Rho GTPases through an immunoglobin-like domain and inhibit GDP/GTP exchange [201;202]. Additionally, RhoGDIs contain a hydrophobic pocket which accommodates the isoprenoid group attached to the GTPase [203]. Masking of the lipid group allows the Rho/RhoGDI complex to exist in the cytoplasm, sequestering the GTPases from membrane associated activators and effectors.

The main function of Rho family GTPases is to regulate the assembly and organization of the actin cytoskeleton. A large number of cellular processes depend on Rho-mediated actin cytoskeleton dynamics, including cytokinesis, phagocytosis, pinocytosis, cell migration, and axon guidance [204-207]. The majority of Rac and Cdc42 effectors contain a CRIB (Cdc42/Rac-interactive binding) domain that recognizes the switch regions of the GTPase [208;209]. Other Rac/Cdc42 effectors utilize other regulatory domains to bind the switch regions of the GTPases (*e.g.* the PH domain of PLC-β). Many Rho effectors contain a REM (Rho effector homology) region, which contains a three leucine-zipper like motif [210]. In context of the current work, several emerging Rho family effectors involved in phospholipid metabolism are especially intriguing. The ability of Rho GTPases to regulate PLC enzymes, though a relatively new observation, increases the complexity of Rho family

GTPase signaling. Additionally, several PI kinases are regulated by Rho family GTPases, which may result in amplified PLC signaling because of increased substrate levels [211].

Rab, Ran, and Arf GTPases

The largest family of Ras GTPases are the Rab proteins (Ras-like proteins in the brain), with 61 total members [212]. Rab proteins are localized to specific intracellular compartments based on prenylation and divergent carboxy-terminal sequences. The localization of specific Rab GTPases is essential for the regulation of intracellular vesicular transport and trafficking proteins between different organelles in the endocytic and secretory pathways [213]. Ran GTPases (Ras-like nuclear) are the most abundant small GTPases in the cell and are involved in nucleocytoplasmic transport of RNA and proteins [214]. Regulated activation of Ran proteins have also been implicated in mitotic spindle assembly, DNA replication, and nuclear envelope assembly [215]. Arf proteins are localized to the Golgi apparatus and are involved in vesicle formation. The regulation of Arf proteins is somewhat unique in that when bound to GDP, Arf proteins are cytoplasmic, and upon nucleotide exchange, the myristoylated amino-terminal helix is exposed, allowing association with lipid membranes [216].

1.6.2 Heterotrimeric G proteins

In the late 1950s and early 1960, Sutherland and Rall were able to show hormone stimulated production of cAMP. However, the mechanism of hormone-promoted cAMP production remained elusive [217]. Years later, Rodbell and colleagues discovered the requirement for GTP in adenylyl cylase simulation [218]. Finally, Ross and Gilman bridged the gap by showing G protein-mediated communication between β-adrenergic receptor activation and stimulation of adenylyl cyclase [219]. There are 16 known Gα genes encoding

23 known Gα-subunits divided into four major classes: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ [99]. In addition to the core "Ras-like" domain, Gα subunits contain a helical domain resulting in an additional switch III loop. When bound to GDP, Gα subunits form a heterotrimer through association with an obligate Gβγ heterodimer. The heterotrimeric complex of Gα and Gβγ negatively regulates signaling due to the tight association of the proteins in the GDP-bound state, with Gβγ dimers serving as guanine nucleotide dissociation inhibitors, or GDIs. There are five ~35 kDa Gβ subunits and fourteen ~8 kDa Gγ subunits, which associate in various combinations to form heterodimers. Both Gα- and Gγ-subunits are modified by attachment of lipid moieties, resulting in membrane associations of both Gα- and Gγ-subunits. All Gα-subunits, with the exception of transducin, have an amino-terminal 16-carbon palmitate attached through a thioester bond to a cysteine residue [220]. Additionally, $G\alpha_i$ family members undergo co-translational N-myristolyation to an amino-terminal glycine, followed by removal of the initiator methionine. Three Gγ-subunits have a 15-carbon farnesyl moiety attached (Gγ1, Gγ8, and Gγ11) and the remaining Gγ-subunits have a 20 carbon geranylgeranyl group attached.

Heterotrimeric G proteins are associated with signaling directly downstream of the approximately 865 genes that encode GPCRs. The specificity of G proteins for GPCRs is conferred through five amino acids at the carboxy-terminus of Gα-subunits. Extracellular ligand binding to GPCRs causes an intracellular conformational change of the receptor stimulating GEF activity on Gα-subunits. The GEF activity of the receptor most likely promotes the release of GDP by stabilizing the nucleotide-free state of the Gα subunit, which then binds GTP due to the molar excess of GTP over GDP in cells. GTP binding causes conformational changes within the three switch regions of Gα leading to the dissociation of

Gβγ. Activated Gα subunits and liberated Gβγ subunits initiate downstream signals by interacting with specific effector proteins. Signaling is terminated by hydrolysis of GTP to GDP by the Gα subunit and reassociation of the heterotrimer complex. Gα-subunits display the highest intrinsic rate of hydrolysis among G proteins (k_{cat} ~ 2-5 min^{-1}) due the presence of a conserved arginine residue that is absent in Ras-family GTPases, which stabilizes the transition state of GTP hydrolysis [221].

The rate of GTP hydrolysis by Gα-subunits is not fast enough to account for physiological rates of signal transduction. Several Gα effector proteins, including PLC-β1, act as GAPs to increase the rate of GTP hydrolysis by Gα-subunits [222]. Additionally, a family of RGS proteins negatively regulate heterotrimeric G proteins signaling by increasing the rate of GTP hydrolysis at least 50-fold [223-225]. RGS proteins bind directly to the switch regions of Gα-subunits, locking the protein in the transition state for GTP hydrolysis. RGS proteins are often multifunctional proteins containing elaborate domains not involved in GAP activity. Most relevant to this work are the Rho family GEFs containing an RGS domain that serve as specific GAPs for $G\alpha_{12/13}$ family members, but also contain a RhoGEF domain to activate Rho family GTPases.

1.7 Research Goals

Phospholipase C enzymes generate the second messengers $Ins(1,4,5)P_3$ and DAG by hydrolyzing the minor membrane lipid $PtdIns(4,5)P_2$. The complexity of inositol lipid signaling has recently been extended with the identification of Ras superfamily GTPase-mediated regulation of PLC isozymes. Perhaps the most unique example of functionally complex regulation of phospholipase C occurs in the recently discovered isozyme PLC-ε, which is activated *in vivo* after overexpression of various small GTPases within the Rho and

Ras families through at least two distinct domains. Measuring inositol phosphate accumulation in cells overexpressing PLC isoforms with potential activators provides a powerful tool for identifying pathways that regulate PLC activity. However, coexpression experiments cannot unquestionably identify proteins that regulate PLC function via direct protein-protein interactions. The overall goals of the following research is to use purified proteins to establish unambiguously the direct activation of PLC-ε by small GTPases within the Rho and Ras families and to establish the enzymatic consequences of the interaction of multiple G proteins with PLC-ε**.** To this end, we will attempt to purify a catalytically active fragment of PLC-ε and determine the enzymatic characteristics of the enzyme. Regulation of the activity of PLC-ε by various G proteins will be studied by reconstitution of purified proteins in a model phospholipid vesicle system. These experiments will provide the first information regarding proteins that directly interact with PLC-ε to regulate the functional activity of the enzyme.

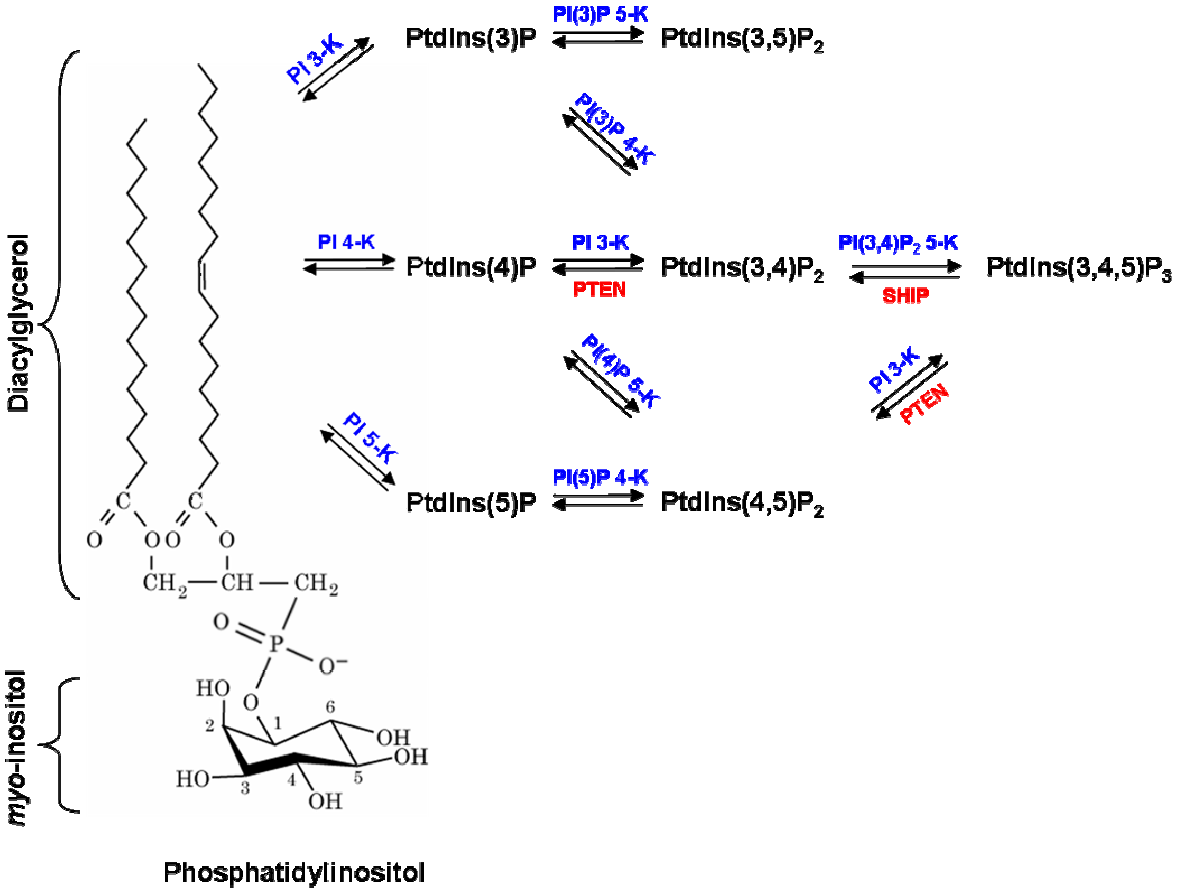

Figure 1.1. Synthesis and metabolism of phosphoinositides. Phosphatidylinsitol is the precursor to all of the phosphoinositides. The basic structure of phosphatidylinositol is composed of a di-esterified glycerol lipid moiety connected via a di-ester phosphate bond to the D1 position of *myo*-inositol. The five free hydroxyl groups are regulated by the attachment of mono-ester phosphate groups at various positions on the inositol ring. Several lipid kinases (*blue*) recognize specific hydroxyl groups on the inositol ring and transfer a γ-phosphate from ATP to the inositol ring. The action of the lipid kinases is antagonized by several lipid phosphatases (*red*) which remove specific phosphate groups from the inositol headgroup.

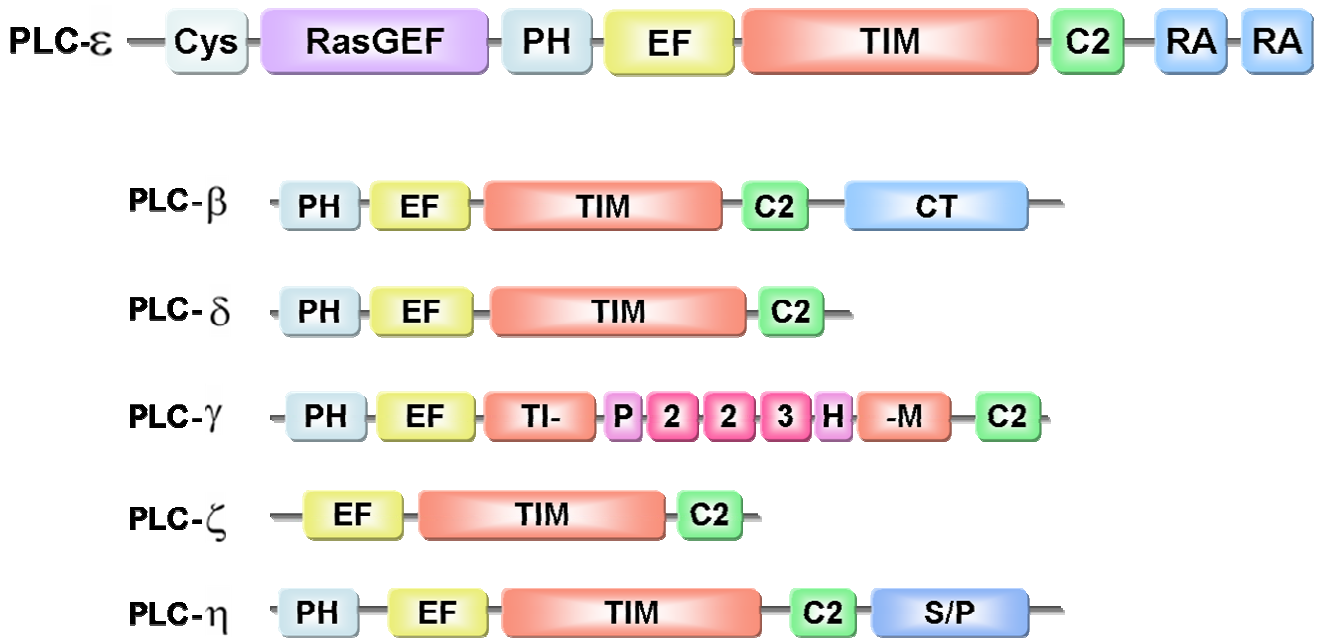

Figure 1.2. Domain organization of mammalian phospholipase C (PLC) family members. The six subfamilies of PLCs share common structural and regulator motifs, which are denoted by similar color. All PLC isozymes contain the canonical domains found in the evolutionarily conserved PLC-δ, including a pleckstrin homology (PH) domain (excluding PLC-ζ), a series of four EF-hand domains, a catalytic TIM barrel, and a C2 domain. Elaboration of the conserved catalytic regions by various modular domains gives rise to the different PLC subfamilies. PLC-ε contains a Ras guanine nucleotide exchange domain (RasGEF), tandem carboxy-terminal Ras association (RA) domains, and a Cysteine-rich (Cys) region. The four PLC-β isozymes contain a carboxy-terminal (CT) domain necessary for dimerization and regulation by $G\alpha_q$. The catalytic region of PLC-γ is interrupted by the presence of a split PH domain and an array of Src-homology (SH) 2 and 3 domains, which confer enzyme activity downstream tyrosine kinases. PLC-η contains a region rich in Ser/Pro (S/P), however, the role of this region in enzyme regulation is not determined.

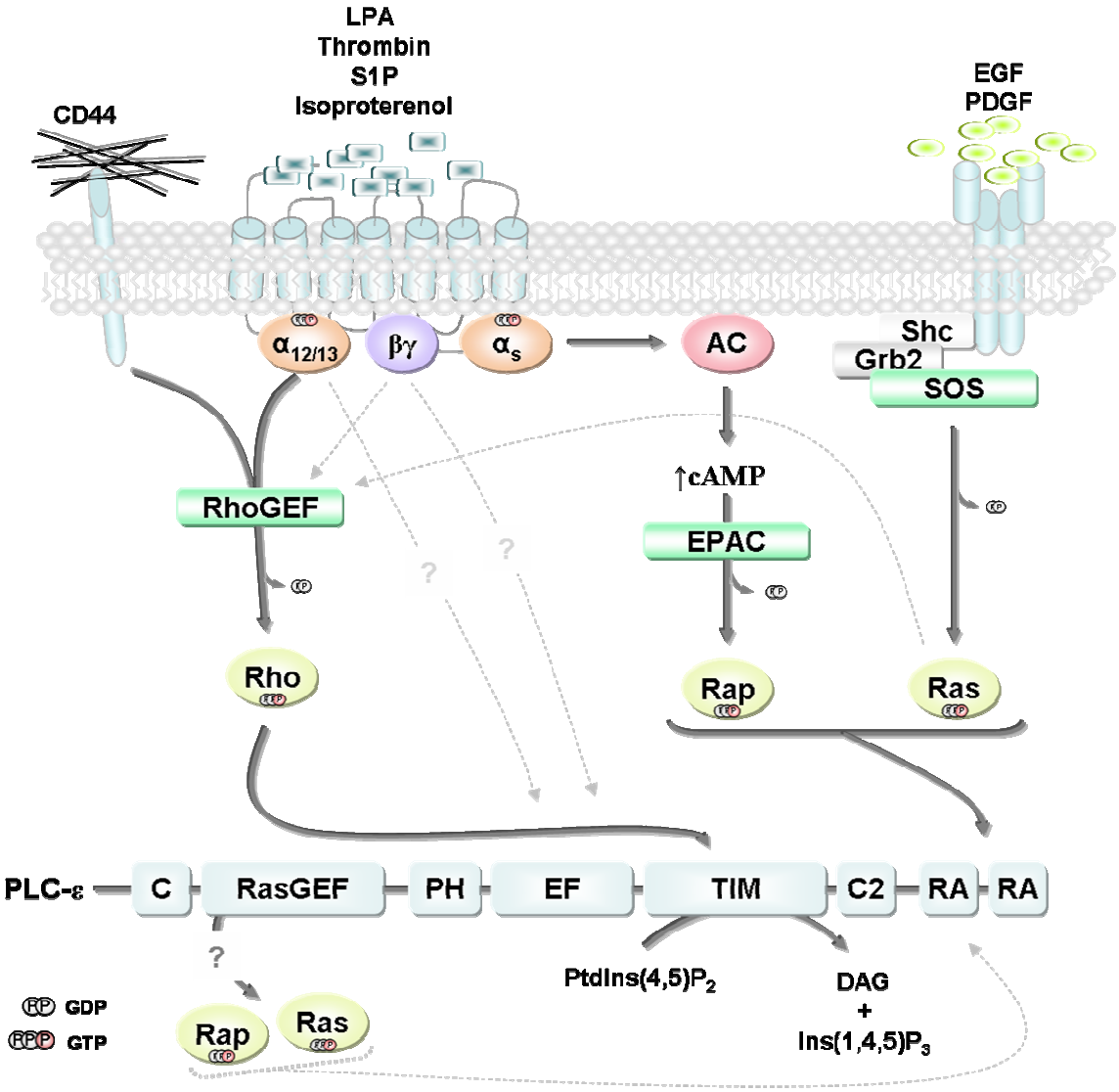

Figure 1.3. PLC-ε links heterotrimeric and small G protein signaling pathway in the generation of second messengers. The phospholipase activity of PLC-ε is downstream of an ever increasing number of signaling pathways integrating heterotrimeric and small G proteins. Activation of transmembrane receptors that activate Gα12/13, Rho, and Ras GTPases are implicated in the activation of PLC-ε. More well characterized pathways are indicated by bolder lines with significant evidence for the direct modulation of PLC-ε activity by several Ras family proteins, as well as Rho family members. Proposed regulatory pathways (dashed grey lines) involved the undefined activation of PLC-ε by heterotrimeric $G\alpha_{12/13}$ and/or Gβγ-subunits. Additionally, PLC-ε potentially regulates a positive feedback loop RasGEF-promoted activation of Ras GTPases activated PLC-ε through binding the carboxy-terminal RA domains.

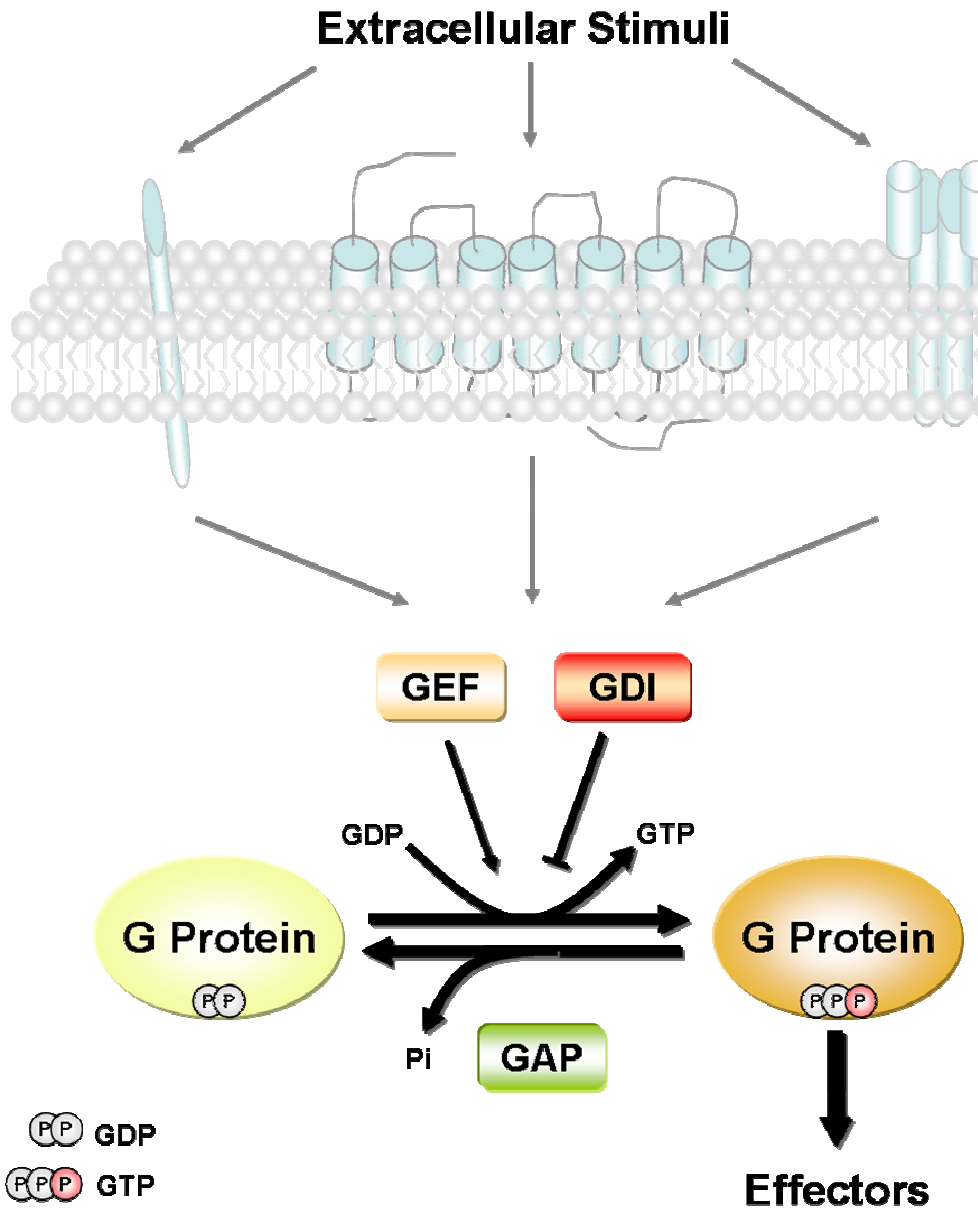

Figure 1.4. Model of regulated nucleotide cycling of G proteins. An large number of extracellular stimuli interact with transmembrane receptors to activate guanine nucleotide exchange factors (GEFs) resulting in the exchange of GDP for GTP on heterotrimeric and monomeric GTPases. The action of GEFs is inhibited by guanine dissociation inhibitors (GDIs) that bind GDP-bound GTPases and inhibit nucleotide exchange and/or sequester GTPases in the cytoplasm. Once activated, GTP-bound G proteins recognize downstream effectors to initiate diverse signaling cascades. The cycle returns to the basal state when the the GTPase hydrolyzes the γ-phosphate of GTP. Because of the relatively slow intrinsic rate of hydrolysis, the majority of G proteins are aided by GTPase-accelerating proteins (GAPs).

Table 1.1. Tissue distribution and function of mammalian PLC isoforms (adapted from [226])

Isozyme	Tissue Distribution	Function in KO mice	Ref.
PLC-β1	brain (cerebrum, hippocampus) adrenal gland, lung, parotid gland	induction of epilepsy	[55;65]
PLC-β2	hematopoietic cells (neutrophil, platelet)	chemoattractant-induced superoxide production	[96]
PLC-β3	brain, liver, parotid gland, platelet	μ-opioid-mediated responses	[88;97]
PLC-β4	brain (cerbellum, retina)	visual response, ataxia, long-term depression	[65;98;227]
PLC-δ1	brain, heart, lung, testis		[55]
PLC-δ3	skeletal muscle, heart, brain		[55]
PLC-δ4	testis, brain, skeletal muscle	acrosome reaction in sperm	[89;90;228]
PLC-γ1	lung, thymus, brain (neuron)	embryonic lethality	[55;117;229]
PLC-γ2	spleen, thymus, lung	B cell development	[55]
PLC-ε	lung, liver, heart, skeletal muscle	cardiac function, isoproterenol response, calcium response	[129;130;156]
PLC-ζ	sperm		[119]
PLC-η1	brain (hippocampus, Purkinje cells)		[124]
PLC-η2	brain (hippocampus, cerbral cortex, olfactory bulb)		[122]

CHAPTER 2

DIRECT ACTIVATION OF PURIFIED PHOPHOLIPASE C EPSILON BY RHOA STUDIED IN RECONSTITUTED PHOSPHOLIPID VESICLES

2.1 Abstract

Phospholipase C-ε (PLC-ε) was shown recently to be a downstream effector of Rho GTPases, and we have utilized an *in vitro* phospholipid vesicle reconstitution system with purified proteins to show this regulation to be direct. This chapter describes high-level expression of a hexa-histidine-tagged fragment of PLC-ε encompassing the catalytic core of the enzyme through the tandem RA domains using a recombinant baculovirus and High Five insect cells. The recombinant protein is purified to homogeneity using metal chelate affinity and size exclusion chromatography. The small GTPase RhoA also is expressed to high levels in a lipidated form after baculovirus expression in High Five cells and is purified to near homogeneity after detergent extraction and metal chelate affinity chromatography. The capacity of GTPγS-bound RhoA to stimulate the phospholipase activity of PLC-ε is assessed by reconstitution of the RhoA in PtdIns(4,5)P_2 substrate-containing phospholipid vesicles.

2.2 Introduction

An extensive array of extracellular signaling molecules including hormones, neurotransmitters, and growth factors elicit their physiological effects through receptor-mediated stimulation of phospholipase C (PLC) [48;56]. PLC enzymes respond by hydrolyzing the minor membrane phospholipid PtdIns(4,5)P_2 into the second messenger

molecules inositol (1,4,5)-trisphosphate and diacylglycerol, which are responsible for the release of Ca^{2+} from intracellular stores and the activation of protein kinase C, respectively.

The six PLC isoforms (PLC-β, -γ, -δ, -ε, -ζ, and –η ; Fig 1) are characterized by the presence of conserved X- and Y-boxes, which fold together to form a triose-phosphate isomerase (TIM) barrel and the catalytic core of the enzyme [76]. These isozymes are differentially elaborated with structural/regulatory domains outside of the catalytic region. For example, the two PLC-γ isozymes contain SH2 and SH3 domains and are subject to regulation by tyrosine kinases [112;113], and the four PLC-β isozymes contain a long carboxy terminal domain that confers capacity to bind and be activated by Gα subunits of Gq family of heterotrimeric G proteins [101-103].

Kataoka and colleagues first identified PLC-ε as a Ras-binding protein in *C. elegans* [127], and subsequent work from the laboratories of Kataoka [133], Lomasney [129], and Kelley and Smrcka [130] revealed the presence of PLC- ε in mammalian tissues. Two Ras-associating (RA) domains in the carboxyl terminus of PLC- ε underlie direct regulation of this signaling protein by Ras and Rap [129;133;187] and a conserved CDC25 guanine nucleotide exchange (GEF) domain in the amino terminus provides a poorly understood mechanism whereby PLC- ε acts as an upstream regulator of Ras, Rap, and potentially other Ras superfamily GTPases [129;139]. Although heterotrimeric G proteins of the Gq family do not regulate the activity of PLC- ε, this isozyme is activated by $G\alpha_{12}$ [129], $G\alpha_{13}$ [131], and Gβγ-subunits [131]. Regulation by these heterotrimeric G protein subunits may not be direct, and the fact that several RhoGEFs are effectors of $G\alpha_{12/13}$ [144;145] suggested to us that Rho might be responsible for activation of PLC-ε by $G\alpha_{12/13}$. Indeed, RhoA, RhoB, and RhoC, but not Rac or Cdc42, markedly stimulate the lipase activity of PLC-ε after coexpression in COS-7

cells [142]. Whereas truncation of the carboxy terminal RA domains of PLC-ε result in loss of regulation by Ras, the capacity of both $G\alpha_{12/13}$ and Rho to activate PLC- ε is retained. Expression of C3 botulinum toxin, which ADP-ribosylates and inactivates Rho, results in concomitant loss of capacity of Rho, $G\alpha_{12}$, $G\alpha_{13}$, or LPA or thrombin receptor agonists (which activate $G\alpha_{12/13}$ signaling pathways) to activate PLC-ε [147]. These data are consistent with the idea that heterotrimeric G protein-coupled receptors that activate $G\alpha_{12/13}$ regulate PLC-ε through activation of a RhoGEF and conversion of Rho to its GTP-bound form. Pull down experiments illustrating GTP-dependent interaction of RhoA with PLC-ε also were consistent with this possibility [230].

To address whether PLC-ε is directly activated by Rho, we have developed an *in vitro* system that allows quantification of the enzymatic activity of purified PLC-ε and the capacity of PLC-ε to function as a direct effector for RhoA [231]. This article describes in detail methods for purifying to homogeneity a catalytically active fragment of PLC-ε, as well as methodology for the purification of lipidated RhoA. In both cases we have generated a recombinant baculovirus, and expressed the PLC isozyme or RhoA to high levels using an insect cell expression system. We also present methodology for quantification of the activity of this Rho effector enzyme using PtdIns(4,5)P_2 substrate-containing phospholipid vesicles. Similar methodology for study of regulation of PLC-β2 by Rac has been described previously [232].

2.3 Materials and Methods

Virus Preparation

Baculovirus encoding a fragment of PLC-ε encompassing the EF hand domains through tandem RA domains (PLC-ε EF-RA2: amino acids 1258 through 2215) was prepared

by PCR amplification of the desired regions of rat PLC-ε and subsequent ligation into pFastBacHTb (Invitrogen), which incorporates a hexa-histidine affinity tag at the amino terminus of the coding sequence. The human monomeric GTPase RhoA also was cloned into pFastBacHTb for viral propagation. Viruses are amplified according to the manufacturer's instructions, and the titer of the working stock viruses was approximately 7 x 10^7 pfu/ml. Working stock viruses are stored at 4°C in the dark. Additional aliquots of P1 and P2 viral stocks are frozen at -80°C for long-term storage.

Cell Culture

High Five cells (Invitrogen) are maintained in shaker flasks at 27°C with Express Five SFM supplemented with 10 mM L-glutamine. Cell density is monitored daily and maintained between 0.8 and 4 x 10^6 cells/ml. A day prior to infection, cells are diluted to a density of 0.8 x 10^6 cells/ml in a final volume of 1 L per 4 L shaker flask. Cells are grown overnight in shaker flasks at 150 rpm and 27°C to reach a target density of ~2.0 x 10^6 cells/ml the following day.

PLC-ε EF-RA2 Purification

Overexpression of recombinant protein is somewhat variable using a baculovirus expression system and optimal expression conditions are determined empirically. In our experience, the PLC-ε EF-RA2 fragment is expressed to high levels after 48 hours of infection using a multiplicity of infection (MOI, number of virus particles per cell) of 1.

Four liters of High Five cells (~2.0 x 10^6 cells/ml) are infected with working stock virus encoding hexa-histidine-tagged PLC-ε EF-RA2. Forty-eight hours postinfection, cells are harvested by centrifugation at 1,000*g* for 10 min in a J6 Beckman swinging bucket centrifuge at 4°C. All buffers and subsequent steps during the purification should be at 4°C.

Cells are resuspended in 100 ml of lysis buffer (lysis buffer: 20 mM HEPES, pH 8, 300 mM NaCl, 15 mM imidazole, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol (β-ME), 10 % glycerol, and 2 EDTA-free complete protease inhibitor tablets (Roche Applied Sciences)) and lysed using an EmulsiFlex C5 cell homogenizer (Avestin). Cell lysate is cleared of intact cells and nuclei by low-speed centrifugation (500*g*, 15 min, J6 Beckman centrifuge). The supernatant from the low-speed spin is centrifuged at 150,000g for 45 min in a Type 60Ti rotor in an ultracentrifuge (Beckman). Although the majority of recombinant protein exists in the pellet after the ultracentrifugation step, sufficient PLC-ε EF-RA2 for subsequent purification is recovered in the soluble fraction. The supernatant is diluted to a final volume of 150 ml with lysis buffer and passed through a 5 μM syringe filter to remove any residual debris.

Metal Chelate Affinity Chromatography

Wash a 5-ml HiTrap Chelating HP column (GE Healthcare) with 5 column volumes of de-ionized water using a disposable syringe with luer fittings to remove the ethanol in which the column is stored. The column is then charged with Ni^{2+} by injecting a single column volume (5 ml) of 0.1 M $NiSO_4$ with a disposable syringe. Connect the column to the FPLC and equilibrate with the following washes: 5 column volumes of starting buffer (starting buffer: lysis buffer minus protease inhibitors), 5 column volumes of elution buffer (elution buffer: 20 mM HEPES, pH 8, 300 mM NaCl, 1 M imidazole, 1 mM $CaCl_2$, 1 mM β-ME , and 10% glycerol), and 5 column volumes of starting buffer.

The soluble fraction of the whole cell lysate containing approximately 500 μg of PLC-ε EF-RA2 per liter of cells is loaded onto the chelating column at a rate of 1 ml/min, maintaining a column pressure of < 0.5 mPa. The column is washed with 10 column volumes of starting buffer, followed by 10 column volumes of 50 mM imidazole (5% elution

buffer) to remove proteins nonspecifically bound to the Ni^{2+} charged resin. Ultraviolet absorbance levels measured at A_{280} should return to near baseline during the 5% elution buffer wash. Recombinant protein is eluted using a 50 mM to 1 M imidazole gradient (5-100% elution buffer) over 10 column volumes, collecting 2.5 ml fractions. A peak of absorbance measured at A_{280} will indicate fractions containing the recombinant protein, which is confirmed using Coomassie-stained 7.5 % SDS-PAGE gels (Fig 2).

Fractions containing recombinant protein are pooled and hexa-histidine-tagged tobacco etch virus (TEV) protease is added to the sample to cleave the hexa-histidine tag from the PLC-ε fragment. This mixture is dialyzed overnight against 4 L of dialysis buffer (dialysis buffer: 20 mM HEPES, pH 8, 300 mM NaCl, 1 mM $CaCl_2$, and 10% glycerol) at 4^oC to dilute the imidazole used to elute the recombinant protein from the metal chelate column. The amount of TEV protease used should be sufficient to completely cleave the hexa-histidine tag from the PLC-ε fragment. Because individual TEV protease preparations have varying specific activities, the amount of enzyme required for complete cleavage is determined empirically. Purification of hexa-histidine-tagged TEV protease was described previously [233].

A small amount of precipitation may appear in the sample following overnight dialysis. This material is removed by centrifuging the sample for 10 min at 2,000*g*. The sample is then loaded onto a 1 ml HiTrap Chelating HP column using the FPLC. The 1 ml column is prepared in the same manner as the 5 ml HiTrap Chelating HP column. A flow rate of 0.5 ml/min is used to load the column, keeping the column pressure < 0.5 mPa. Although the hexa-histidine tag is cleaved from PLC-ε EF-RA2 by TEV protease, this PLC-ε fragment possesses a low affinity for the Ni^{2+}-charged resin and is retained on the column

during the loading procedure. The loaded column is washed with 10 column volumes of dialysis buffer. The cleaved PLC-ε fragment is eluted from the column using a 50 mM imidazole (5% elution buffer) wash over 10 column volumes, collecting 1 ml fractions. The presence of a non-cleavable hexa-histidine tag on TEV protease results in retention of this enzyme by the column during the elution of PLC-ε. However, the TEV can be eluted with a 5 column volume, 50 mM to 1 M imidazole (5-100% elution buffer) gradient. Other nonspecific proteins also will be eluted over this range. The presence of the recombinant proteins is confirmed using Coomassie-stained SDS-PAGE (Fig 2).

Sephacryl S-300 Gel-Filtration Chromatography

Fractions from the second metal chelate affinity column purification step containing recombinant PLC-ε EF-RA2 are pooled and applied in a volume of 10 ml to a HiPrep 26/60 Sephacryl S-300 High Resolution column (26 x 600 mm, 320 ml bed volume) (GE Healthcare) equilibrated with buffer A (buffer A: 20 mM HEPES, pH 8, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM DTT, and 5% glycerol). This step is performed to ensure the recombinant PLC-ε EF-RA2 is monodispersed and not aggregated, however, this step does not significantly increase the purity of the sample. A flow rate of 1 ml/min is used to elute proteins and 5 ml fractions are collected starting at injection of the sample. The elution volume (V_e) of the purified PLC is approximately 162 ml as determined by a peak of protein absorbance measured at A_{280}, which is verified with Commassie-stained SDS-PAGE gels (Fig 2). These results indicate that PLC-ε EF-RA2 exists as a 117 kDa monomer relative to protein standards used to calibrate the S-300 column. Fractions containing the recombinant protein are pooled and concentrated using a 50,000 MWCO PES centrifugal filtering device (Vivascience) to a concentration of 1-2 mg/ml. Aliquots of 25-50 μl are snap frozen and

stored at -80°C. Repeated freeze-thaw or freezing of diluted samples should be avoided as the protein may lose enzyme activity. Under optimum conditions, the purification process should take no more than 72 hr between cell lysis and the freezing of aliquots. Typical yields of near homogeneous, monodispersed 117 kDa PLC-ε EF-RA2 are 1-2 mg per 4 L of baculovirus-infected High Five cells.

Prenylated RhoA Purification

Cell Infection and Membrane Protein Extraction

One liter of High Five cells (~2.0 x 10^6 cells/ml) are infected with baculovirus encoding hexa-histidine-tagged RhoA at an MOI of 1. Cells are harvested by low speed centrifugation (1,000*g* for 15 min in J6 swinging bucket centrifuge, Beckman) approximately forty-eight hours after infection. The cell pellet is resuspended in 25 ml of ice-cold lysis buffer (lysis buffer: 20 mM HEPES, pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM β-ME, 5% glycerol, plus one EDTA-free complete protease inhibitor tablet (Roche)) and lysed using an EmulsiFlex C5 cell homogenizer (Avestin). All subsequent steps are carried out at 4°C. The cell lysate is cleared of intact cells and nuclei by low-speed centrifugation (500*g*, 15 min, J6 Beckman centrifuge). Membranes containing prenylated RhoA are harvest by centrifugation of the slow speed supernatant at 150,000*g* for 45 min.

The pelleted membranes are resuspended in 20 ml of extraction buffer (extraction buffer: 20 mM HEPES, pH 8,150 mM NaCl, 5 mM $MgCl_2$, 1 mM β-ME, 5% glycerol, 1% sodium cholate, and one EDTA-free complete protease inhibitor tablet) and homogenized using approximately 20 strokes with a glass/glass dounce homogenizer. The protein concentration of the sample is determined using a Bradford assay, and the sample is diluted to a final protein concentration of 5 mg of protein/ml in extraction buffer. Membrane

proteins are extracted by continuous stirring of the resuspended membrane/detergent mixture at 4°C for 1 hr. Solubilized membrane proteins are recovered by ultracentrifugation of the detergent extracted membranes at 100,000*g* in a Type 60Ti rotor for 1 hr. The clarified supernatant is diluted 3-fold in lysis buffer to achieve a final sodium cholate concentration of approximately 0.3%. Dilution of the detergent sample is important since sodium cholate is an ionic detergent and may interfere with protein binding to the Ni^{2+} charged resin.

Metal Chelate Affinity Chromatography

A 1 ml HiTrap Chelating HP column is prepared as previously described. Using an FPLC, the diluted supernatant containing recombinant RhoA is loaded onto the column at a rate of 0.5 ml/min, maintaining a column pressure of < 0.5 mPa. The column is washed with subsequent steps of 10 column volumes of buffer A (buffer A: 20 mM HEPES, pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM β-ME, 0.3% sodium cholate, and 5% glycerol), and 10 column volumes of 5% buffer B (buffer B: buffer A + 1 M imidazole). Recombinant RhoA is eluted using a 50 to 750 mM imidazole gradient (5-75% buffer B) over 20 column volumes, collecting 1 ml fractions over the gradient. Absorbance measured at A_{280} is used to identify fractions containing recombinant protein, and the precence of RhoA is verified using 12.5% SDS-PAGE Coomassie-stained gels. Fractions containing the recombinant RhoA are pooled and concentrated using a 10,000 MWCO PES centrifugal filtering device (Vivascience).

The concentration of RhoA is determined by replicating the reconstitution assay conditions described below in the presence of [^{35}S]GTPγS. Briefly, approximately 50 pmoles (estimated from Coomassie-stained SDS-PAGE gels) was reconstituted with PE:PtdIns(4,5)P_2-containing vesicles in a final volume of 60 µl (see below for buffer

conditions). The assay includes a 10-fold molar excess of unlabeled GTPγS and approximately 100,000 cpm/assay of [^{35}S]GTPγS. The reaction is incubated at 25°C for 30 min, allowing the intrinsic exchange of GTPγS for GDP on RhoA, and terminated by the addition of 4 ml of stop buffer (stop buffer: 20 mM HEPES, pH 8, 120 mM NaCl, and 25 mM $MgCl_2$). The amount of [^{35}S]GTPγS bound RhoA is quantified by filtering samples over 0.45 μM nitrocellulose filters and liquid scintillation counting of the nitrocellulose membranes.

Phospholipase C assay

Prepare phospholipid vesicles by combining 30 nmoles/assay L-α-phosphatidylethanolamine (PE), 3 nmoles/assay PtdIns(4,5)P_2, and ~10,000 cpm/assay [^{3}H]PtdIns(4,5)P_2, in a 12 x 75 mm glass borosilicate tube and drying the lipids under a constant stream of nitrogen. [^{3}H]PtdIns(4,5)P_2 is isolated from [^{3}H]-inositol labeled turkey erythrocytes as previously described [103] or obtained from commercial sources. The dried lipids are resuspended in 20 μl of 20 mM HEPES, pH 7.4, per assay, and the mixture is probe-sonicated with approximately five 10 sec pulses. Once a homogeneous mixture of lipids is observed the solution is vortexed and placed on ice.

Assays are performed in a final volume of 60 μl in 12x75 mm conical polypropylene tubes. Reaction mixtures are prepared in an ice water bath and each assay includes: 1) 10 μl of 6X assay buffer (6X assay buffer: 120 mM HEPES, pH 7.4, 420 mM KCl, 12 mM DTT, 18 mM EGTA, and 17.1 mM $CaCl_2$ (this concentration of EGTA and $CaCl_2$ will yield a final free calcium concentration of 1 μM)); 2) 20 μl of phospholipid vesicles giving 30 nmoles/assay (500 μM) of PE and 3 nmoles/assay (50 μM) PtdIns(4,5)P_2; 3) 10 μl of 60 μM GDP or GTPγS; 4) 9 μl of 1 mg/ml fatty acid free bovine serum albumin (FAF-BSA); and 5)

10 μl of purified RhoA diluted in buffer A from the FPLC purification. The tubes are vortexed and incubated at 25°C for 20 min to allow GDP or GTPγS to bind to RhoA. Reactions are placed on ice and cooled to 4°C prior to the addition of PLC enzyme.

The amount of PLC enzyme used is determined empirically since different purified fractions potentially exhibit varying specific activities. If 10,000 cpm of PtdIns(4,5)P_2 is used per assay, the amount of hydrolysis observed by PLC-ε in the absence of G protein should be approximately 100-200 cpm above background (usually ~ 50 cpm). Under any given condition, the total number of counts hydrolyzed should not exceed 30% of the starting [^{3}H]PtdIns(4,5)P_2. Given the specific activity of the purified fragment, 1-2 ng of purified protein usually is sufficient to achieve the desired levels of enzyme activity. PLC-ε is diluted to 1 ng/μl in 1 mg/ml FAF-BSA, and the assay is initiated by the addition of 1 μl of enzyme to each tube, which are then vortexed, placed in a 25°C water bath, and incubated for 10 min. Final assay conditions are 500 μM PE, 50 μM PtdIns(4,5)P_2, 20 mM HEPES, pH 7.4, 70 mM KCl, 2 mM DTT, 3 mM EGTA, 2.85 mM $CaCl_2$, 0.16 mg/ml FAF-BSA, and 0.05% sodium cholate (final sodium cholate concentration is below the critical micelle concentration for sodium cholate).

The reaction is terminated by the addition of 200 μl of ice cold 10% trichloroacetic acid and 100 μl of 10 mg/ml FAF-BSA, which will precipitate proteins and non-hydrolyzed lipids. The tubes are vortexed and centrifuged for 10 min at 3,000*g* in a J6 swinging bucket centrifuge at 4°C. This step separates the soluble [^{3}H]Ins(1,4,5)P_3 cleavage product from uncleaved [^{3}H]PtdIns(4,5)P_2. [^{3}H]Ins(1,4,5)P_2 is quantified by liquid scintillation counting of the reaction supernatant.

The specific activity of the phospholipid substrate in cpm/mol is determined by dividing the total cpm of [^{3}H]PtdIns(4,5)P_2 used per assay by the total number of moles of nonradioactive PtdIns(4,5)P_2 used per assay (labeled substrate is not included in the total pmol of PtdIns(4,5)P_2 since there is a substantial molar excess of unlabeled substrate). A concentration of 50 μM PtdIns(4,5)P_2 in a final assay volume of 60 μl gives 3,000 pmoles of substrate per assay. The specific activity is approximately 3.3 cpm/pmol if 10,000 cpm of [^{3}H]PtdIns(4,5)P_2 is used per assay. Dividing this number by the assay duration in minutes will give a final value of pmol/min.

2.4 Acknowledgements

This work was supported by National Institutes of Health grants GM29536, GM57391, and GM65533. JS recognized support from the Pew Charitable Trusts.

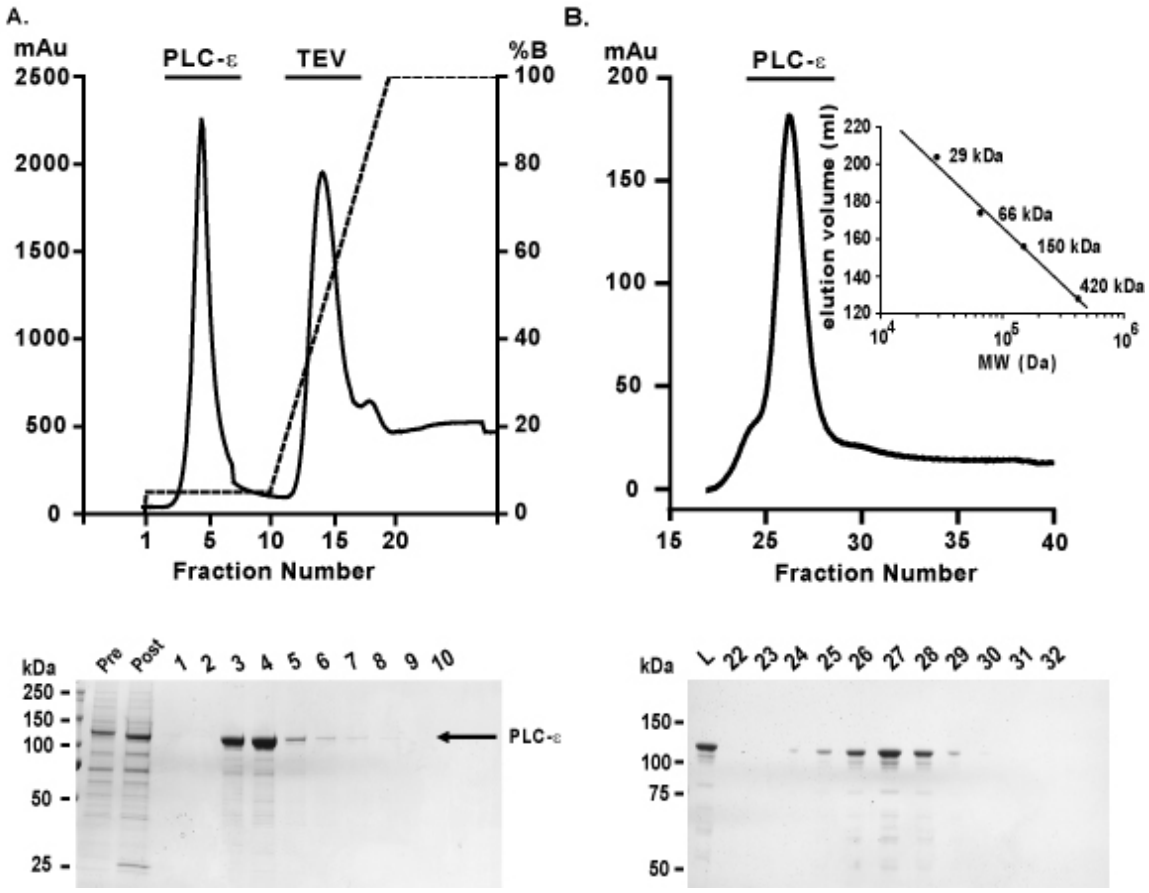

Figure 2.1. Purification of PLC-ε EF-RA2 with metal chelate and size exclusion chromatography. *A, above*, the left-hand *y* axis and solid line indicate mAU (A_{280}) and the right-hand *y* axis and hatched line indicate the percentage of elution buffer (1 M imidazole). The absorbance peak at 5% elution buffer corresponds to the cleaved PLC-ε EF-RA2 fragment and the second absorbance peak indicates the elution of hexa-histidine-tagged TEV protease and other non-specific proteins. *Below*, a Coomassie-stained SDS-PAGE gel showing the uncleaved, pooled fractions from the initial metal chelate elution (*Pre*), TEV protease cleaved PLC-ε EF-RA2 (*Post*) at a lower molecular weight, and elution of 1 ml fractions of cleaved PLC-ε from the second metal chelate column at 50 mM imidazole (5% elution buffer) over a range of 10 ml. Note the presence of TEV protease as a band at ~25 kDa in the post-cleavage lane. *B, above,* elution profile of PLC-ε EF-RA2 after injection onto an S-300 size exclusion column measuring ultraviolet absorbance (A_{280}) versus volume (ml). Protein standards were used to calibrate the S-300 column (inset). *Below*, a load sample (*L*) and samples of 5 ml fractions containing the monomeric PLC-ε EF-RA2 fragment eluted from the S-300 column is shown on a Coomassie-stained SDS-PAGE gel.

CHAPTER 3

RHOA ACTIVATES PURIFED PHOSPHOLIPASE C-EPSILON BY A GUANINE NUCLEOTIDE-DEPENDENT MECHANISM

3.1 Abstract

Phospholipase C-epsilon (PLC-ε) is a recently identified PLC isoform activated by subunits of heterotrimeric G proteins ($G\alpha_{12}$, $G\alpha_{13}$, and $G\beta\gamma$), as well as by the low molecular weight GTPases, Rho and Ras. To define the enzymatic activity and substrate specificity of PLC-ε, as well as its potential direct activation by Rho-family GTPases, a major fragment of PLC-ε encompassing the catalytic core (EF-hand repeats through the tandem RA domains; ~118 kDa) was purified to near homogeneity and assayed after reconstitution under various conditions. Similar to the enzymatic profiles of previously purified PLC-β isozymes, the purified fragment of PLC-ε maximally hydrolyzed PtdIns(4)P at a rate of approximately 10 μmol per milligram protein per minute, exhibited phospholipase activity dependent upon the concentration of free calcium, and favored PtdIns(4,5)P_2 as substrate relative to other phosphoinositides. Furthermore, in mixed detergent phospholipid micelles, RhoA stimulated the phospholipase activity of the PLC-ε fragment in both a concentration-dependent and GTPγS-dependent manner. This activation was abolished by deletion of a unique ~65 amino acid insert within the catalytic core of PLC-ε. Although Rac1 activated purified PLC-β2 in a guanine nucleotide-dependent manner, Rac1 failed to promote guanine nucleotide-dependent activation of purified PLC-ε. These results indicate that PLC-ε is a direct downstream

effector for RhoA and that RhoA-dependent activation of PLC-ε is dependent on a unique insert within the catalytic core of the phospholipase.

3.2 Introduction

Phospholipase C (PLC) isozymes respond to a variety of extracellular signaling molecules to stimulate hydrolysis of PtdIns(4,5)P_2 into the second messengers Ins(1,4,5)P_3 and diacylglycerol [48]. The generation of these two second messengers triggers the release of intracellular Ca^{2+} and stimulation of protein kinase C isozymes, respectively. Five different isoforms of PLC have been identified, each with distinct mechanisms of activation. The PLC-β isozymes are directly activated by heterotrimeric G protein α subunits of the G_q family [101-103] and by Gβγ subunits [104-106], and were shown more recently to be activated by the small GTPase Rac [17;109;110;234]. Activation of receptor and cytosolic tyrosine kinases promotes activation of PLC-γ via phosphorylation and translocation [56;112;113]. The mechanisms by which extracellular stimuli regulate PLC-δ [94;235] and the recently identified PLC-ζ [119;236] are less well understood, although both are believed to require the mobilization of Ca^{2+}.

The fifth family member of PLC isozymes, PLC-ε, initially was identified in *C. elegans* as a Ras-binding protein [127]. Mammalian PLC-ε subsequently was identified as a phospholipase C isozyme containing multiple Ras interaction domains [129;130;133] including a CDC25 homology domain in the amino terminus and tandem Ras-associating (RA) domains at the carboxyl terminus. Co-expression of GTPase deficient, constitutively active Ras mutants with PLC-ε results in accumulation of inositol phosphates in COS-7 cells [130]. Transient coexpression with $G\alpha_{12}$, $G\alpha_{13}$ [129;131], or Gβγ [131] also promotes phospholipase activity of PLC-ε. More recently, a screen of Rho-family GTPases revealed a

marked activation of PLC-ε when coexpressed with RhoA, RhoB, or RhoC, but not with Rac or Cdc42 [142]. This activation was dependent upon a unique ~65 residue insert within the Y box of the catalytic TIM-barrel of PLC-ε and co-precipitation studies indicated an interaction between PLC-ε and RhoA. Although these results suggested that PLC-ε is a direct, downstream effector of Rho GTPases, other interpretations are also consistent with the data.

To provide incontrovertible evidence for direct activation of PLC-ε by GTP-bound Rho GTPases, we have overexpressed and purified a large fragment of the phospholipase (PLC-ε EF-RA2; ~118 kDa) and present here the enzymatic characterization of this fragment reconstituted with phospholipids and GTPases. Relative to previously purified PLC isozymes, purified PLC-ε EF-RA2 exhibited similar specific phospholipase activity and phosphoinositide substrate preferences. Moreover, our analyses illustrate direct, guanine nucleotide-dependent activation of PLC-ε by RhoA and further implicate the catalytic core of PLC-ε as the effector region necessary for RhoA-stimulated phospholipase activity.

3.3 Materials and Methods

Materials

The open reading frame of rat PLC-ε was a kind gift from Grant Kelley, State University of New York, Syracuse, NY. A PLC-ε specific antibody was generated by BioSource against a predicted exposed sequence (aa 1523-1540; LKAHQTPVDILKQKAHQL) directly C-terminal to the X box of the catalytic core. [^{3}H]-labeled phosphoinositide substrates were prepared from [^{3}H]inositol-labeled turkey erythrocytes as previously described [103]. His_6-tagged TEV protease was purified as previously described [233].

Purification of PLC-ε EF-RA2

A fragment of rPLC-ε encompassing approximately half the EF-hand repeats through the second RA domain (amino acids 1258 through 2215) was amplified from full length rPLC-ε by PCR and subcloned into NcoI/XhoI-digested pFastBacHT, which incorporates a His_6-tag and tobacco etch virus (TEV) protease site at the amino terminus of the expressed enzyme. Baculovirus encoding PLC-ε EF-RA2 was generated using the Bac-to-Bac method (Invitrogen). HighFive insect cells at a density of ~2.0 x 10^6 cells/ml were infected with virus encoding PLC-ε EF-RA2 at a multiplicity of infection of 1.0. After forty-eight hours of incubation at 27 ^oC, cells were harvested by low speed centrifugation and resuspended in 100 ml of buffer A (20 mM HEPES, pH 8.0, 300 mM NaCl, 1 mM $CaCl_2$, and 10% glycerol) including 2 EDTA-free Complete protease inhibitor tablets (Roche Applied Biosciences). Cells were lysed using an EmulsiFlex C5 cell homogenizer (Avestin) and the lysate was centrifuged at 100,000*g* for 45 min. The clarified supernatant was diluted to a final volume of 150 ml and loaded onto a 5-ml HighTrap metal chelate column (Amerhsam Biosciences) charged with Ni^{2+}. Purification steps included a 10 column volume buffer A wash, a 10 column volume, 3% buffer B wash (buffer A + 1M imidazole), and recombinant protein was eluted with 10 column volumes of 40% buffer B collected in 5 ml fractions. Fractions containing the recombinant protein were dialyzed overnight against buffer A in the presence of His_6-tagged TEV protease to remove the His_6-tag from PLC-ε EF-RA2. The cleaved protein was subjected to a second passage over a Ni^{2+}-charged metal chelate column and eluted with 5 mM imidazole as a final purification step. Fractions containing the purified protein were pooled and concentrated using a 50,000 MWCO PES centrifugal filtering device (Vivascience). The final concentration of the recombinant protein was determined by

a Bradford assay relative to bovine serum albumin standards. Purification of avian PLC-βt was previously described [237]. PLC-β2 PH-C2 is a fragment of full length human PLC-β2 that encompasses amino acid residues 1 through 801 and was purified as previously described [17].

Purification of FLAG-tagged PLC-ε constructs

Construction of mammalian expression vectors for PLC-ε EF-Ct and PLC-ε EF-CΔY was previously described [142]. PLC-ε EF-Ct spans amino acids 1198 through 2281 and PLC-ε EF-CΔY spans amino acids 1198 through 2281 but lacks 62 amino acids (residues 1667-1728) that occur as an approximately 70 amino acid insert that is conserved in all orthologues of PLC-ε. COS-7 cells were seeded on 150 mm dishes at a density of 5.0×10^6 cells per dish and maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 ^oC in an atmosphere of 90% air/10% CO_2. sixty μl of FuGENE 6 (Roche Applied Science) transfection reagent was used to transfect 20 μg of each PLC-ε vector construct per dish according to manufacturer's protocol. The overexpressed recombinant PLC isozymes were purified using anti-FLAG M2 affinity gel according to the manufacturer's protocol (Sigma). Briefly, approximately 36 hours after transfection, growth medium was removed from the cells and the dishes were rinsed with PBS (10 mM phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4). After aspiration of the PBS, 1 ml of lysis buffer was added per dish, and the dishes were incubated for 15 min with shaking. Cells were scraped from dishes, and the lysate was centrifuged at 12,000*g* for 10 min. The resultant supernatant was subjected to a single gravity flow passage over a 1 ml bed volume of anti-FLAG M2 affinity gel and washed with 10 bed volumes of TBS (50 mM Tris, pH 7.5, 150 mM NaCl) to

remove unbound proteins. Recombinant protein was then collected in six 500 μl elutions with 100 μg/ml FLAG peptide in TBS.

Purification of Small GTPases

The coding sequences of human monomeric GTPases were amplified by PCR and ligated into BamHI/XhoI-digested pFastBacHT. Baculovirus encoding the Rho-family GTPases was produced using the Bac-to-Bac system (Invitrogen). Recombinant proteins were expressed in HighFive insect cells at a density of ~2.0 x 10^6 cells/ml after infection with baculovirus at multiplicity of infection of 1.0. The cells were harvested after 48 h at 27 ^oC by low speed centrifugation and resuspended in 50 ml buffer A (20 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 5% glycerol) with 1 EDTA-free Complete protease inhibitor tablet. Membranes of virus-infected cells were harvested by high speed centrifugation at 100,000 x *g* of the cell lysate for 1 h. Lipidated, membrane-bound small GTPases were solubilized by detergent extraction of the harvested membranes in buffer A containing 1% purified sodium cholate and 1 EDTA-free Complete protease inhibitor tablet for 1 h at 4 ^oC. Solubilized membrane proteins were recovered by centrifugation of the extracted membranes at 100,000*g* for 1 h. The clarified supernatant was diluted with buffer A to achieve a final detergent concentration of 0.3% and subsequently loaded onto a HighTrap metal chelate column charged with Ni^{2+} sulfate. Recombinant protein was collected over an imidazole gradient running from zero to 1 M over 20 column volumes. Fractions containing the recombinant GTPase were pooled and concentrated using 10,000 MWCO PES centrifugal filtering device (Vivascience). The concentrations of the individual GTPases were determined by quantifying the binding of [^{35}S]GTPγS under the same buffer conditions used for reconstitution of the GTPases in phospholipid vesicles described below.

Phospholipase C Assay

Two different procedures were utilized for measuring phospholipase activity of PLC enzymes. Initial experiments measuring maximum enzymatic activity were carried out in the presence of a detergent-substrate mixture. The second assay employed combined phospholipid vesicles with purified GTPases in cholate-containing buffers prior to the addition of PLC-ε. Assays were performed interchangeably using either [^{3}H]PtdIns(4)P or [^{3}H]PtdIns(4,5)P_2 as substrate as indicated in the figure legends.

Maximum enzymatic activity was determined as previously described with minor modification [103]. Briefly, PLC isozymes were incubated in final buffer conditions including 10 mM HEPES, pH 7.4, 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 5.8 mM $MgSO_4$, 0.5% cholate, 100 μM free calcium, 0.16 mg/ml fatty acid free bovine serum albumin (FAF-BSA), 100 μM PtdIns(4)P (Avanti Polar Lipids), and ~10,000 cpm/assay [^{3}H]PtdIns(4)P in a final volume of 60 μl. Assays were at 30 ^{o}C for 10 min and were terminated by addition of 200 μl of 10% trichloroacteic acid and 100 μl of 10 mg/ml FAF-BSA. Centrifugation of the reaction mixture separated aqueous [^{3}H]Ins(1,4)P_2 from precipitated [^{3}H]PtdIns(4)P, and soluble [^{3}H]Ins(1,4)P_2 was quantified by liquid scintillation counting.

Most experiments to determine enzyme characteristics and potential regulation by small GTPases utilized PE- and PtdIns(4,5)P_2-containing lipid vesicles in assays that were previously described [238], with minor modifications . Briefly, recombinant proteins were presented in lipid vesicles containing 50 μM PtdIns(4,5)P_2, 500 μM phosphatidylethanolamine (PE) (Avanti Polar Lipids), and approximately 10,000 cpm [^{3}H]PtdIns(4,5)P_2 per assay. Final buffer conditions were 20 mM HEPES, pH 7.4, 70 mM

KCl, 3 mM EGTA, 2 mM dithiothreitol, 0.16 mg/ml FAF-BSA, and 1 μM free Ca^{2+} in a final volume of 60 μl. The concentration of free calcium was calculated using WinMAXC software (Stanford University). All reactions were performed at 25 ^{o}C for 10 min after the addition of the indicated PLC enzyme. Experiments designed to determine the substrate selectivity of PLC-ε EF-RA2 utilized 1 nmol of each PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2, along with 30 nmol of PE per assay, changing only the [^{3}H]-labeled phosphoinositide substrate for each condition.

For experiments determining the effects of small GTPases on phospholipase activity, 10 μl of purified G-protein was reconstituted in phospholipid vesicles, resulting in a final cholate concentration of 0.05%. The small GTPases were loaded with nucleotide by incubation with 10 μM GDP or 10 μM GTPγS under assay conditions for 20 min at 25 ^{o}C prior to the addition of PLC. Assays were initiated by the addition of PLC enzyme and incubations were for 10 min at 25 ^{o}C. Reactions were terminated by the addition of 200 μl of 10% trichloroacetic acid and 100 μl of mg/ml FAF-BSA. [^{3}H]Ins(1,4,5)P_3 was quantified by liquid scintillation counting of the soluble fraction after centrifugation of the reaction mixture.

Vesicle incorporation assays

To verify whether purified, lipidated GTPases partitioned into phospholipids vesicles, sucrose loaded vesicles were prepared at the same ratio of PE and PtdIns(4,5)P_2 used in reconstitution experiments analyzing PLC enzyme activity. Briefly, 500 μM PE and 50 μM PtdIns(4,5)P_2 per assay were combined in a glass borosilicate tube and dried under a stream of nitrogen. The dried lipid film was resuspended in a 0.2 M sucrose, 20 mM HEPES, pH 7.4 solution and water bath sonicated for 20 min at room temperature. Approximately 0.2 μg

of bacterially or Sf9 expressed, purified GTPase (prespun at >100,000 x *g* for 40 min) was added to 20 µl of sucrose loaded vesicles. The proteins lipid mixture was incubated for 30 min on ice to allow partitioning of proteins into lipid vesicles. Vesicle were diluted to a final volume of 60 µl and final buffer conditions were 20 mM HEPES, pH 7.4, 70 mM KCl, 3 mM EGTA, 2 mM dithithreitol, 0.16 mg/ml fatty acid-free bovine serum albumin, 10 µM free calcium, 0.08% sodium cholate. The mixture was spun at >100,000 x *g* for 45 min to sediment sucrose loaded vesicles, the pellet was washed 1X with assay buffer, and the mixture was spun again for 30 min. The resulting supernatant and washed aspirated pellets subjected to SDS-PAGE and immunoblotted with anti-6xHis antibody to detect purified GTPase. Control experiments included protein added to 0.2 M sucrose in the absence of phospholipid vesicles and the same sedimentation protocol.

3.4 Results

Purification of PLC-ε EF-RA2

A fragment of PLC-ε (Fig 3.1A) encompassing approximately half the EF-hand repeats through second RA domain (amino acids 1258-2215) was purified after overexpression from a recombinant baculovirus in HighFive insect cells as described under Experimental Procedures. The soluble fraction of the enzyme bound efficiently to a metal chelate column as a first purification step. After TEV protease cleavage of the affinity tag, a second passage over a metal chelate column yielded a nearly homogeneous preparation of the recombinant protein (Fig 3.1B). The purified PLC-ε fragment migrated as a species of approximately 118 kDa on SDS-PAGE, as seen by Coomassie staining and immunoblot analysis with a PLC-ε specific antibody. Contaminating proteins observed by Coomassie staining were not immunoreactive, suggesting that the impurities were not breakdown

products of the PLC-ε fragment. Typical yield of purified PLC-ε was 0.5-1.0 mg per liter of baculovirus-infected insect cells. Multiple attempts to purify full-length PLC-ε after insect cell expression have yielded insufficient amounts of purified protein.

PLC activity of PLC-ε EF-RA2

The purified PLC-ε fragment exhibited phospholipase C activity in assays utilizing a detergent and lipid mixture containing 50 μM [^{3}H]PtdIns(4)P as substrate (Fig 3.1C). Enzymatic activity was dependent on the amount of purified phospholipase and was linear over a range of concentrations. The observed enzymatic activity of approximately 10 μmol/min/mg of protein was similar to that of the widely studied purified avian PLC-βt [64;103;105;239]. Subsequent assays were performed utilizing PE- and PtdIns(4,5)P_2-containing vesicles either in the absence of detergent when examining PLC-ε activity alone, or in the presence of 0.05% sodium cholate when reconstituting PLC-ε with vesicles containing purified small GTPases. Phosphoinositide hydrolysis was linear for at least ten minutes under the conditions of these assays (Fig 3.2A). Similar to other PLC isozymes, enzymatic activity of PLC-ε EF-RA2 was dependent on the concentration of free calcium, with an EC_{50} value of approximately 65 ± 11 nM (n = 3) and maximum activity observed at a concentration of approximately 1 μM free Ca^{2+} (Fig 3.2B). Kinetic analyses of PLC-ε EF-RA2 were performed utilizing PE- and PtdIns(4,5)P_2-containing vesicles presented at various concentrations in the bulk medium (see Experimental Procedures). These experiments and Hofstee analyses revealed a V_{max} of ~6.5 μmol/min/mg of protein and a K_m value for PtdIns(4,5)P_2 of approximately 6.0 μM (n = 3) (Fig 3.2C). The phospholipid substrate selectivity of PLC-ε EF-RA2 was determined by using PE- and PtdIns-containing vesicles composed of an equal ratio of PtdIns : PtdIns(4)P : PtdIns(4,5)P_2. The recombinant PLC-ε

fragment hydrolyzed phosphoinositides with a rank order of selectivity of PtdIns(4,5)P_2 > PtdIns(4)P > PtdIns (Fig 3.2D).

Activation of Recombinant PLC-ε by RhoA

Previous studies indicated that the Rho family members, RhoA, RhoB, and RhoC induce stimulation of phospholipase activity when transiently coexpressed with PLC-ε in COS-7 cells [142]. To determine whether the stimulation of PLC-ε by Rho family GTPases is direct, RhoA was expressed from a recombinant baculovirus in insect cells, purified to near homogeneity from detergent-extracted membranes, and reconstituted in substrate-containing phospholipid vesicles. As illustrated in the time course assay in Fig 3.2A, recombinant RhoA (300 nM) stimulated phospholipase activity of PLC-ε by 2.5 fold in the presence of GTPγS, but had no effect in the presence of GDP. To further assess this phenomenon, RhoA was reconstituted over a wide range of concentrations in substrate-containing phospholipid vesicles, and PtdIns(4,5)P_2 hydrolysis was quantified in the presence of the purified PLC-ε fragment (Fig 3.3). The amount of recombinant PLC-ε used (approximately 2 ng) for reconstitution with the GTPases was adjusted to achieve a basal activity of 5-10 pmol/min. RhoA caused a 2-3 fold stimulation of PLC-ε in all experiments. Stimulation was GTPγS-dependent and half-maximal activation was observed at a concentration of RhoA of 58 ± 18 nM (n = 4). The calcium dependence for activation of PLC-ε was not changed in the presence of GTPγS-activated RhoA (Fig 3.2B). GTPγS-dependent activation of PLC-ε was not observed with non-lipidated RhoA purified from bacteria suggesting that carboxy-terminal isoprenylation of RhoA may be required for activation (data not shown).

The selectivity for activation of PLC-ε by Rho-family GTPases was examined by assessing the potential capacity of purified Rac1 to activate PLC-ε following purification of

the lipidated form of the GTPase from baculovirus infected insect cells. At relatively high concentrations (300 nM), Rac1 caused an increase in PLC-ε activity. However, no guanine nucleotide dependence was observed (Fig 3.4). Lower concentrations of Rac1 had no effect on PLC-ε either in the absence or presence of GTPγS (data not shown), and the increased activity observed at higher GTPase concentrations may result from contaminating proteins that accompany the purification. Although Rac1 had no effect on PLC-ε activity, this Rho family GTPase caused an approximate 2-fold, GTPγS-dependent stimulation of PLC-β2 PH-C2 (Fig 3.4), consistent with previously published data [17;109;110].

Activity of the unique Y box insert of PLC-ε

Previous transient expression studies in COS-7 cells indicated that a unique amino acid insert within the Y box of the catalytic TIM-barrel of PLC-ε not present in other PLC isozymes is required for activation of PLC-ε by Rho, $G\alpha_{12}$, and $G\alpha_{13}$ [142]. To further determine the importance of the Y region insert in PLC-ε, two FLAG-tagged fragments of PLC-ε were purified after overexpression in COS-7 cells (Fig 3.5, inset). Both constructs of PLC-ε spanned the EF-hand repeats through the C-terminus, with one fragment lacking 62 amino acids within the Y box (Fig 3.5A).

Analyses of the purified PLC-ε fragments in the absence of RhoA indicated that removal of the insert within the Y box decreased basal enzymatic activity of the enzyme approximately 20-fold. Therefore, when reconstituted with RhoA-containing phospholipid vesicles, the amount of purified PLC-ε fragment added was normalized to give a basal lipase activity of approximately 5 pmol/min with each construct. Under these conditions, 300 nM purified RhoA caused an approximate 2-fold stimulation of the PLC-ε EF-Ct fragment, and this activation was entirely GTPγS-dependent. In contrast, no GTPγS-dependent activation

by RhoA was observed with the PLC-ε fragment lacking the 62 amino acid Y box insert (Fig 3.5B). These data further support *in vivo* data suggesting that the Y-insert region is necessary for RhoA-dependent activation of PLC-ε.

3.5 Discussion

The recent identification of PLC-ε as a downstream effector of Ras and Rho adds a new level of complexity to phospholipid signaling involving monomeric GTPases. In this study, we illustrate the direct, GTP-dependent stimulation of PLC-ε in phospholipid vesicles reconstituted with purified RhoA. These data also extend previous *in vivo* studies in which phosphoinositide hydrolysis by PLC-ε promoted by Rho was shown to be dependent on the presence of a unique ~65 amino acid sequence within the Y box of the catalytic TIM-barrel of PLC-ε. PLC-ε with this region deleted retained phospholipase activation by Gβγ or H-Ras in coexpression experiments in COS-7 cells; however, Rho-, $G\alpha_{12}$-, and $G\alpha_{13}$-dependent activation was not observed [142].

A subfamily of RhoGEFs (*i.e.* p115RhoGEF, LARG, PDZ-RhoGEF) activated by $G\alpha_{12}$ and $G\alpha_{13}$ provides a direct link between stimulation of G-protein coupled receptors and the activation of Rho GTPases [143-146]. Because deletion of the unique insert within the Y box of PLC-ε causes loss of responsiveness to Rho, $G\alpha_{12}$, and $G\alpha_{13}$, we hypothesize that activation of PLC-ε by $G\alpha_{12}$ and $G\alpha_{13}$ occurs indirectly by promoting Rho activation. This suggestion is supported by the fact that C3 toxin, which ADP-ribosylates and inactivates Rho, prevented activation of PLC-ε by Rho, $G\alpha_{12}$, or $G\alpha_{13}$, or by agonist activation of a lysophosphatidic acid receptor, but does not inhibit the activation of PLC-β isozymes by $G\alpha_q$ (M. Hains, M. Wing, T.K. Harden, unpublished data). Direct activation of PLC-ε by Rho does not rule out the possibility of direct activation of PLC-ε by $G\alpha_{12}$ or $G\alpha_{13}$. However, our

combined *in vivo* and *in vitro* data suggest that $G\alpha_{12}$ and $G\alpha_{13}$ mostly likely activate PLC-ε indirectly via a subfamily of RhoGEFs typified by p115RhoGEF.

Although the complete set of determinants within PLC-ε required for interaction with Rho have yet to be identified, our current results, combined with previous *in vivo* data [142], have unambiguously identified a region within the catalytic core of PLC-ε as necessary for the direct stimulation of the phospholipase activity of PLC-ε by GTP-bound Rho. Sequence analyses of the catalytic regions of PLC-ε have not revealed obvious regions of homology to other known Rho-binding domains [211]. In a similar vein, the PH domain of PLC-β2 binds GTP-activated Rac [17]. However, the current experiments directly illustrate that the PH domain of PLC-ε is not required for activation by RhoA. These results suggest that eventual structural determination of the interface will define a novel mode of effector modulation by Rho GTPases.

The mechanisms whereby Rho-family GTPases activate PLC isozymes remain a point of conjecture. Our unpublished studies suggest that prenylation of RhoA is required for stimulation of PLC-ε since soluble bacterial RhoA failed to activate PLC-ε. GTP-dependent binding of the lipidated GTPase to PLC isozymes may promote phospholipase activity by causing translocation of the lipase to the phospholipid bilayer. However, a more active role of GTPases may involve conformational changes in PLC isozymes that enhance phospholipase activity. Perhaps the strongest case for GTPase-stimulated phospholipase activity occurs with the Rho-promoted activation of PLC-ε studied here, whereby binding of Rho within the catalytic core of PLC-ε is readily envisioned to induce conformational changes favorable for lipase activity. Cooperative mechanisms of Rho-dependent activation

of PLC-ε are also possible, whereby Rho induces the lipase activity of PLC-ε through both spatial and conformational regulation of the enzyme.

The addition of PLC-ε as a direct effector in Rho-dependent signaling expands the role of Ras superfamily proteins in phospholipid signaling and metabolism. Historically, Rho family GTPases have been studied as regulators of actin dynamics, gene transcription, cell-cycle progression, and cell adhesion [211]. More recently, Rho has been shown to increase PtdIns(4,5)P_2 levels in cells through activation of phosphatidylinositol 4-phosphate 5-kinase [240;241] and to negatively regulate diacylglycerol kinase activity [242]. Rho may potentiate inositol lipid signaling by increasing PtdIns(4,5)P_2 substrate levels and increasing PKC activity by inhibition of phosphorylation of DAG. PLC-ε potentially is involved in several signal transduction pathways involving multiple Ras family GTPases. The data reported here illustrate PLC-ε to be an effector for GTP-bound RhoA, and future experiments will more completely define the binding regions within PLC-ε and the physiological role played by PLC-ε dependent pathways in Rho-activated signaling.

3.6 Acknowledgements

This work was supported by National Institutes of Health grants GM29316, GM57391, and GM65533. JS recognized support from the Pew Charitable Trusts. The authors are indebted to Gary Waldo for purification of PLC-βt, Mark Jezyk for purification of PLC-β2 PH-C2, and David Bourdon for production of [^{3}H]-labeled phosphoinositides.

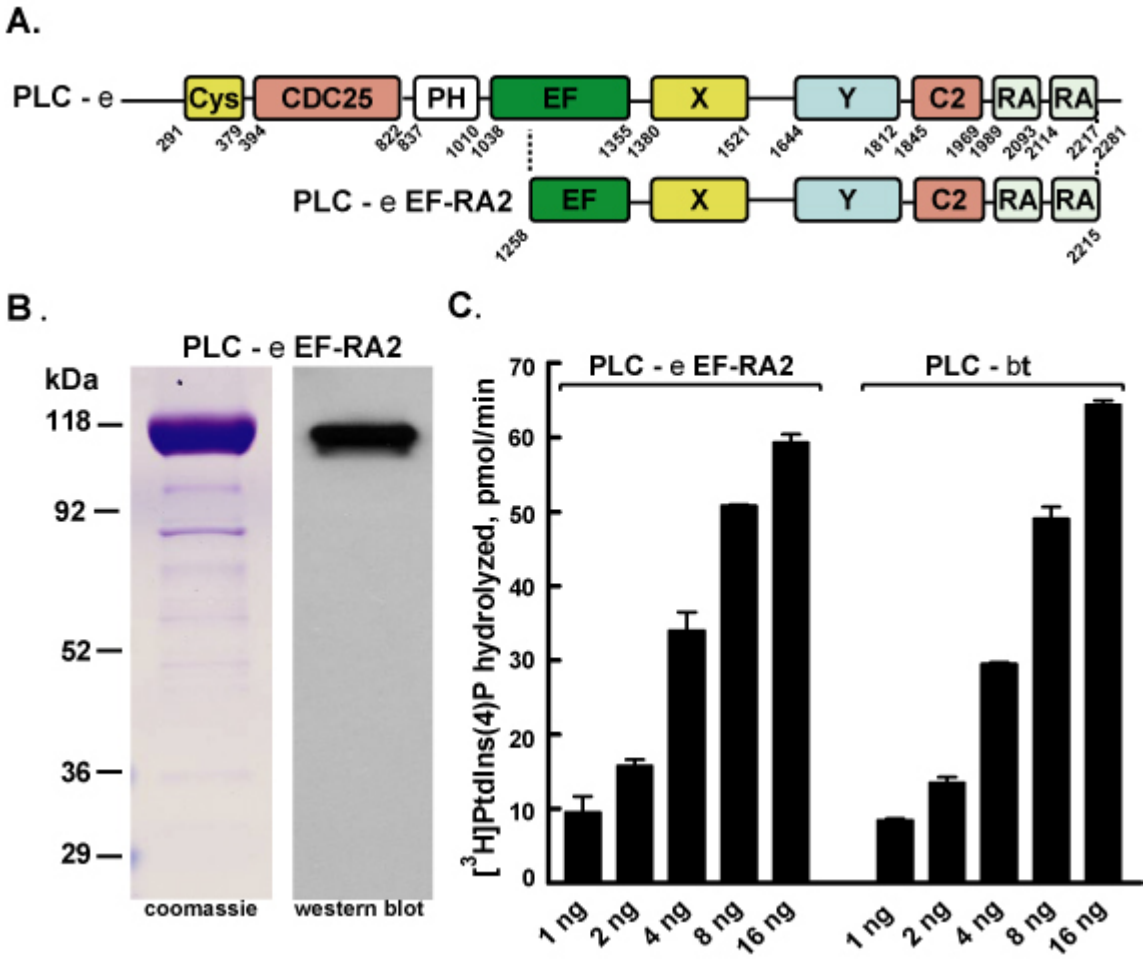

Figure 3.1. Purification and functional activity of PLC-ε EF-RA2. *A,* schematic representation of full-length PLC-ε (2281 total amino acids; top) and PLC-ε EF-RA2 (residues 1258 – 2215; bottom). PLC-ε EF-RA2 lacks the amino-terminal cysteine-rich (Cys), CDC25, and PH domains while retaining approximately half the EF-hand repeats, as well as the catalytic TIM-barrel (X and Y regions), C2 domain, and tandem RA domains. *B,* PLC-ε EF-RA2 was purified as described under Experimental Procedures and final samples of recombinant protein were analyzed by SDS-PAGE using a 4-15% polyacrylamide gradient. Gels were either Coomassie-stained (left) or transferred to nitrocellulose and immunoblotted with an antibody specific to the X-Y linker region of PLC-ε (right). *C,* the specific activity of the purified PLC-ε fragment was measured using [^{3}H]PtdIns(4)P as substrate in the presence of 0.5% sodium cholate for 10 minutes at 30 ^{o}C (left) and compared to the specific activity of purified avian PLC-βt (right).

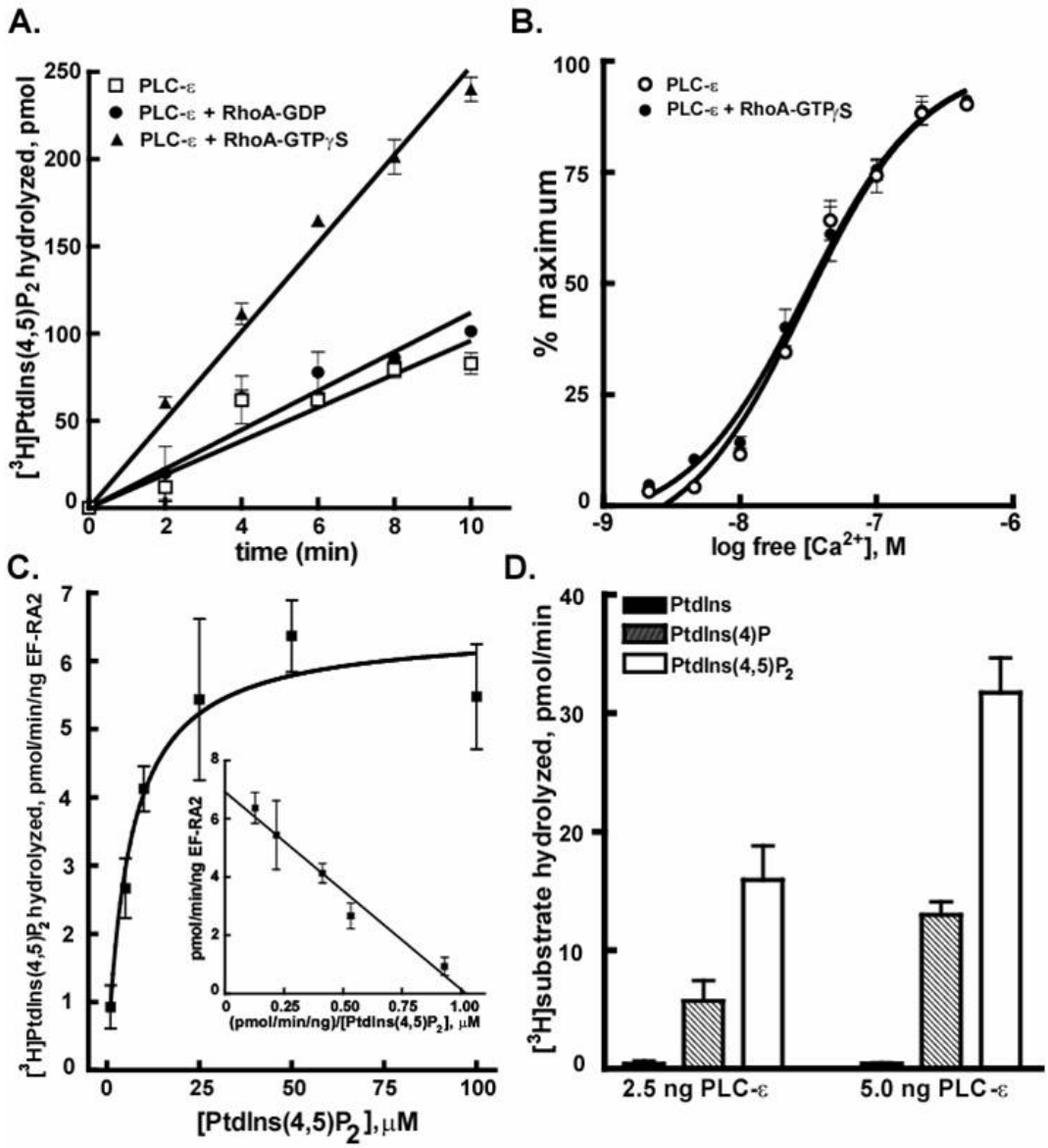

Figure 3.2. Characterization of the phospholipase activity of PLC-ε EF-RA2. *A,* time course of PtdIns(4,5)P_2 hydrolysis in the presence PLC-ε EF-RA2 (10 ng) was tested either in the absence (open squares) or presence of 300 nM inactive (GDP-bound) RhoA (closed circles) or activated (GTPγS-bound) RhoA (closed triangles). *B,* PLC-ε EF-RA2 was incubated with phospholipid vesicles containing [^{3}H]PtdIns(4,5)P_2 substrate under varying calcium concentrations in the absence of RhoA (open circles) or presence of 300 nM GTPγS-bound RhoA (closed circles). *C,* the lipase activity of PLC-ε EF-RA2 (2 ng) was assayed with increasing concentrations of PtdIns(4,5)P_2 substrate. The data shown are the mean ± S.E.M. of three separate experiments and inset is a Hofstee plot of the data. *D,* the substrate selectivity of PLC-ε EF-RA2 was tested by presentation of either [^{3}H]PtdIns (black bars), [^{3}H]PtdIns(4)P (dashed bars), or [^{3}H]PtdIns(4,5)P_2 (open bars) with equal amounts of each cold substrate per assay as described under Experimental Procedures. Enzyme activity was tested with two different concentrations of the purified enzyme.

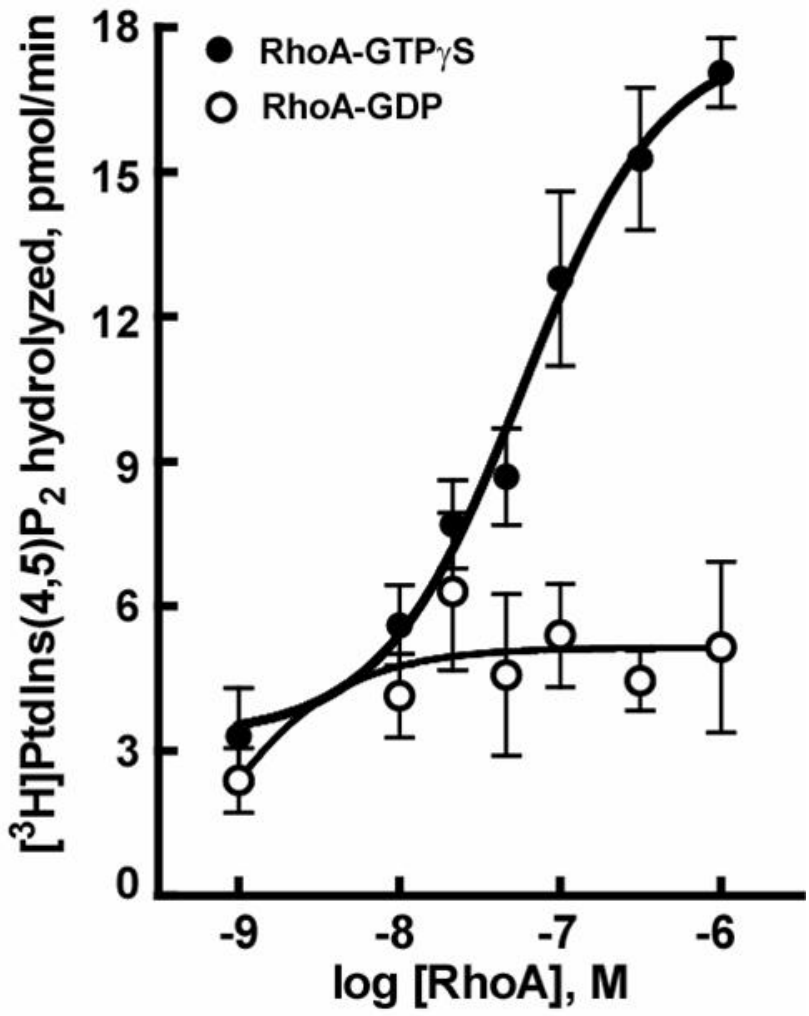

Figure 3.3. Activation of PLC-ε by RhoA. Increasing concentrations of recombinant RhoA purified from detergent-extracted HighFive insect cell membranes were reconstituted in phospholipid vesicles as described in Experimental Procedures. Assays included PLC-ε EF-RA2 in mixed detergent phospholipid micelles containing [^{3}H]PtdIns(4,5)P_2. After a 20 min pre-incubation of RhoA with either 10 μM GDP (open circles) or 10 μM GTPγS (closed circles), PLC-ε EF-RA2 (1-2 ng) was added and the assay was performed for 10 min at 25 ^{o}C. Basal activity of PLC-ε EF-RA2 in the absence of RhoA (5-15 pmol/min) was subtracted to give the final values. The results presented are the mean ± S.E.M. of four individual experiments.

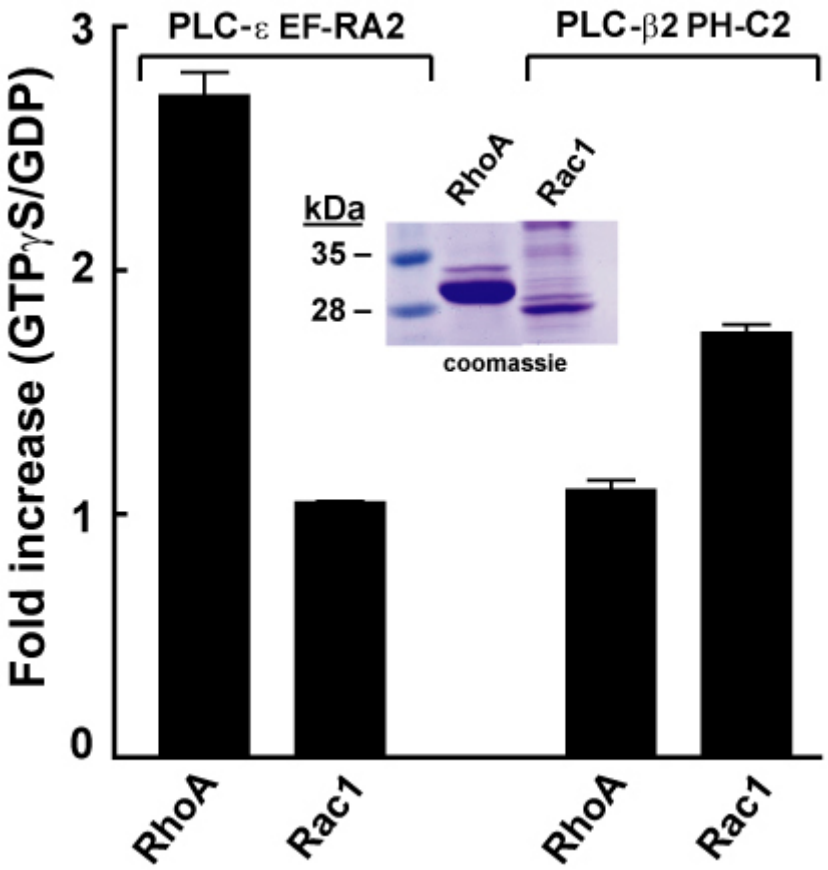

Figure 3.4. Stimulation of PLC isozymes by small GTPases. RhoA and Rac1 were purified from detergent extracted HighFive insect cell membranes after expression from recombinant baculoviruses as described under Experimental Procedures. Inset, purified proteins were resolved by SDS-PAGE and gels were stained with Coomassie blue. Purified PLC-ε EF-RA2 (2 ng) or PLC-β2 PH-C2 (2 ng) were incubated with mixed detergent phospholipid micelles reconstituted with 300 nM of each monomeric GTPase in the presence of 10 μM GDP or 10 μM GTPγS. Data are presented as fold increase of activity observed in the presence of GTPγS after subtraction of the basal PLC activity.

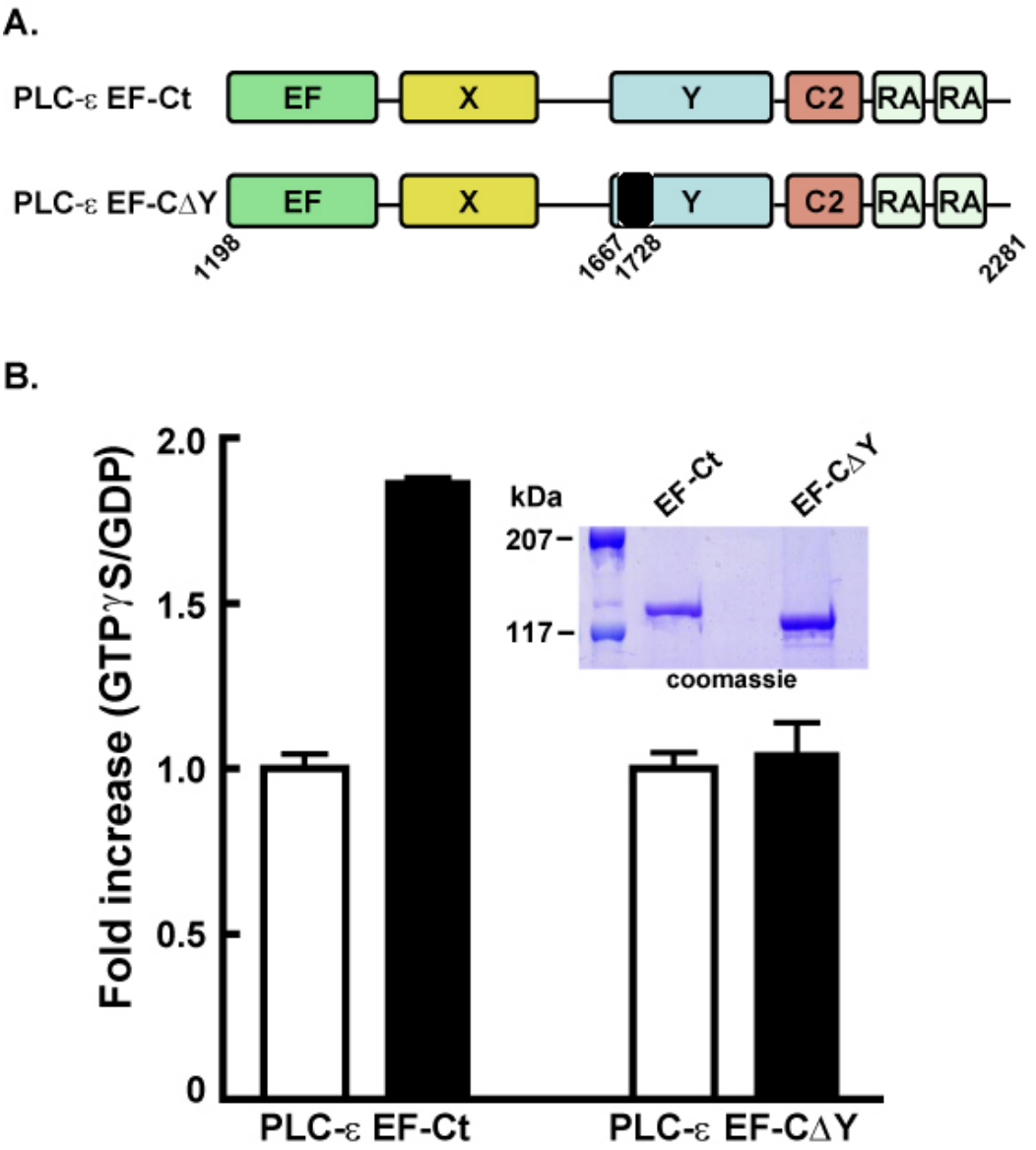

Figure 3.5. Loss of Rho-dependent regulation in a PLC-ε fragment lacking a conserved insert within the Y box. *A,* domain architecture of two PLC-ε fragments that span the EF-hand repeats through the carboxy-terminus. Both constructs span amino acids 1198 through 2281; however, PLC-ε EF-CΔY lacks residues 1667-1728 within the Y box (dark region). *B,* both PLC-ε fragments were purified as described in Experimental Procedures and analyzed by SDS-PAGE using a 4-15% polyacrylamide gradient (inset). PLC-ε EF-Ct (1 ng) and PLC-ε EF- CΔY (20 ng) were incubated with detergent-lipid vesicles reconstituted with 300 nM RhoA in the presence of 10 μM GDP or 10 μM GTPγS using [^{3}H]PtdIns(4,5)P_2 as substrate. The data are presented as the fold increase in phospholipase activity in the presence of GTPγS-bound RhoA over GDP-bound RhoA after subtraction of basal PLC activity.

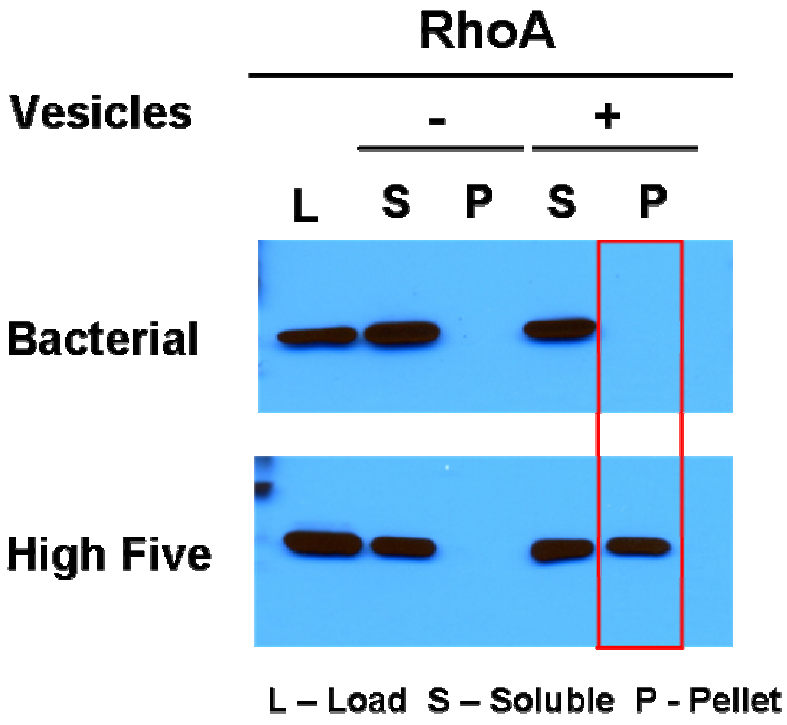

Figure 3.6. Association of purified RhoA with sucrose-loaded phospholipids vesicles. The ability of purified, lipidated RhoA (High Five) versus soluble (Bacterial) RhoA to associated with phospholipid vesicles was tested by addition of proteins to sucrose-loaded vesicles (+) compared to proteins added to sucrose in the absence of lipids (-). Supernatant (S) and pelleted lipid fractions (P) were subjected to SDS-PAGE and proteins were visualized by western blotting with anti-His antibody. The input of total protein (L) is shown as a reference. Data is representative of several G proteins (Rac1, RhoA, Rap1A, Rap2B) tested under the same conditions.

CHAPTER 4

DIRECT, INDEPENDENT, AND DUAL REGULATION OF A RHO AND RAS REGULATED PHOSPHOLIPASE EFFECTOR

4.1 Abstract

Phospholipase C-epsilon (PLC-ε) is an elaborate PLC isoform involved in a diverse set of signaling pathways, including pathways downstream of Ras and Rho family GTPases. To determine whether PLC-ε is regulated directly and dually by Rho and Ras family GTPases, we have used a combination of *in vivo* and *in vitro* assays to quantify regulated phospholipase activity. RhoA and H-Ras markedly stimulated inositol phosphate accumulation in PLC-ε expressing cells and required distinct regions of interaction to regulate enzyme activity. PLC-ε-dependent inositol phosphate accumulation observed in the presence of a single Ras superfamily GTPase was exceeded by coexpression of maximally activating amounts of RhoA and H-Ras together in cells expressing PLC-ε. Purified PLC-ε EF-RA2 also displayed increased activity when presented to PtdIns(4,5)P_2-containing phospholipid vesicles reconstituted with purified, GTPγS-loaded K-Ras or RhoA. Mutation of two critical lysine residues within the second Ras-association domain of PLC-ε prevented K-Ras-dependent activation but retained activation by RhoA. Furthermore, when maximally activated by purified RhoA or K-Ras, PLC-ε was further activated by increasing concentrations of the complementary GTPase. Moreover, PLC-ε displays an increased sensitivity to K-Ras activation in the presence of a maximally activating concentration of RhoA. These results indicate that PLC-ε is a direct downstream and dual effector of

RhoA and Ras GTPases mediated via interactions within distinct regions of the enzyme.

4.2 Introduction

Receptor activation by a variety of hormones, neurotransmitters, growth factors, and other extracellular signaling molecules promotes activation of inositol lipid-specific phospholipase C (PLC) enzymes to catalyze the hydrolysis of PtdIns(4,5)P_2 into diacylglycerol and inositol (1,4,5)-trisphosphate [48]. These second messenger molecules are responsible for the stimulation of protein kinase C isozymes and the mobilization of intracellular Ca^{2+}, respectively [53;54]. The subsequent depletion of cellular PtdIns(4,5)P_2 is also involved in cell signaling as various structured domains, including some PH, FERM, ENTH, and PX domains, specifically bind inositol lipids to promote membrane localization and/or regulate functional activity [5;7]. PtdIns(4,5)P_2 binding proteins are implicated in several cell signaling events including membrane trafficking, changes in the actin cytoskeleton, and ion channel and transporter function [7].

The six PLC subfamilies (PLC-β, -γ, -δ, -ε, -ζ, and -η) all contain conserved X- and Y-boxes, which fold together to form a catalytic triose-phosphate isomerase (TIM) barrel. Elaboration of the catalytic core with various protein binding and/or regulatory domains gives rise to multiple modes of regulation and different PLC subfamilies. PLC-β isozymes are activated by heterotrimeric Gα subunits of the G_q family [101-103], Gβγ subunits [104-106], and the small GTPase Rac [17;71;109;110]. The region linking the catalytic X- and Y-boxes of PLC-γ isoforms encompass a split PH domain, two SH2 domains, and an SH3 domain, resulting in regulation by tyrosine phosphorylation and translocation to the plasma membrane [56;112;113]. More recently, Rac GTPases have been shown to specifically activate PLC-γ2, but not PLC-γ1 [116]. Emerging evidence suggests the recently identified

PLC-η is regulated by heterotrimeric Gβγ subunits [123]. The regulation of PLC-δ [94;235] and PLC-ζ [119;236] are less well understood, although both are believed to be sensitive to lower levels of intracellular calcium compared to other PLC isozymes.

The sixth family member of PLC isozymes, PLC-ε, was initially identified in *C. elegans* as a Ras-binding protein [127]. Subsequent cloning of the mammalian homolog of PLC-ε confirmed the presence of conserved Ras-interacting domains, including tandem carboxy-terminal Ras-associating (RA) domains and an amino-terminal CDC25 homology domain [129;130;133]. Coexpression of constitutively active Ras-family GTPases, including Ras and Rap family members, with PLC-ε results in an increased accumulation of inositol phosphates in COS-7 cells [130;140;142]. *In vitro* studies with various Ras proteins and PLC-ε has yielded conflicting results regarding the possibility of direct regulation of PLC-ε by Ras family GTPases [130;133;141]. In addition to Ras regulation of PLC-ε activity, coexpression of PLC-ε with $G\alpha_{12}$, $G\alpha_{13}$ [129;131], or Gβγ [131] also promotes increased phospholipase activity in COS-7 cells. Finally, we have identified PLC-ε as a direct effector of Rho family GTPases, specifically RhoA, RhoB, and RhoC [142;231]. The combination of *in vivo* coexpression of PLC-ε with Rho GTPases and *in vitro* reconstitution experiments with purified proteins confirmed PLC-ε as a direct downstream effector of Rho GTPases.

Direct activation of PLC-ε by Ras superfamily GTPases has not been unambiguously substantiated in studies with purified proteins. The goals of this study were to establish incontrovertible evidence regarding the direct regulation of PLC-ε by Rho and Ras family GTPases and to show that Rho and Ras interact with distinct regions of PLC-ε to independently and dually regulate PLC-ε enzyme activity. Coexpression of RhoA and Ras GTPases with various PLC-ε constructs confirms previous studies suggesting that

independent sites are involved in Rho- versus Ras-mediated enzyme regulation. Additionally, coexpression of RhoA and H-Ras resulted in PLC-ε-dependent increases in inositol phosphate accumulation that were markedly greater than that observed with expression of PLC-ε with either GTPase individually. To begin an unambiguous evaluation of the potential dual regulation of PLC-ε by two different Ras superfamily GTPases, we have examined this signaling system using *in vitro* reconstitution of purified proteins in model PtdIns(4,5)P_2-containing phospholipid vesicles. Our *in vitro* analyses illustrate that RhoA and K-Ras individually are sufficient to increase the activity of a purified fragment of PLC-ε (PLC-ε EF-RA2) in both a GTPγS- and concentration-dependent manner. Point mutations made at two critical lysine residues in the second RA domain of PLC-ε results in loss of K-Ras- but not RhoA-dependent enzyme activity and confirm *in vivo* data suggesting the sites of interaction on PLC-ε for RhoA and Ras GTPases are mutually exclusive. Additionally, when PLC-ε is activated by a maximally effective concentration RhoA or K-Ras, the activity of PLC-ε is increased further by the addition of increasing concentrations of K-Ras or RhoA, respectively. Moreover, when maximally activated by RhoA, PLC-ε displays an increased sensitivity to K-Ras.

4.3 Materials and Methods

Materials

The open reading frame of rat PLC-ε in pCMV-script was a kind gift from Grant Kelley, State University of New York, Syracuse, NY. Hemagglutinin A-tagged and GTPase deficient mutants of Ras-family GTPases were obtained from the Gutherie Institute (Sayre, PA). His_6-tagged TEV protease was purified as previously described [233].

Construction of Mammalian PLC-ε Expression Vectors

PLC-ε fragments were constructed as previously described [142]. Briefly, full-length rPLC-ε cDNA was used as a template to PCR amplify the selected regions following standard protocols and ligated in-frame into pCMV-myc (Clontech). Constructs were confirmed by sequencing. The specific constructs used and amino acid ranges are as follows: CDC25-Ct, 509-2281; CDC25-RA1, 509-2113; and EF-CtΔY, 1198-2281 lacking residues 1667-1728.

Transfection of COS-7 Cells and Quantification of Inositol Phosphate Accumulation

COS-7 cells were maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 ug/ml streptomycin at 37°C in an atmosphere of 90% air/10% CO_2. Cells were seeded in 12-well culture dishes at a density of ~65,000 cells per well 24 h prior to transfection. The indicated DNA vectors were transfected using FuGENE 6 (Roche Applied Science) transfection reagent according to the manfucaturer's protocol. The total amount of DNA used per well was 700 ng and included empty vector to maintain an equal amount of DNA per well. Approximately 24 h after transfection, the culture medium was changed to inositol-free Dulbecco's modified Eagle's medium (ICN Biomedicals) containing 1 μCi/well *myo*-[2-^{3}H(N)]inositol (American Radiolabeled Chemicals). Metabolic labeling was allowed to proceed for 12-18 h. Accumulation of [^{3}H]-labeled inositol phosphates was quantifiated following a 60 min incubation in the presence the 10 mM LiCl to inhibit inositol monophosphatases. The reaction was stopped by aspiration of the culture medium and subsequent addition of 50 mM formic acid followed by neutralization with 150 mM NH_4OH. [^{3}H]-inositol phosphates were isolated and quantified using Dowex chromatography as described previously [243].

Purification of PLC-ε fragments

PLC-ε EF-RA2 (amino acids 1258-2215) was amplified from full-length rPLC-ε and subcloned into NcoI/XhoI-digested pFastBacHT, which incorporates an amino-terminal hexahistidine tag and tobacco etch protease site for cleavage of the affinity tag. Site-directed mutagenesis was used to generate PLC-ε EF-RA2 (K>E), a construct with two point mutations at lysine residues 2150 and 2152, both mutated to glutamic acid. Baculoviruses encoding each PLC-ε construct were generated using the Bac-to-Bac method per the manufacture's instructions (Invitrogen). PLC-ε EF-RA2 and PLC-ε EF-RA2 (K>E) were purified as described previously with minor modifications [231;244]. HighFive cells were grown in 4 liter shaker flasks at 27°C to a density of ~2.0 X 10^{6} cells/ml and infected with virus encoding either PLC-ε EF-RA2 or PLC-ε EF-RA2 (K>E) at a multiplicity of infection of 1.0. Cells were harvested 48 h after infection by low speed centrifugation and resuspended to a final volume of 150 ml in buffer A (20 mM HEPES, pH 8.0, 300 mM NaCl, 1 mM $CaCl_2$, 10% glycerol, and EDTA-free Complete protease inhibitor tablets (Roche Applied Science)). The resuspended cells were lysed by passage through an EmulsiFlex C5 homogenizer (Avestin). Intact cells and nuclei were removed from the lysate by low speed centrifugation at 500 x *g* for 15 min. The supernatant from the low-speed centrifugation was clarified by centrifugation at 100,000*g* for 1 h. The resulting supernatant was diluted to a final volume of 150 ml and loaded onto a 5-ml HisTrap metal chelate column (GE Healthcare) charged with Ni^{2+}. The loaded column was washed with 10 column volumes of buffer A, followed by a 10 column volume, 3% buffer B wash (buffer A + 1 M imidazole). Recombinant protein was eluted using a 3-50% buffer B linear gradient over 20 column volumes collecting 5 ml fractions. Fractions containing recombinant protein were pooled, and hexahistidine-tagged TEV protease was added to the sample to cleave the hexahistidine-

tag from the PLC-ε fragment. The sample was dialyzed overnight against buffer A to remove the imidazole. The cleaved PLC-ε fragment was subjected to a second passage over a Ni^{2+}-charged 1-ml HisTrap column, and the protein was eluted with buffer containing 5 mM imidazole, which separates of the cleaved PLC-ε fragment from the hexahistidine-tag and TEV protease. Fractions containing the recombinant protein were pooled and concentrated using a 50,000 MWCO PES centrifugal filtering device (Sartorius). PLC-β2 PH-C2 (amino acids 1-801) was purified as described previously [71].

Purification of Monomeric GTPases

Full-length human monomeric GTPases were amplified by PCR and ligated into BamHI/XhoI-digested pFastBacHT, which incorporates an amino-terminal hexahistidine tag. Baculoviruses of each individual GTPase were produced using the Bac-to-Bac system per manufacture's protocol. HighFive cells at a density of ~2.0 X 10^6 cells/ml were infected with baculovirus at an MOI of ~1.0. 48 h after infection, cells were harvest at 27°C by low speed centrifugation and resuspended in a final volume of 100 ml of buffer A (20 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 5% glycerol, 1 mM DTT, one EDTA-free Complete protease inhibitor tablet). Cells were lysed using an Emulsiflex C5 homogenizer. Cell lysate was cleared of intact cells and nuclei by low speed centrifugation at 500*g* for 15 min. Membranes of virus-infected cells were harvested from the resulting supernatant by high speed centrifugation at 100,000*g* for 1 h. Post-translationally modified, membrane bound GTPases were extracted from the harvested membranes after resuspension in buffer A containing 1% sodium cholate and one EDTA-free Complete protease inhibitor at a final protein concentration of 5 mg/ml. Resuspended membranes were subjected to rapid stirring

for 1 h at 4°C. Solubilized membrane proteins were separated from the particulate fraction by centrifugation at 100,000*g* for 1 h.

Ni-NTA agarose (Qiagen) was added to the resulting supernatant and incubated for 1 h at 4°C. The resulting slurry mixture was added to a gravity flow column and washed with 10 CV of buffer A containing 500 mM NaCl. The column was then equilibrated with buffer A containing 10 mM NaCl, and recombinant protein was eluted in 10 CV buffer B (20 mM HEPES, pH 8.0, 10 mM NaCl, 1 mM $MgCl_2$, 400 mM imidazole, 5% glycerol, 0.5% sodium cholate). Sample containing the eluted protein was diluted to 50 ml in buffer B and applied to a 1 ml HiTrap Q column (GE Healthcare). The loaded column was washed with 10 CV of buffer B and protein was eluted using a linear NaCl gradient from 10 mM to 500 mM over 25 CV. Fractions containing the recombinant protein were pooled and applied directly to a 1-ml HisTrap column (GE Healthcare) charged with Ni^{2+}. The loaded column was washed with 10 column volumes of buffer C (20 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% sodium cholate, 5% glycerol) and 10 column volumes of 5% buffer D (buffer C + 1 M imidazole). Protein specifically bound to the column was eluted with a 0 to 50% buffer D gradient over 20 CV collecting 1 ml fractions. Fractions containing recombinant GTPase were pooled and concentrated using a 10,000 MWCO PES centrifugal filtering device. All steps were completed within one day with no freezing of the sample between column purifications. The concentrations of the individual GTPases were determined by quantifying the binding of [^{35}S]GTPγS under identical buffer and assay conditions described below for reconstitution of the GTPases with phospholipid vesicles.

Phospholipase C Reconstitution Assay

Experiments to determine PLC enzyme regulation by G proteins were performed as previously described with minor modifications [231;244]. Briefly, phospholipid vesicles were created by combining 600 uM PE (Avanti Polar Lipids), 50 uM PtdIns(4,5)P_2, and ~10,000 cpm of [^{3}H]PtdIns(4,5)P_2 (Perkin Elmer) per assay into a glass borosilicate tube and drying the mixture under a stream of nitrogen. Vesicles were created by resuspension of the dried lipids in 20 mM HEPES, pH 7.4, followed by probe sonication. Assay were performed in a final volume of 60 µl with final buffer conditions containing 20 mM HEPES, pH 7.4, 70 mM KCl, 3 mM EGTA, 2 mM dithithreitol, 0.16 mg/ml fatty acid-free bovine serum albumin, 10 µM free calcium, and 0.08% sodium cholate. Purified GTPases were diluted 6-fold into the assay, resulting in a final cholate concentration of 0.03%. The GTPases were loaded with nucleotide in the absence of PLC by incubating the G protein/lipid suspension for 30 min at 30°C with 10 µM GDP or 10 µM GTPγS. Reactions were initiated by the addition of purified PLC and incubations were at 30°C for 5-10 min as indicated in the figure legends. Reactions were terminated by the addition of 200 µl of 10% tricholoroacetic acid and 100 µl of 10 mg/ml fatty acid-free bovine serum albumin. Soluble [^{3}H]inositol phosphates were quantified by liquid scintillation counting of the soluble fraction after centrifugation of the reaction mixture.

4.4 Results

Activation of PLC-ε by Small GTPases

Our previous studies suggested that Rho and Ras GTPases regulate the activity of PLC-ε through specific and separate regions of the enzyme [142]. These observations were extended by a series of experiments in COS-7 cells transiently transfected with DNA encoding RhoA or H-Ras with various constructs of PLC-ε. Activity observed with PLC-ε

lacking the amino-terminal region (CDC25-Cterm) was markedly increased by coexpression with either RhoA or H-Ras (Fig 4.1B). Removal of the RA2 domain of PLC-ε (CDC25-RA1) resulted in an isozyme that was not activated by H-Ras, but retained RhoA-promoted inositol phosphate accumulation. PLC-ε lacking an insertion within the Y-box that is unique to PLC-ε isozymes (EF-CtΔY) was not activated by coexpression with RhoA; however, removal of the Y-box insert did not affect the ability of PLC-ε to be regulated by H-Ras [142]. These experiments confirm previously identified regions required for GTPase-dependent regulation of PLC-ε and suggest that PLC-ε potentially is dually regulated by both Rho and Ras GTPases.

To assess the ability of PLC-ε to be regulated simultaneously by Rho and Ras GTPases, full-length PLC-ε was expressed in COS-7 cells with various amounts of RhoA or H-Ras DNA to determine the amount of DNA required for maximal PLC-ε stimulation (data not shown). These maximal activating amounts of RhoA and H-Ras then were transiently expressed together with PLC-ε in COS-7 cells. As illustrated in Fig 4.2, coexpression of RhoA and H-Ras together resulted in PLC-ε-dependent inositol phosphate accumulation significantly greater than that observed in the presence of either GTPase individually. Western blots confirmed that the levels of GTPase or PLC-ε expression from the indicated amounts of DNA were not changed by coexpression with various other expression vectors.

Selective G Protein Regulation of PLC-ε Activity

To unequivocally illustrate that GTPases specifically increase the activity of PLC-ε via direct protein-protein interactions, various G proteins (*i.e.* RhoA, K-Ras, Rac1, and Gβγ) were overexpressed in insect cells, harvested from detergent extracted membranes, and purified to homogeneity as described under "Experimental Procedures." K-Ras was used as

the model Ras GTPase in these studies because it was readily purifed in ample amounts as an active and lipidated protein. Purified, lipidated GTPases were reconstituted in [^{3}H]PtdIns(4,5)P_2 substrate-containing phospholipid vesicles and their capacity to activate purified PLC-ε was examined. Both RhoA (125 nM) and K-Ras (125 nM) markedly increased the enzyme activity of purified PLC-ε EF-RA2 and the stimulatory effects of these GTPases occurred following preloading of the GTPases with GTPγS, but not with GDP (Fig 4.3A). Soluble GTPases purified from bacteria failed to activate PLC isozymes (data not shown).

In contrast to the activation observed with RhoA or K-Ras, purified, GTPγS-loaded Rac1 did not increase the activity of PLC-ε. GTPγS-bound Rac1 did increase the activity of purified PLC-β2 PH-C2 (Fig 4.3B), consistent with previously published data [110;231]. Under the same assay conditions, GTPγS-bound RhoA and K-Ras did not activate purified PLC-β2 PH-C2. A small increase in PLC activity occurred with RhoA and K-Ras, as well as Rac1, in the presence of GDP and we attribute this to effects resulting from reconstitution of various amounts of protein in the vesicle preparation, often leading to changes in overall enzyme activity.

Results from our previous studies illustrated increased inositol phosphate accumulation in COS-7 cells transiently transfected with Gβγ dimers and PLC-ε [131]. To assess if the observed increase in lipase activity is due to a direct interaction between Gβγ and PLC-ε, purified $G\beta_1\gamma_2$ was reconstituted in phospholipid vesicles, and inositol lipid hydrolysis was measured in the presence of purified PLC-ε EF-RA2 or PLC-β2 PH-C2. As illustrated in Fig 3C, purified, lipidated $G\beta_1\gamma_2$ (200 nM) increased the activity of purified PLC-β2 PH-C2 by ~10-fold, but had no effect on PLC-ε EF-RA2 activity. Various other

concentrations of purified $G\beta_1\gamma_2$ ranging from 1 nM to 500 nM also did not increase the activity of purified PLC-ε EF-RA2 (data not shown), suggesting the increased enzyme activity observed when PLC-ε is coexpressed with Gβγ dimers *in vivo* is not due to a direct interaction. Additionally, when $G\beta_1\gamma_2$ was reconstituted with GTPγS-bound RhoA or K-Ras in phospholipid vesicles, the activity of purified PLC-ε EF-RA2 was not increased above the level observed in the presence of RhoA or K-Ras alone (data not shown).

Regulation of PLC-ε by RhoA and K-Ras Through Interactions with Distinct Regions

Interaction and regulation of PLC-ε with various Ras-family GTPases in intact cells is dependent on the presence of the second Ras-associating domain of PLC-ε [130;133;142]. In contrast, RhoA-dependent regulation of PLC-ε requires only the catalytic core of the enzyme and is dependent on the presence of a unique insert within the Y-box of the PLC-ε catalytic region [142;231]. To determine whether PLC-ε is directly regulated through distinct interactions of the enzyme with RhoA and K-Ras, a double-mutation in the second RA2 domain of PLC-ε EF-RA2 (K>E), previously shown by Kelley *et al.* to inhibit activation of PLC-ε by Ras GTPases *in vivo*, was introduced into the wild-type PLC-ε EF-RA2 construct [130]. The double mutation in the RA domain of PLC-ε results in a charge reversal of both lysine residues, creating a negatively charged surface potential, which ostensibly repels the negatively charged potential of Ras family switch regions. Additionally, amino acid 2150 was recently shown to form contacts with the switch I region of H-Ras [135]. The PLC-ε EF-RA2 (K>E) fragment was purified to homogeneity using the same methodology used to purify wild-type PLC-ε EF-RA2.

Analyses of the purified PLC-ε fragments in the absence of RhoA or K-Ras indicated that the double mutation within the second RA domain of PLC-ε EF-RA2 (~3.0 pmol/min/ng

protein) had little effect on basal phospholipase activity compared to wild-type PLC-ε EF-RA2 (~4.9 pmol/min/ng protein). When reconstituted with RhoA or K-Ras, wild-type PLC-ε EF-RA2 activity was increased in the presence of RhoA and K-Ras in a GTPγS-dependent manner (Fig 4.4). In contrast, whereas the PLC-ε EF-RA2 double mutant retained stimulation by GTPγS-bound RhoA, enzyme activity was not increased by GTPγS-bound K-Ras over a wide range of concentrations. These data support the combined *in vivo* and *in vitro* data suggesting that two distinct sites of interaction exist in PLC-ε for RhoA and K-Ras, and that the second RA domain is essential for Ras-dependent activation of PLC-ε.

Dual Regulation of PLC-ε by RhoA and K-Ras

Results from the previous experiment confirm that PLC-ε is regulated by RhoA and K-Ras in a GTPγS-dependent manner through interactions with distinct regions of PLC-ε. To test whether a combination of the two GTPases increases the activity of PLC-ε above that observed with a single GTPase, RhoA and K-Ras were co-reconstituted in the same phospholipid vesicle preparation, and the phospholipase activity of purified PLC-ε EF-RA2 was assessed. At maximally activating concentrations of either RhoA or H-Ras (300 nM) preloaded with GTPγS, the rate of substrate hydrolysis of wild-type PLC-ε ER-RA2 increased approximately 2.5-fold over that observed with PLC-ε alone (Fig 4.5, Table 4.1). When maximally activating concentrations of RhoA (300 nM) and K-Ras (300 nM) were combined in the same vesicles, the enzymatic rate of PLC-ε EF-RA2 was approximately additive with the stimulation observed with RhoA or K-Ras individually (Fig 4.5). Inositol lipid hydrolysis was linear for at least 8 min under the assay conditions utilized.

To further assess the regulation of PLC-ε by both RhoA and K-Ras, each individual GTPase was reconstituted over a wide range of concentrations in phospholipid vesicles with

wild-type PLC-ε EF-RA2 (Fig 4.6). The enzyme activity of purified PLC-ε was increased in a concentration-dependent manner by GTPγS-bound RhoA or K-Ras. At maximal concentration of either RhoA or H-Ras (300 nM), the rate of substrate hydrolysis of wild-type PLC-ε ER-RA2 increased by approximately 2.5-3 fold (Fig 4.6, Table 4.1). Under these assay conditions, RhoA (~20 nM) and K-Ras (~15 nM) exhibited similar EC_{50} values for activation of PLC-ε EF-RA2. Additionally, when the same concentration range of RhoA was examined in the presence of a maximally activating concentration of K-Ras (300 nM), the resulting activity was essentially additive, and little or no change in the observed EC_{50} of RhoA was observed (Fig 4.6A). Concentration-dependent activation of PLC-ε by K-Ras was observed in the presence of a maximally activating concentration of RhoA (300 nM). However, in contrast to the lack of effect of K-Ras on the RhoA activation curve, the presence of 300 nM RhoA resulted in an approximately three fold decrease in the EC_{50} observed for K-Ras (Table 4.1). Thus, dual, independent, and additive activation of PLC-ε occurs in response to purified RhoA and K-Ras. Additionally, maximal activation of the isozyme by RhoA results in an increase in sensitivity to activation by K-Ras.

4.5 Discussion

The presence of tandem carboxy-terminal RA domains has implicated PLC-ε as a potential downstream efffector of activated Ras GTPases; however, *in vitro* studies provide conflicting results regarding the ability of Ras family proteins to directly regulate PLC-ε enzyme activity [130;133;135]. In this study, we demonstrate the direct, GTPγS- and concentration-dependent stimulation of purified PLC-ε in phospholipid vesicles reconstituted with purified, lipidated K-Ras. These data also confirm previous studies suggesting association with the RA2 domain is required for Ras-regulated activity, since mutation of two

critical lysine residues within this region results in the loss of Ras-promoted enzyme activity. However, mutations within the second RA domain of PLC-ε did not affect Rho-promoted activation *in vivo* or *in vitro*. These data substantiate our *in vivo* coexpression experiments implicating PLC-ε as a direct downstream effector of Rho and Ras GTPases through interactions with distinct regions of PLC-ε.

Determination of the individual regions of PLC-ε required for Rho and Ras family regulation, as well as apparent additive stimulation of PLC-ε activity upon coexpression with Rho and Ras in COS-7 cells, suggested potential dual regulation of PLC-ε by Rho and Ras family GTPases. Indeed, when purified PLC-ε EF-RA2 was added to phospholipid vesicles reconstituted with maximally activating concentrations of RhoA and K-Ras, the phospholipase activity of PLC-ε EF-RA2 exceeded that observed with either GTPase individually. Additionally, when maximally activated by RhoA, PLC-ε displayed increased sensitivity to K-Ras activation, with an approximately three-fold leftward shift in the apparent EC_{50} compared to the activation observed with K-Ras alone. These data suggest that K-Ras and RhoA ultimately regulate PLC-ε activity by different mechanisms. Herein, we have identified PLC-ε as a unique enzyme that is regulated dually by two separate Ras superfamily GTPases through interactions with distinct regions of the effector protein.

The observed additive activity of PLC-ε by Rho and Ras family GTPases suggests the binding of both GTPases does not cause in a transfer of energy resulting in a conformational change within the active site of the enzyme. The relatively large size of PLC-ε allows the enzyme to bind various GTPases through distinct regions while the binding of one GTPases would not affect the binding of a complimentary GTPase at a more distant site. However, although the activity of both GTPases together is additive, our data suggests that activation of

PLC-ε by RhoA increases the sensitivity to K-Ras activation. These data would suggest that RhoA binding to PLC-ε either orients the enzyme at the membrane in a favorable position for K-Ras to bind the RA2 domain of PLC-ε; or that RhoA binding relieves inhibition of the RA domains. Our hypothesis is that the binding of Rho or Ras GTPases to PLC-ε are mutually exclusive events and that the binding of PLC-ε to membrane associated GTPases results in an increased ability of the enzyme to associate with lipid membranes. Under these conditions, the binding of a single GTPases would reduce the ability of PLC-ε to dissociate from the membrane, thereby lowering the off rate between the enzyme and membrane. Upon binding to both Rho and Ras GTPases, the off rate for the PLC-ε/membrane event is reduced even further, essentially locking the enzyme at the membrane and increasing the activity observed with either GTPase alone.

Other mechanisms of dual regulation of PLC-ε by multiple GTPases are possible; however, our kinetic data suggests the binding events are mutually exclusive events. If the activity of PLC-e were greater than additive in the presence of both GTPases, we would hypothesize that the binding of both GTPases would result in a combined effort to activate the enzyme. For instance, the binding of Rho and Ras to a similar region near the catalytic site could move the X-Y linker region of the enzyme, thereby relieving the autoinhibition and ultimately leading to synergistic activation. Conversely, the binding of both GTPases could reduce the activity observed with a single GTPase, which would suggest a either a similar binding site for both GTPases or that the binding of either GTPase would reduce the binding of the other GTPase. Finally, it would also be possible for the activity of PLC-e to remain at the level of either GTPase individually in the presence of both GTPases. This scenario

would suggest a shared binding site for both Rho and Ras GTPases, or that the off rate for the enzyme/membrane association can not be increased in the presence of both GTPases.

Many signaling cascades emanate from activation of the over 150 Ras superfamily GTPases. However, dual regulation of a single protein by RhoA and Ras GTPases to our knowledge does not exist. Our current understanding of PLC-ε regulation places the enzyme at a point of convergence for several signaling pathways downstream of both heterotrimeric and multiple Ras superfamily GTPases. However, the spatial and temporal regulation of PLC-ε by multiple GTPase signaling pathways has yet to be delineated. Other well characterized effectors of multiple Ras superfamily GTPases, also involved in phospholipid metabolism, are class I PI3Ks. Ras and Rac GTPases interact with distinct subunits of PI3Ks to regulate functional activity; Ras GTPases binding to the p110 subunit, and Rac GTPases binding to the p85 subunit of PI3Ks [182;245;246].

The mechanisms by which Ras superfamily GTPases activate PLC-ε have yet to be elucidated. Our studies suggest that lipidation of GTPases is essential for the activation of PLC isozymes in reconstitution assays because bacterially expressed soluble GTPases failed to activate PLC-ε. The requirement for lipidation suggests activated Ras superfamily GTPases recruit PLC-ε to phospholipid membrane surfaces resulting in increased enzyme activity. Other potential mechanisms of PLC-ε regulation by Ras superfamily GTPases involve the initiation of conformational changes within the enzyme upon binding of activated GTPases. Bunney and colleagues proposed a mechanism of PLC-ε autoinhibition whereby the RA domains fold back onto the catalytic regions of the enzyme, which is relieved upon binding to Ras and translocation to the plasma membrane [135]. Additionally, several lines of evidence have implicated the highly variable X-Y linker region in autoinhibition of PLC

enzymes by occluding the active site of several isozymes [60;70;71]. Our identification of a unique sequence in the Y-box of PLC-ε that is required for RhoA-promoted PLC-ε activity suggests the potential for Rho GTPases to relieve this autoinhibition by binding PLC-ε proximal to the X-Y linker region.

Mechanistic insights into the regulation of PLC-ε by Ras superfamily GTPases will be aided by structural determination of PLC-ε in complex with G protein activators. The recent solved crystal structure of PLC-β2 in complex with Rac1 revealed direct binding of activated Rac1 to the PH domain of PLC-β2 [71]. Comparing the structure of PLC-β2 alone to PLC-β2 bound to Rac1 suggests little or no change in the conformation of the catalytic region of PLC-β2. Thus, binding to Rac1 results in membrane localization and orients the catalytic region of PLC-β2 in a favorable position to facilitate increased substrate access. Similar mechanisms of PLC-ε regulation by Ras superfamily GTPases are readily envisioned; however, binding to Ras superfamily GTPases could also induce conformational changes in the catalytic site of PLC-ε not observed with PLC-β2 and Rac1.

Two decades ago, two groups independently suggested a role for Ras family proteins in the regulation of phosphoinositide metabolism [125;126]. The identification of PLC-ε as a direct and dual effector of Rho and Ras adds a new level of complexity to the role of Ras superfamily proteins in phospholipid signaling and metabolism. Because PLC-ε is cooperatively regulated by interactions with both Rho and Ras, we envision physiological scenarios in which PLC-ε activity is simultaneously activated by multiple GTPases resulting in increased phospholipid metabolism. Future experiments will attempt to elucidate the spatial and temporal regulation of PLC-ε by multiple GTPase signaling pathways, as well as

provide structural information regarding the molecular mechanisms by which Ras superfamily GTPases regulate the function of PLC-ε.

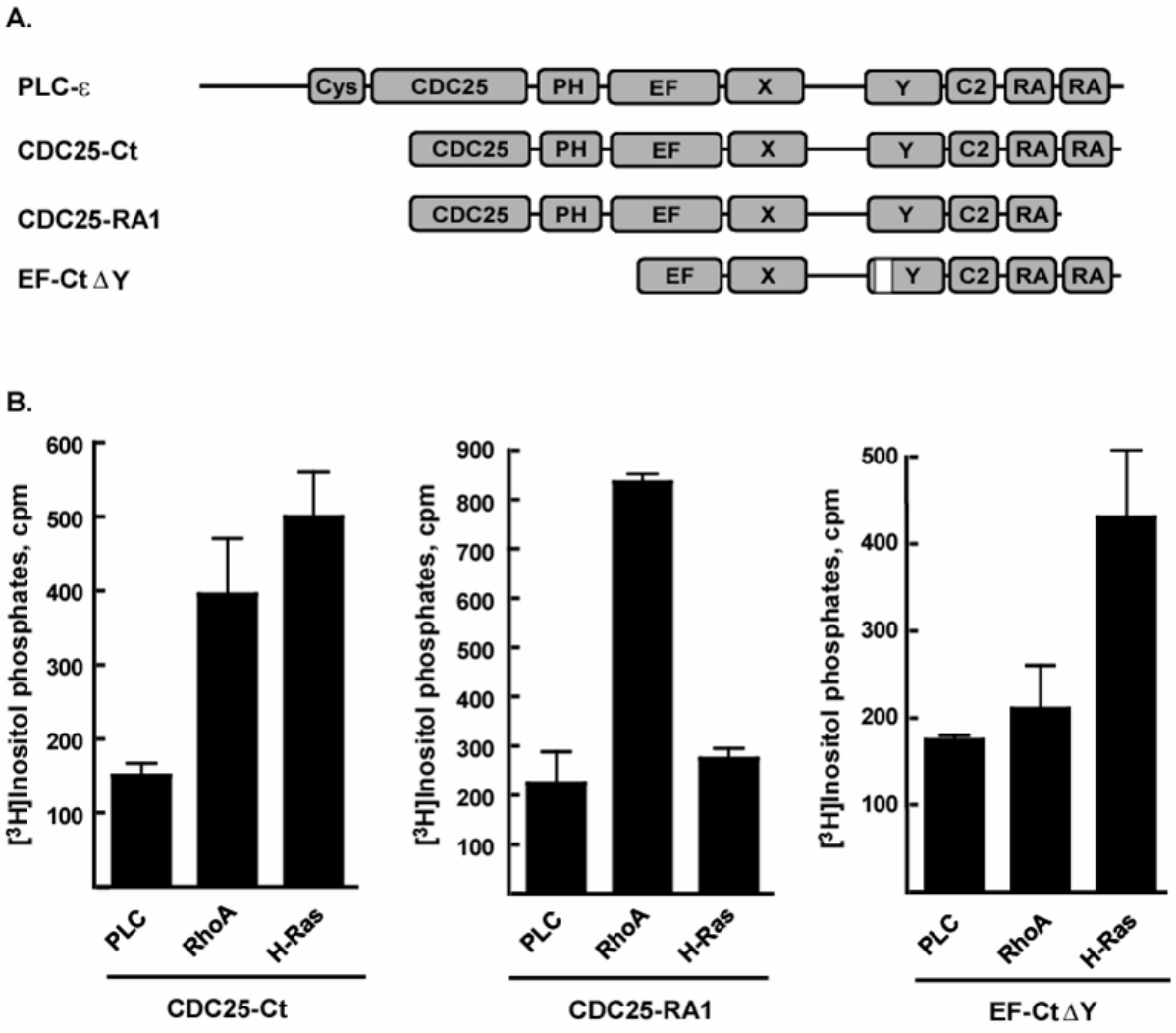

Figure 4.1. RhoA and H-Ras activation of PLC-ε requires individual domains. *A,* The specific PLC-ε constructs used and amino acid ranges are as follows: CDC25-Ct, 509-2281; CDC25-RA1, 509-2113; and EF-CtΔY, 1198-2281 lacking residues 1667-1728. *B*, constitutively active mutants of RhoA (100 ng) or H-Ras (100 ng) were expressed in COS-7 cells in the absence and the presence of the indicated PLC-ε constructs (50 ng each). [^{3}H]Inositol phosphate accumulation was quantified as described under "Experimental Procedures" and [^{3}H]inositol phosphate accumulation for each G protein in the absence of PLC-ε was subtracted from the corresponding samples expressing PLC-ε. Data shown are the mean ± S.D. for triplicate samples, and the results are representative of three or more experiments in each case.

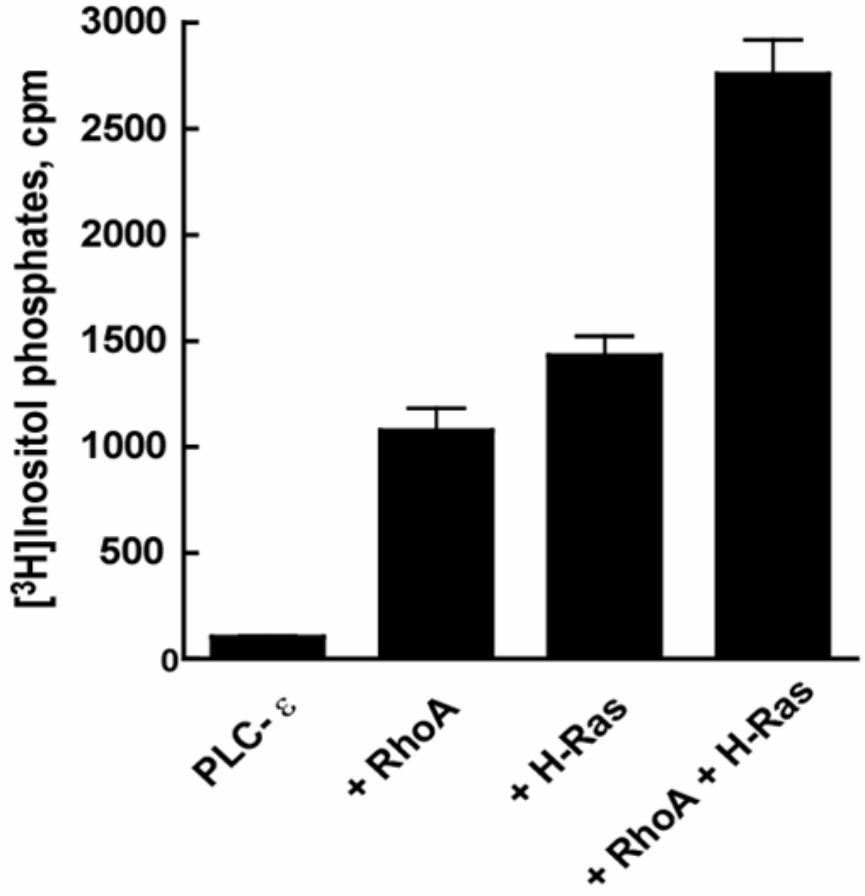

Figure 4.2. Dual activation of PLC-ε in cells overexpressing RhoA and H-Ras. Full-length FLAG-PLC-ε was expressed alone in COS-7 cells, or expressed in the presence of RhoA (100 ng) and H-Ras (100 ng) individually or in combination. The amounts of DNA used were optimized to give maximal PLC-ε-dependent stimulation for each individual GTPase. [^{3}H]Inositol phosphate accumulation was quantified as described under "Experimental Procedures" and [^{3}H]inositol phosphate accumulation for each G protein in the absence of PLC-ε was subtracted from the corresponding samples expressing PLC-ε. Data shown are the mean ± S.D. for triplicate samples, and the results are representative of three or more experiments in each case.

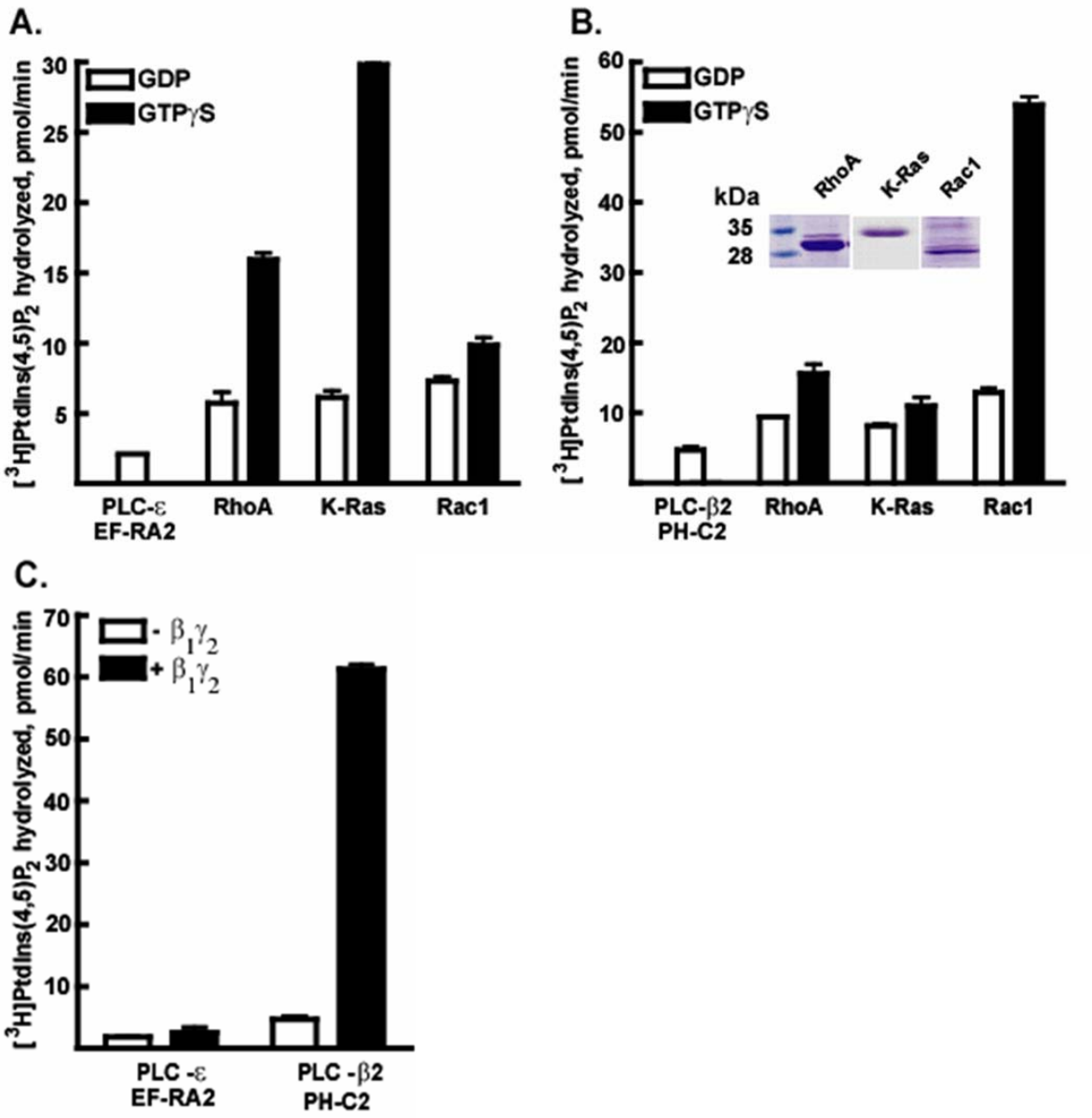

Figure 4.3. Specificity of G protein regulation of PLC isozymes. *A,* monomeric GTPases were purified from detergent-extracted HighFive insect cell membranes as described under "Experimental Procedures." Purified RhoA (125 nM), K-Ras (125 nM), or Rac1 (125 nM) were reconstituted in phospholipids vesicles containing [^{3}H]PtdIns(4,5)P_2 and incubated with 10 μM GDP (*open bars*) or 10 μM GTPγS (*black bars*) for 30 min at 30°C prior to the addition of PLC. The assay was initiated by the addition of purified PLC-ε EF-RA2 (2 ng) and incubated at 30°C for 8 min. *B,* purified PLC-β PH-C2 (5 ng) was added to phospholipid vesicles containing GDP-bound (*open bars*) or GTPγS-bound (*black bars*) RhoA (125 nM), K-Ras (125 nM), or Rac1 (125 nM). Incubations were performed for 8 min at 30°C. Inset, purified GTPases were resolved by SDS-PAGE and gells were stained with Coomassie blue. *C,* PLC-ε EF-RA2 or PLC-β PH-C2 was added to phospholipids vesicles in the absence (*open bars*) or resence (*black bars*) of purified G$\beta_1\gamma_2$ (100 nM). The results presented are the mean ± S.E. of a representative experiment performed three times.

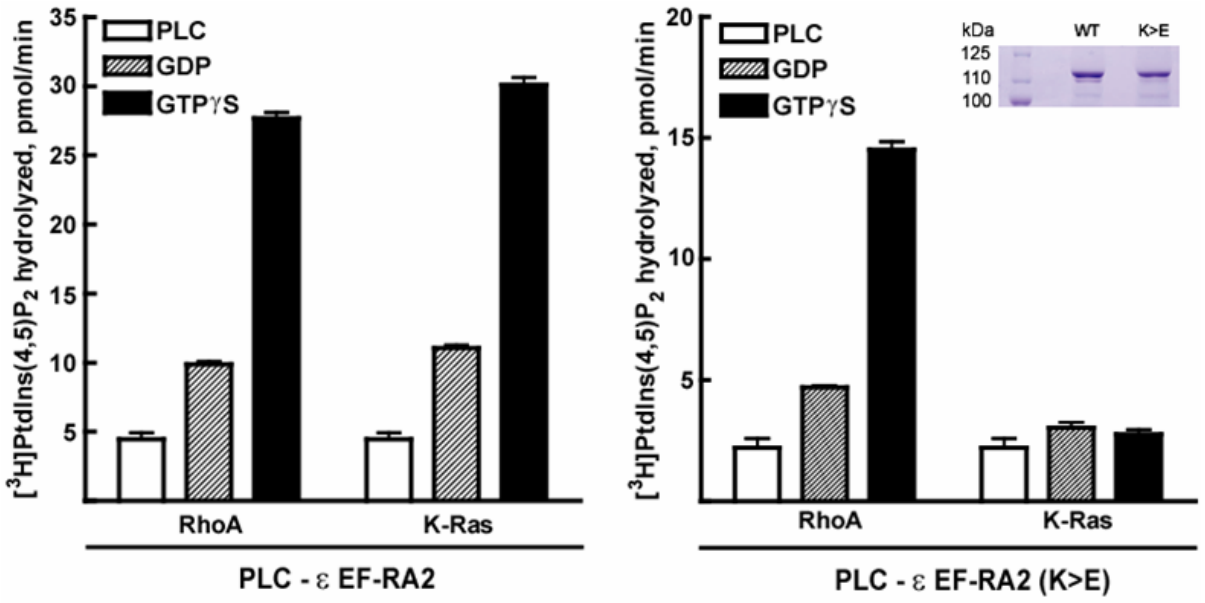

Figure 4.4. Direct *in vitro* activation of PLC-ε by RhoA and K-Ras through distinct domains. Wild-type PLC-ε EF-RA2 (*left*) or PLC-ε EF-RA2 (K2150E, K2152E) (*right*) were reconstituted in phospholipid vesicles containing [^{3}H]PtdIns(4,5)P_2 substrate. Assays were performed in the absence of G-proteins (*open bars*) or in the presence of maximally activating concentrations of GDP-bound (*dashed bars*) or GTPγS-bound (*black bars*) RhoA (300 nM) or K-Ras (300 nM). G-proteins were preincubated in the presence of 10 μM GDP or 10 μM GTPγS to allow nucleotide exchange. The assay was initiated by the addition of PLC enzyme and incubations were for 8 min at 30°C. Data are presented as the mean ± S.E. of an individual experiment representative of three individual experiments. Inset, purified PLC-ε EF-RA2 proteins were resolved by SDS-PAGE and gels were stained with Coomassie blue.

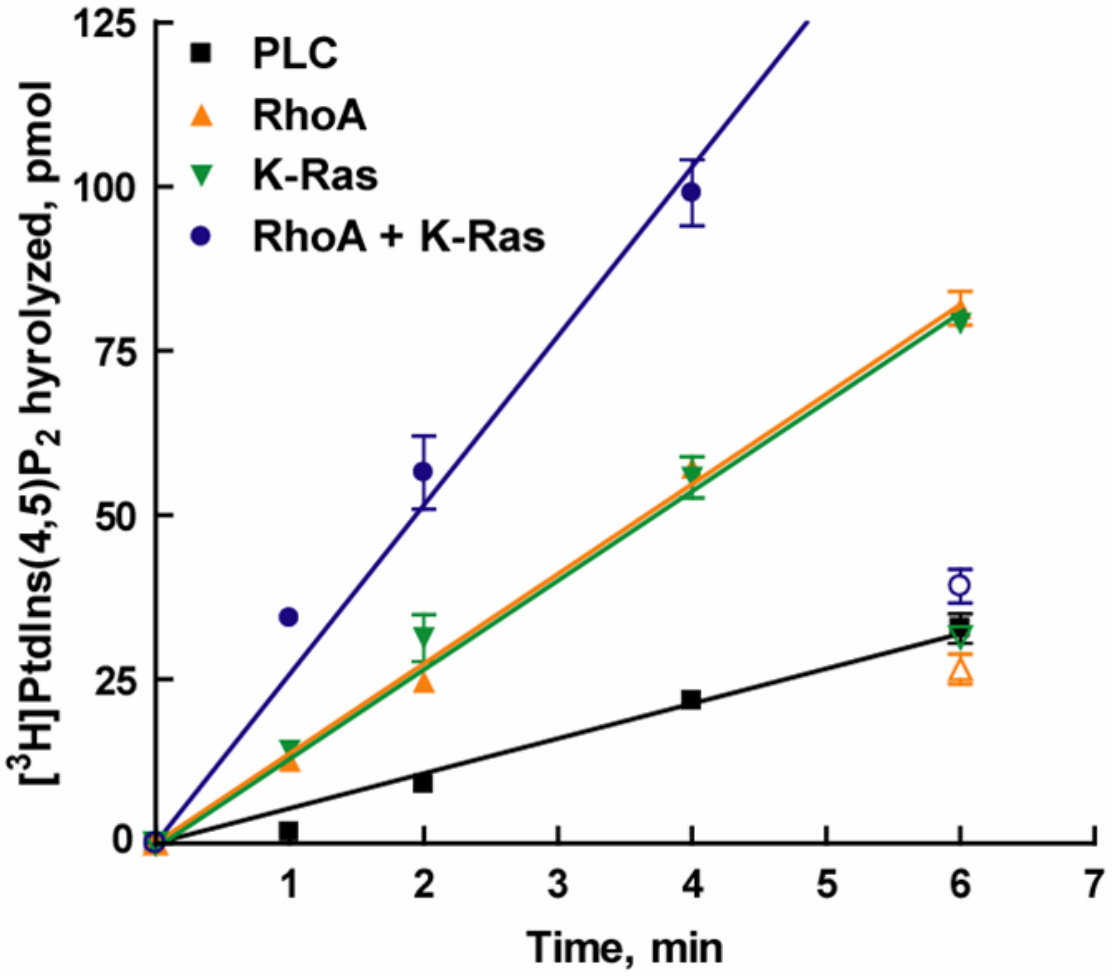

Figure 4.5. Time course of PtdIns(4,5)P_2 hydrolysis by purified PLC-ε. The time dependent stimulation of purified, wild-type PLC-ε EF-RA2 (1 ng) was tested in phospholipid vesicles reconstituted with maximally activating concentrations (300 nM) of RhoA (*orange closed upward triangles*) or K-Ras (*green closed downward triangles*), or vesicles containing 300 nM of both RhoA and K-Ras (*blue closed circles*). GTPases reconstituted in phospholipids vesicles were preincubated in the presence of 10 μM GTPγS prior to PLC addition for 30 min at 30°C to allow nucleotide exchange. The activity of PLC-ε ER-RA2 in the absence of G proteins is also represented (*black closed squares*). The ability of GDP-bound RhoA (*orange open upward triangles*), K-Ras (*green open downward triangles*), or RhoA and K-Ras (*blue open circles*) to regulate PLC-ε EF-RA activity are represented at the 6 min time point The results presented are representative of five individual experiments and are presented as the mean ± S.E.

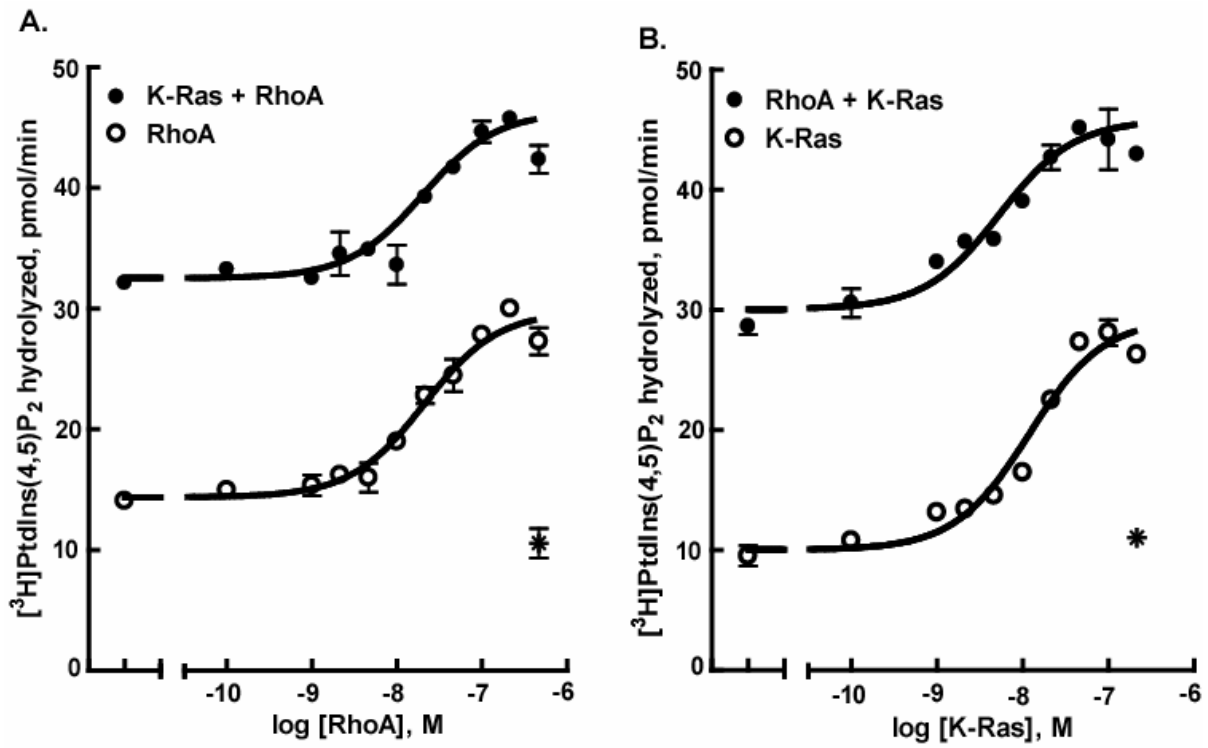

Figure 4.6. Direct dual and independent regulation of PLC-ε by purified RhoA and K-Ras. *A,* increasing concentrations of RhoA were reconstituted in phospholipid vesicles containing [^{3}H]PtdIns(4,5)P_2 in the absence (*open circles*) or presence of 300 nM K-Ras (*closed circles*). After a 30-minute preincubation of RhoA or RhoA/K-Ras containing micelles in the presence of 10 μM GTPγS, PLC-ε EF-RA2 (2 ng) was added and the incubation was continued for 8 min at 30°C. *B,* increasing concentrations of K-Ras were reconstituted in phospholipid vesicles in the absence (*open circles*) or presence of 300 nM RhoA (*closed circles*). Assay was performed for 8 min at 30°C. Purified PLC-ε EF-RA2 was added to phospholipid vesicles containing maximally activating concentrations of RhoA and K-Ras preloaded with GDP (*asterisk*).

TABLE 4.1
RhoA and K-Ras promoted activation of PLC-ε

	Rate (pmol/min)	EC50 (nM)
PLC-ε EF-RA2	12.2 ± 2.1	
+ RhoA	29.8 ± 0.9	21.3 ± 3.4
+ RhoA + K-Ras*	44.3 ± 1.1	19.3 ± 0.5
+ K-Ras	29.3 ± 1.2	14.9 ± 2.6
+ K-Ras + RhoA*	45.8 ± 0.8	5.6 ± 0.7

* Concentration effect curves generated in the presence of 300 nM of the noted GTPase

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

All signal transduction pathways regulating the action of PLC isoforms result in the depletion of PtdIns(4,5)P_2 and the generation of second messenger molecules. Whereas PLC activation was once thought to simply occur downstream of heterotrimeric G proteins or tyrosine kinase phosphorylation, emerging mechanisms of PLC regulation and the identification of novel PLC isoforms suggest common, yet specific roles for individual PLC isoform activation. Measuring inositol phosphate accumulation in cells overexpressing PLC isoforms is a powerful tool for identifying potential mechanisms of enzyme regulation; however, this method is not without limitations. Coexpression studies are useful for identifying protein involved in signaling pathways that regulate PLC activity, but these assays cannot unambiguously demonstrate the regulation of PLC enzymes via direct protein-protein interactions. *In vitro* reconstitution of purified protein components circumvents some of the pitfalls of cell-based systems for studying PLC regulation, but also requires the ability to purify sufficient amounts of soluble, active protein. To this end, we have purified and characterized a catalytically active fragment of PLC-ε encompassing the EF-hand repeats through the second RA2 domain (Chapter 2). The purified enzyme displayed characteristics shared among all PLC isoforms, and more importantly, allowed us to identify PLC-ε as a direct effector of activated RhoA (Chapter 3). These data, combined with previous experiments [147], have provided definitive evidence for the regulation PLC-ε activity

downstream of activated GPCRs coupled to $G\alpha_{12/13}$, the activation of Rho-family GEFs, and the subsequent activation of Rho GTPases.

My experiments have also helped to resolve conflicting studies concerning Ras-regulated PLC-ε activation by showing the direct, RA2-dependent regulation of PLC-ε by purified Ras family GTPases (Chapter 4). The regulation of PLC-ε activity by Rho and Ras family proteins through interactions with distinct regions of the enzyme directed us to explore the potential dual regulation of PLC-ε by both Ras subfamilies. Indeed, my experiments show that when PLC-ε is activated by a maximally activating concentration of either RhoA or K-Ras, enzyme activity is increased even further by the addition of the complementary GTPase. Herein, we have identified one of the first enzymes directly regulated by two different subclasses of Ras superfamily GTPases. Another well characterized effector of both Ras and Rho family GTPases are the class I PI3Ks, which are activated by both Ras and Rac GTPases; however, these GTPases regulate activity through interactions with distinct subunits of PI3K [245;246]. The future aims of this research are to continue to define the molecular basis of PLC-ε regulation by Rho and Ras family GTPases with the ultimate goal of understanding the mechanisms whereby various GTPases individually and cooperatively regulate PLC-ε activity.

Whereas transient transfection of cells limits the ability to detect direct protein-protein regulation of PLC enzymes, reconstitution experiments fail to address the biology associated with PLC activation *in vivo*. For instance, although *in vitro* experiments suggest PLC-ε is regulated individually and dually by Rho and Ras family GTPases, these experiments do not address the physiological consequences of PLC-ε regulation by various GTPases. Dual regulation of PLC-ε by both Rho and Ras GTPases would allow intricate

spatial regulation of PtdIns(4,5)P_2 hydrolysis at cellular localizations where both GTPases were activated. Coexpression of PLC-ε with RhoA and H-Ras in COS-7 cells suggests a similar additive increase in enzyme activity observed *in vitro*. However, it is not known if the observed increased phospholipase activity is due to varied localization of PLC-ε to multiple intracellular membranes, or if RhoA and Ras cooperate at the same intracellular membranes to regulate PLC-ε activity. To address this issue, immunohistological detection of PLC-ε could be used to visualize PLC localization after overexpression with constitutively active RhoA, various Ras proteins, or Rho and Ras proteins. Localization of PLC-ε at similar membranes in the presence of Rho and/or Ras proteins would suggest dual regulation of PLC-ε occurs in a physiologically relevant context. Ras GTPases have been implicated as upstream regulators of Rho activation, suggesting Ras activation could potentially result in a transient increase in PLC-ε activity, followed by sustained PtdIns(4,5)P_2 hydrolysis resulting from activation of Rho GTPases. Live cell imagining could also be used to show the spatial and temporal regulation of PLC-ε in response to receptor mediated activation of Rho, Ras, or Rho and Ras in combination.

Defining the regions within PLC-ε necessary for interaction with various Ras superfamily GTPases, as well as the potential mechanisms by which G proteins stimulate PLC-ε enzyme activity are essential steps for ascertaining physiological consequences of these interactions. Ras and Rho family proteins are involved in several mammalian physiologies including cell proliferation, cell differentiation, cytoskeletal changes, and vesicular trafficking. An emerging role for Ras superfamily GTPases involves the regulation of phospholipid metabolism through the regulation of PI kinases and phospholipases. The ability of Rho GTPases to regulate the function of PLC-ε could result in sustained calcium

release and PKC activation as Rho GTPases also activate PI(4)P-5K. Moreover, several proteins involved in actin polymerization and vesicular trafficking require PtdIns(4,5)P_2 for activation, suggesting site specific Rho and Ras regulation of PLC-ε could negatively regulate pathways involved in cytoskeletal changes. The oncogenic properties of mammalian Ras proteins, combined with Kataoka's inactivated PLC-ε mouse model, suggests PLC-ε may be intricately involved pathways leading to cell proliferation and tumorgenesis. Current studies in Dr. Channing Der's lab are exploring the proliferative effects of hyperactive PLC-ε by expressing PLC-ε lacking the X-Y linker region. As both Rho and Ras GTPases are involved in cell cycle progession, it would also be interesting to determine if PLC-ε-dependent changes in phosphoinositide levels, calcium release, and/or PKC activation are involved in the ability of Ras superfamily GTPases to regulate cell cycle progression.

Although we have identified PLC-ε as a direct effector of GTP-bound RhoA, the site of RhoA interaction has yet to be delineated. Sequence analysis does not identify regions of homology shared among other Rho family effectors, suggesting that determination of the Rho-binding site within PLC-ε will reveal a novel Rho-interacting motif. Removal of a unique sequence within the Y-box of PLC-ε results in the loss of RhoA-mediated activation. However, GTP-dependent binding of RhoA to mutated PLC-ε was still observed using GST-pulldown assays ([142], chapter 3). Collectively, these experiments suggest the insert region of PLC-ε may be part of, or proximal to, the RhoA binding site within PLC-ε. One method for identifying the sequence required for RhoA binding is to create short overlapping peptides corresponding to the sequence of PLC-ε. This methodology was previously utilized by Sankaran and colleagues to identify the region PLC-β that interacts with Gβγ-subunits [247]. The peptides could be tested for the ability to block Rho-mediated PLC-ε activation in

reconstitution experiments and the specificity of the peptides could be tested by showing inability to block K-Ras activation. Additionally, peptides corresponding to the region within the RA2 domain of PLC-ε required for K-Ras activation, derived from the crystal structure provided by Bunney and colleagues [135], could be used as an experimental control and provide evidence regarding the feasibility of the proposed experiments.

The most significant future aim of these studies is to obtain structural information regarding the mechanisms by which Rho and Ras family proteins regulate the functional activity of PLC-ε. Several attempts have been made to crystallize the fragment of PLC-ε used in functional experiments described throughout this research, but with little success. Regardless, the domains encompassed within the purified PLC-ε fragment remain the most intriguing crystallography targets as the EF-RA2 regions confer regulation by both Rho and Ras family GTPases. Various constructs of other PLC isoforms (*i.e.* PLC-β2 PH-C2, full-length PLC-γ1) have yielded significantly greater quantities of purified proteins, which may be possible for PLC-ε by redefining the amino acid boundaries encompassed by expression vectors. For instance, including the PH domain, or the short sequence following the RA2 domain, may aid in protein solubility resulting in increased yields of purified protein after overexpression in insect cells.

Definitive answers regarding the mechanisms of PLC-ε regulation require structural information obtained by comparing structures of PLC-ε isolated during different states of "activation." Based on experiments described throughout this work, there are several obvious targets for crystallography: (i) PLC-ε in the absence of activators, (ii) PLC-ε in complex with activated RhoA, (iii) PLC-ε in complex with one of several Ras GTPases, and (iv) PLC-ε in complex with both RhoA and Ras. Perhaps the most beneficial crystal target is

PLC-ε in the absence of an activator, which will provide evidence regarding the basal state of the enzyme to compare with structures of PLC-ε in complex with G protein activators. Although crystal structures of the catalytic core of two PLC isoforms have already been solved, the crystal structure of PLC-ε EF-RA2 will substantiate *in vivo* experiments suggesting the X-Y linker region of PLC-ε autoinhibits enzyme activity, and provide evidence to support our hypothesis that occlusion of the active site by the X-Y linker serves as a general mechanism to autoinhibit several, if not all, PLC isoforms.

Solving the crystal structure of PLC-ε in complex with RhoA will answer several questions regarding the regulation of PLC-ε by Rho GTPases, perhaps making this the most intriguing crystal target. First, because the site of RhoA interaction is currently unknown, solving the crystal structure of a PLC-ε/RhoA complex will detail specific contacts made between the two proteins. This will be especially important if scanning peptides produced for the experiments described above fail to identify the site within PLC-ε that binds RhoA. Secondly, because RhoA interacts within the catalytic region of PLC-ε, there is an increased probability that complex formation results in conformational changes within PLC-ε to confer increased enzymatic activity. Moreover, because of the apparent autoinhibition of PLC-ε through occlusion of the active site by the X-Y linker, RhoA could potentially result in the relief of autoinhibition by binding to regions within or adjacent to the linker region of PLC-ε. Finally, the role of the "Y-loop insert" region in RhoA-regulated enzyme activity could be determined by comparing the structure of PLC-ε alone to PLC-ε in complex with RhoA.

Although the recent NMR and crystal structures of the RA domains of PLC-ε have provided detailed analysis regarding the interaction between the RA2 domain of PLC-ε and H-Ras, these data do not address the enzymatic consequences of the observed interaction

[135]. Based on their crystallographic and experimental data, Bunney and colleagues propose that the RA domains fold back on the catalytic region of PLC-ε resulting in autoinhibition of enzyme activity, which is relieved upon Ras binding and translocation of the enzyme to the plasma membrane. Solving the crystal structure of PLC-ε alone and in complex with activated H-Ras would provide convincing evidence regarding the plausibility of this mechanism of PLC-ε regulation. Alternatively, binding of Ras family proteins to the RA domains could simply recruit and tether PLC-ε to the plasma membrane, which would be supported by a crystal structure suggesting the RA domains are loosely tethered to the catalytic region of the enzyme.

The crystal structure of PLC-β2/Rac1 suggests association with Rac1 serves to localize PLC-β2 to the plasma membrane and position the enzyme in an orientation favorable for substrate hydrolysis. However, complex formation between Rac1 and PLC-β2 does not result in significant conformational changes within the catalytic regions of the enzyme [71]. Comparison of the PLC-β/Rac complex with PLC-ε/RhoA and/or Ras would provide significant information concerning the regulation of various PLC isozymes by Ras superfamily GTPases. Whether PLC isozymes have evolved a collective affinity for Ras superfamily proteins to confer membrane localization, or if individual PLC isozymes are affected differently through interactions with G proteins will provide specific details on the mechanisms of Ras superfamily PLC activation.

Several mouse models have begun to identify physiologies associated with PLC-ε activity [154-156]. Mice expressing a catalytically inactive PLC-ε, or mice lacking the entire PLC-ε gene, exhibit various phenotypes. However, the upstream signaling pathways mediating the various observed physiologies have yet to be determined. Defining critical

amino acids required for Rho and/or Ras interaction with PLC-ε will allow targeted mutation of PLC-ε at these amino. Mutated PLC-ε constructs could be expressed in systems ranging from overexpression in COS-7 cells to mice expressing various mutated PLC-ε constructs, with the goal of defining the physiologies associated with signal-specific PLC-ε regulation.

Although PLC-ε research is relatively new within the field of PLC signaling, the efforts of several groups have made great strides in delineating pathways involved in PLC-ε enzyme regulation. The ability to be regulated by such a large number of upstream signals places PLC-ε at the nexus of converging signaling pathways derived from both heterotrimeric and monomeric G protein signaling. In addition to responding to several upstream signals, PLC-ε is unique among PLC isoforms operating as both an initiator and recipient of Ras GTPase signals, potentially leading to an autofeedback mechanism of regulation. Given the increasing number of signaling pathways involved in the regulation of PLC-ε, it is probable that PLC-ε is critically involved in several mammalian physiologies. The studies presented here and the proposed future directions will contribute to the further understanding of unique pathways that regulate the activity of PLC-ε.

Reference List

1. Downes CP, Gray A, Lucocq JM. Probing phosphoinositide functions in signaling and membrane trafficking. *Trends Cell Biol.* 2005; **15**:259-268.

2. Fruman DA, Meyers RE, Cantley LC. Phosphoinositide kinases. *Annu.Rev.Biochem.* 1998; **67**:481-507.

3. Rohrschneider LR, Fuller JF, Wolf I, Liu Y, Lucas DM. Structure, function, and biology of SHIP proteins. *Genes Dev.* 2000; **14**:505-520.

4. Leslie NR, Downes CP. PTEN: The down side of PI 3-kinase signalling. *Cell Signal.* 2002; **14**:285-295.

5. Lemmon MA. Phosphoinositide recognition domains. *Traffic.* 2003; **4**:201-213.

6. Vanhaesebroeck B, Leevers SJ, Ahmadi K *et al*. Synthesis and function of 3-phosphorylated inositol lipids. *Annu.Rev.Biochem.* 2001; **70**:535-602.

7. Niggli V. Regulation of protein activities by phosphoinositide phosphates. *Annu.Rev.Cell Dev.Biol.* 2005; **21**:57-79.

8. Hilgemann DW. Getting ready for the decade of the lipids. *Annu.Rev.Physiol* 2003; **65**:697-700.

9. Haslam RJ, Koide HB, Hemmings BA. Pleckstrin domain homology. *Nature* 1993; **363**:309-310.

10. Mayer BJ, Ren R, Clark KL, Baltimore D. A putative modular domain present in diverse signaling proteins. *Cell* 1993; **73**:629-630.

11. Musacchio A, Gibson T, Rice P, Thompson J, Saraste M. The PH domain: a common piece in the structural patchwork of signalling proteins. *Trends Biochem.Sci.* 1993; **18**:343-348.

12. Rebecchi MJ, Scarlata S. Pleckstrin homology domains: a common fold with diverse functions. *Annu.Rev.Biophys.Biomol.Struct.* 1998; **27**:503-528.

13. Rameh LE, Arvidsson A, Carraway KL, III *et al*. A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J.Biol.Chem.* 1997; **272**:22059-22066.

14. Garcia P, Gupta R, Shah S *et al*. The pleckstrin homology domain of phospholipase C-delta 1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. *Biochemistry* 1995; **34**:16228-16234.

15. Falasca M, Logan SK, Lehto VP, Baccante G, Lemmon MA, Schlessinger J. Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J.* 1998; **17**:414-422.

16. Lodowski DT, Barnhill JF, Pitcher JA, Capel WD, Lefkowitz RJ, Tesmer JJ. Purification, crystallization and preliminary X-ray diffraction studies of a complex between G protein-coupled receptor kinase 2 and Gbeta1gamma2. *Acta Crystallogr.D.Biol.Crystallogr.* 2003; **59**:936-939.

17. Snyder JT, Singer AU, Wing MR, Harden TK, Sondek J. The pleckstrin homology domain of phospholipase C-beta2 as an effector site for Rac. *J.Biol.Chem.* 2003; **278**:21099-21104.

18. Jaffe AB, Aspenstrom P, Hall A. Human CNK1 acts as a scaffold protein, linking Rho and Ras signal transduction pathways. *Mol.Cell Biol.* 2004; **24**:1736-1746.

19. Kay BK, Yamabhai M, Wendland B, Emr SD. Identification of a novel domain shared by putative components of the endocytic and cytoskeletal machinery. *Protein Sci.* 1999; **8**:435-438.

20. Kalthoff C, Alves J, Urbanke C, Knorr R, Ungewickell EJ. Unusual structural organization of the endocytic proteins AP180 and epsin 1. *J.Biol.Chem.* 2002; **277**:8209-8216.

21. De Camilli P, Chen H, Hyman J, Panepucci E, Bateman A, Brunger AT. The ENTH domain. *FEBS Lett.* 2002; **513**:11-18.

22. Ford MG, Pearse BM, Higgins MK *et al*. Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science* 2001; **291**:1051-1055.

23. Ford MG, Mills IG, Peter BJ *et al*. Curvature of clathrin-coated pits driven by epsin. *Nature* 2002; **419**:361-366.

24. Stahelin RV, Long F, Diraviyam K, Bruzik KS, Murray D, Cho W. Phosphatidylinositol 3-phosphate induces the membrane penetration of the FYVE domains of Vps27p and Hrs. *J.Biol.Chem.* 2002; **277**:26379-26388.

25. Stahelin RV, Long F, Peter BJ *et al*. Contrasting membrane interaction mechanisms of AP180 N-terminal homology (ANTH) and epsin N-terminal homology (ENTH) domains. *J.Biol.Chem.* 2003; **278**:28993-28999.

26. Ellson CD, Andrews S, Stephens LR, Hawkins PT. The PX domain: a new phosphoinositide-binding module. *J.Cell Sci.* 2002; **115**:1099-1105.

27. Xu J, Liu D, Gill G, Songyang Z. Regulation of cytokine-independent survival kinase (CISK) by the Phox homology domain and phosphoinositides. *J.Cell Biol.* 2001; **154**:699-705.

28. Ellson CD, Gobert-Gosse S, Anderson KE *et al*. PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40(phox). *Nat.Cell Biol.* 2001; **3**:679-682.

29. Kanai F, Liu H, Field SJ *et al*. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat.Cell Biol.* 2001; **3**:675-678.

30. Yu JW, Lemmon MA. All phox homology (PX) domains from Saccharomyces cerevisiae specifically recognize phosphatidylinositol 3-phosphate. *J.Biol.Chem.* 2001; **276**:44179-44184.

31. Gaullier JM, Simonsen A, D'Arrigo A, Bremnes B, Stenmark H, Aasland R. FYVE fingers bind PtdIns(3)P. *Nature* 1998; **394**:432-433.

32. Lawe DC, Patki V, Heller-Harrison R, Lambright D, Corvera S. The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization. *J.Biol.Chem.* 2000; **275**:3699-3705.

33. Wurmser AE, Gary JD, Emr SD. Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. *J.Biol.Chem.* 1999; **274**:9129-9132.

34. Stenmark H, Aasland R, Driscoll PC. The phosphatidylinositol 3-phosphate-binding FYVE finger. *FEBS Lett.* 2002; **513**:77-84.

35. Dumas JJ, Merithew E, Sudharshan E *et al*. Multivalent endosome targeting by homodimeric EEA1. *Mol.Cell* 2001; **8**:947-958.

36. HIGGS HN, Pollard TD. Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. *J.Cell Biol.* 2000; **150**:1311-1320.

37. Lassing I, Lindberg U. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature* 1985; **314**:472-474.

38. Moriyama T, Narita H, Oki M, Matsuura T, Kito M. Purification of polymeric phospholipase Cs from human platelets. *J.Biochem.(Tokyo)* 1990; **108**:414-419.

39. Hamada K, Shimizu T, Matsui T, Tsukita S, Hakoshima T. Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. *EMBO J.* 2000; **19**:4449-4462.

40. Santagata S, Boggon TJ, Baird CL *et al*. G-protein signaling through tubby proteins. *Science* 2001; **292**:2041-2050.

41. Berridge MJ, Downes CP, Hanley MR. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem.J.* 1982; **206**:587-595.

42. Silverstone PH, McGrath BM, Kim H. Bipolar disorder and myo-inositol: a review of the magnetic resonance spectroscopy findings. *Bipolar.Disord.* 2005; **7**:1-10.

43. Stopkova P, Saito T, Fann CS *et al*. Polymorphism screening of PIP5K2A: a candidate gene for chromosome 10p-linked psychiatric disorders. *Am.J.Med.Genet.B Neuropsychiatr.Genet.* 2003; **123**:50-58.

44. Stopkova P, Saito T, Papolos DF *et al*. Identification of PIK3C3 promoter variant associated with bipolar disorder and schizophrenia. *Biol.Psychiatry* 2004; **55**:981-988.

45. Hayakawa Y, Chandra M, Miao W *et al*. Inhibition of cardiac myocyte apoptosis improves cardiac function and abolishes mortality in the peripartum cardiomyopathy of Galpha(q) transgenic mice. *Circulation* 2003; **108**:3036-3041.

46. Zhang X, Hartz PA, Philip E, Racusen LC, Majerus PW. Cell lines from kidney proximal tubules of a patient with Lowe syndrome lack OCRL inositol polyphosphate 5-phosphatase and accumulate phosphatidylinositol 4,5-bisphosphate. *J.Biol.Chem.* 1998; **273**:1574-1582.

47. Bell RM, Hannun YA, Loomis CR. Mechanism of regulation of protein kinase C by lipid second messengers. *Symp.Fundam.Cancer Res.* 1986; **39**:145-156.

48. Berridge MJ, Irvine RF. Inositol phosphates and cell signalling. *Nature* 1989; **341**:197-205.

49. HOKIN MR, HOKIN LE. Enzyme secretion and the incorporation of P32 into phospholipides of pancreas slices. *J.Biol.Chem.* 1953; **203**:967-977.

50. Michell RH. Inositol phospholipids and cell surface receptor function. *Biochim.Biophys.Acta* 1975; **415**:81-47.

51. Berridge MJ. Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu.Rev.Biochem.* 1987; **56**:159-193.

52. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 1992; **258**:607-614.

53. Kishimoto A, Takai Y, Mori T, Kikkawa U, Nishizuka Y. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J.Biol.Chem.* 1980; **255**:2273-2276.

54. Streb H, Irvine RF, Berridge MJ, Schulz I. Release of Ca2+ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* 1983; **306**:67-69.

55. Homma Y, Takenawa T, Emori Y, Sorimachi H, Suzuki K. Tissue- and cell type-specific expression of mRNAs for four types of inositol phospholipid-specific phospholipase C. *Biochem.Biophys.Res.Commun.* 1989; **164**:406-412.

56. Rhee SG, Choi KD. Regulation of inositol phospholipid-specific phospholipase C isozymes. *J.Biol.Chem.* 1992; **267**:12393-12396.

57. Kanematsu T, Yoshimura K, Hidaka K, Takeuchi H, Katan M, Hirata M. Domain organization of p130, PLC-related catalytically inactive protein, and structural basis for the lack of enzyme activity. *Eur.J.Biochem.* 2000; **267**:2731-2737.

58. Koyanagi M, Ono K, Suga H, Iwabe N, Miyata T. Phospholipase C cDNAs from sponge and hydra: antiquity of genes involved in the inositol phospholipid signaling pathway. *FEBS Lett.* 1998; **439**:66-70.

59. Wierenga RK. The TIM-barrel fold: a versatile framework for efficient enzymes. *FEBS Lett.* 2001; **492**:193-198.

60. Ellis MV, Carne A, Katan M. Structural requirements of phosphatidylinositol-specific phospholipase C delta 1 for enzyme activity. *Eur.J.Biochem.* 1993; **213**:339-347.

61. Essen LO, Perisic O, Cheung R, Katan M, Williams RL. Crystal structure of a mammalian phosphoinositide-specific phospholipase C delta. *Nature* 1996; **380**:595-602.

62. Boguslavsky V, Rebecchi M, Morris AJ, Jhon DY, Rhee SG, McLaughlin S. Effect of monolayer surface pressure on the activities of phosphoinositide-specific phospholipase C-beta 1, -gamma 1, and -delta 1. *Biochemistry* 1994; **33**:3032-3037.

63. James SR, Paterson A, Harden TK, Demel RA, Downes CP. Dependence of the activity of phospholipase C beta on surface pressure and surface composition in phospholipid monolayers and its implications for their regulation. *Biochemistry* 1997; **36**:848-855.

64. Morris AJ, Waldo GL, Downes CP, Harden TK. A receptor and G-protein-regulated polyphosphoinositide-specific phospholipase C from turkey erythrocytes. I. Purification and properties. *J.Biol.Chem.* 1990; **265**:13501-13507.

65. Kim D, Jun KS, Lee SB *et al*. Phospholipase C isozymes selectively couple to specific neurotransmitter receptors. *Nature* 1997; **389**:290-293.

66. Ellis MV, James SR, Perisic O, Downes CP, Williams RL, Katan M. Catalytic domain of phosphoinositide-specific phospholipase C (PLC). Mutational analysis of

residues within the active site and hydrophobic ridge of plcdelta1. *J.Biol.Chem.* 1998; **273**:11650-11659.

67. Essen LO, Perisic O, Katan M, Wu Y, Roberts MF, Williams RL. Structural mapping of the catalytic mechanism for a mammalian phosphoinositide-specific phospholipase C. *Biochemistry* 1997; **36**:1704-1718.

68. Cheng HF, Jiang MJ, Chen CL *et al*. Cloning and identification of amino acid residues of human phospholipase C delta 1 essential for catalysis. *J.Biol.Chem.* 1995; **270**:5495-5505.

69. Ellis MV, U S, Katan M. Mutations within a highly conserved sequence present in the X region of phosphoinositide-specific phospholipase C-delta 1. *Biochem.J.* 1995; **307 (Pt 1)**:69-75.

70. Simoes AP, Reed J, Schnabel P, Camps M, Gierschik P. Characterization of putative polyphosphoinositide binding motifs from phospholipase C beta 2. *Biochemistry* 1995; **34**:5113-5119.

71. Jezyk MR, Snyder JT, Gershberg S, Worthylake DK, Harden TK, Sondek J. Crystal structure of Rac1 bound to its effector phospholipase C-beta2. *Nat.Struct.Mol.Biol.* 2006; **13**:1135-1140.

72. Cifuentes ME, Honkanen L, Rebecchi MJ. Proteolytic fragments of phosphoinositide-specific phospholipase C-delta 1. Catalytic and membrane binding properties. *J.Biol.Chem.* 1993; **268**:11586-11593.

73. Bromann PA, Boetticher EE, Lomasney JW. A single amino acid substitution in the pleckstrin homology domain of phospholipase C delta1 enhances the rate of substrate hydrolysis. *J.Biol.Chem.* 1997; **272**:16240-16246.

74. Bae YS, Cantley LG, Chen CS, Kim SR, Kwon KS, Rhee SG. Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate. *J.Biol.Chem.* 1998; **273**:4465-4469.

75. Runnels LW, Jenco J, Morris A, Scarlata S. Membrane binding of phospholipases C-beta 1 and C-beta 2 is independent of phosphatidylinositol 4,5-bisphosphate and the alpha and beta gamma subunits of G proteins. *Biochemistry* 1996; **35**:16824-16832.

76. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. *Annu.Rev.Biochem.* 2001; **70**:281-312.

77. Yamamoto T, Takeuchi H, Kanematsu T *et al*. Involvement of EF hand motifs in the Ca(2+)-dependent binding of the pleckstrin homology domain to phosphoinositides. *Eur.J.Biochem.* 1999; **265**:481-490.

78. Nakashima S, Banno Y, Watanabe T *et al*. Deletion and site-directed mutagenesis of EF-hand domain of phospholipase C-delta 1: effects on its activity. *Biochem.Biophys.Res.Commun.* 1995; **211**:365-369.

79. Essen LO, Perisic O, Lynch DE, Katan M, Williams RL. A ternary metal binding site in the C2 domain of phosphoinositide-specific phospholipase C-delta1. *Biochemistry* 1997; **36**:2753-2762.

80. Lomasney JW, Cheng HF, Roffler SR, King K. Activation of phospholipase C delta1 through C2 domain by a Ca(2+)-enzyme-phosphatidylserine ternary complex. *J.Biol.Chem.* 1999; **274**:21995-22001.

81. Wang T, Pentyala S, Elliott JT *et al*. Selective interaction of the C2 domains of phospholipase C-beta1 and -beta2 with activated Galphaq subunits: an alternative function for C2-signaling modules. *Proc.Natl.Acad.Sci.U.S.A* 1999; **96**:7843-7846.

82. Kouchi Z, Shikano T, Nakamura Y, Shirakawa H, Fukami K, Miyazaki S. The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase Czeta. *J.Biol.Chem.* 2005; **280**:21015-21021.

83. Flick JS, Thorner J. Genetic and biochemical characterization of a phosphatidylinositol-specific phospholipase C in Saccharomyces cerevisiae. *Mol.Cell Biol.* 1993; **13**:5861-5876.

84. Andoh T, Yoko T, Matsui Y, Toh A. Molecular cloning of the plc1+ gene of Schizosaccharomyces pombe, which encodes a putative phosphoinositide-specific phospholipase C. *Yeast* 1995; **11**:179-185.

85. Bennett DE, McCreary CE, Coleman DC. Genetic characterization of a phospholipase C gene from Candida albicans: presence of homologous sequences in Candida species other than Candida albicans. *Microbiology* 1998; **144 (Pt 1)**:55-72.

86. Irino Y, Cho H, Nakamura Y *et al*. Phospholipase C delta-type consists of three isozymes: bovine PLCdelta2 is a homologue of human/mouse PLCdelta4. *Biochem.Biophys.Res.Commun.* 2004; **320**:537-543.

87. Shimohama S, Homma Y, Suenaga T *et al*. Aberrant accumulation of phospholipase C-delta in Alzheimer brains. *Am.J.Pathol.* 1991; **139**:737-742.

88. Banno Y, Okano Y, Nozawa Y. Thrombin-mediated phosphoinositide hydrolysis in Chinese hamster ovary cells overexpressing phospholipase C-delta 1. *J.Biol.Chem.* 1994; **269**:15846-15852.

89. Liu N, Fukami K, Yu H, Takenawa T. A new phospholipase C delta 4 is induced at S-phase of the cell cycle and appears in the nucleus. *J.Biol.Chem.* 1996; **271**:355-360.

90. Lee SB, Rhee SG. Molecular cloning, splice variants, expression, and purification of phospholipase C-delta 4. *J.Biol.Chem.* 1996; **271**:25-31.

91. Ferguson KM, Lemmon MA, Schlessinger J, Sigler PB. Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* 1995; **83**:1037-1046.

92. Lemmon, M. A. Regulatory requirement of signaling molecules to the cell membrane by pleckstrin-homology domains. , Falasca M. Ferguson K. M. and Schlessinger J. Trends Cell Biol. 7, 237-242. 1997.

93. Hershberg RD, Reed GH, Slotboom AJ, deHaas GH. Phospholipase A2 complexes with gadolinium (III) and interaction of the enzyme-metal ion complex with monomeric and micellar alkylphosphorylcholines. Water proton nuclear magnetic relaxation studies. *Biochemistry* 1976; **15**:2268-2274.

94. Kim YH, Park TJ, Lee YH *et al*. Phospholipase C-delta1 is activated by capacitative calcium entry that follows phospholipase C-beta activation upon bradykinin stimulation. *J.Biol.Chem.* 1999; **274**:26127-26134.

95. Feng JF, Rhee SG, Im MJ. Evidence that phospholipase delta1 is the effector in the Gh (transglutaminase II)-mediated signaling. *J.Biol.Chem.* 1996; **271**:16451-16454.

96. Li Z, Jiang H, Xie W, Zhang Z, Smrcka AV, Wu D. Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science* 2000; **287**:1046-1049.

97. Xie W, Samoriski GM, McLaughlin JP *et al*. Genetic alteration of phospholipase C beta3 expression modulates behavioral and cellular responses to mu opioids. *Proc.Natl.Acad.Sci.U.S.A* 1999; **96**:10385-10390.

98. Hirono M, Sugiyama T, Kishimoto Y *et al*. Phospholipase Cbeta4 and protein kinase Calpha and/or protein kinase CbetaI are involved in the induction of long term depression in cerebellar Purkinje cells. *J.Biol.Chem.* 2001; **276**:45236-45242.

99. Simon MI, Strathmann MP, Gautam N. Diversity of G proteins in signal transduction. *Science* 1991; **252**:802-808.

100. Fain JN, Wallace MA, Wojcikiewicz RJ. Evidence for involvement of guanine nucleotide-binding regulatory proteins in the activation of phospholipases by hormones. *FASEB J.* 1988; **2**:2569-2574.

101. Smrcka AV, Hepler JR, Brown KO, Sternweis PC. Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science* 1991; **251**:804-807.

102. Taylor SJ, Chae HZ, Rhee SG, Exton JH. Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. *Nature* 1991; **350**:516-518.

103. Waldo GL, Boyer JL, Morris AJ, Harden TK. Purification of an AlF4- and G-protein beta gamma-subunit-regulated phospholipase C-activating protein. *J.Biol.Chem.* 1991; **266**:14217-14225.

104. Camps M, Carozzi A, Schnabel P, Scheer A, Parker PJ, Gierschik P. Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. *Nature* 1992; **360**:684-686.

105. Boyer JL, Waldo GL, Harden TK. Beta gamma-subunit activation of G-protein-regulated phospholipase C. *J.Biol.Chem.* 1992; **267**:25451-25456.

106. Blank JL, Brattain KA, Exton JH. Activation of cytosolic phosphoinositide phospholipase C by G-protein beta gamma subunits. *J.Biol.Chem.* 1992; **267**:23069-23075.

107. Lee SB, Shin SH, Hepler JR, Gilman AG, Rhee SG. Activation of phospholipase C-beta 2 mutants by G protein alpha q and beta gamma subunits. *J.Biol.Chem.* 1993; **268**:25952-25957.

108. Lee CW, Lee KH, Lee SB, Park D, Rhee SG. Regulation of phospholipase C-beta 4 by ribonucleotides and the alpha subunit of Gq. *J.Biol.Chem.* 1994; **269**:25335-25338.

109. Illenberger D, Schwald F, Pimmer D *et al*. Stimulation of phospholipase C-beta2 by the Rho GTPases Cdc42Hs and Rac1. *EMBO J.* 1998; **17**:6241-6249.

110. Illenberger D, Walliser C, Nurnberg B, Diaz LM, Gierschik P. Specificity and structural requirements of phospholipase C-beta stimulation by Rho GTPases versus G protein beta gamma dimers. *J.Biol.Chem.* 2003; **278**:3006-3014.

111. Kamat A, Carpenter G. Phospholipase C-gamma1: regulation of enzyme function and role in growth factor-dependent signal transduction. *Cytokine Growth Factor Rev.* 1997; **8**:109-117.

112. Wahl MI, Nishibe S, Suh PG, Rhee SG, Carpenter G. Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium. *Proc.Natl.Acad.Sci.U.S.A* 1989; **86**:1568-1572.

113. Meisenhelder J, Suh PG, Rhee SG, Hunter T. Phospholipase C-gamma is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. *Cell* 1989; **57**:1109-1122.

114. Kim JW, Sim SS, Kim UH *et al*. Tyrosine residues in bovine phospholipase C-gamma phosphorylated by the epidermal growth factor receptor in vitro. *J.Biol.Chem.* 1990; **265**:3940-3943.

115. Rebecchi MJ, Pentyala SN. Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev.* 2000; **80**:1291-1335.

116. Piechulek T, Rehlen T, Walliser C, Vatter P, Moepps B, Gierschik P. Isozyme-specific stimulation of phospholipase C-gamma2 by Rac GTPases. *J.Biol.Chem.* 2005; **280**:38923-38931.

117. Ji QS, Winnier GE, Niswender KD *et al*. Essential role of the tyrosine kinase substrate phospholipase C-gamma1 in mammalian growth and development. *Proc.Natl.Acad.Sci.U.S.A* 1997; **94**:2999-3003.

118. Wang S, Lukinius A, Zhou Y *et al*. Subcellular distribution of phospholipase C isoforms in rodent pancreas and gastric mucosa. *Endocrinology* 2000; **141**:2589-2593.

119. Saunders CM, Larman MG, Parrington J *et al*. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* 2002; **129**:3533-3544.

120. Kuroda K, Ito M, Shikano T *et al*. The role of X/Y linker region and N-terminal EF-hand domain in nuclear translocation and Ca2+ oscillation-inducing activities of phospholipase Czeta, a mammalian egg-activating factor. *J.Biol.Chem.* 2006; **281**:27794-27805.

121. Hwang JI, Oh YS, Shin KJ, Kim H, Ryu SH, Suh PG. Molecular cloning and characterization of a novel phospholipase C, PLC-eta. *Biochem.J.* 2005; **389**:181-186.

122. Nakahara M, Shimozawa M, Nakamura Y *et al*. A novel phospholipase C, PLC(eta)2, is a neuron-specific isozyme. *J.Biol.Chem.* 2005; **280**:29128-29134.

123. Zhou Y, Wing MR, Sondek J, Harden TK. Molecular cloning and characterization of PLC-eta2. *Biochem.J.* 2005; **391**:667-676.

124. Stewart AJ, Mukherjee J, Roberts SJ, Lester D, Farquharson C. Identification of a novel class of mammalian phosphoinositol-specific phospholipase C enzymes. *Int.J.Mol.Med.* 2005; **15**:117-121.

125. Cantley LC, Whitman M, Chahwala S *et al*. Oncogenes and phosphatidylinositol turnover. *Ann.N.Y.Acad.Sci.* 1986; **488**:481-490.

126. Wakelam MJ, Davies SA, Houslay MD, McKay I, Marshall CJ, Hall A. Normal p21N-ras couples bombesin and other growth factor receptors to inositol phosphate production. *Nature* 1986; **323**:173-176.

127. Shibatohge M, Kariya K, Liao Y *et al*. Identification of PLC210, a Caenorhabditis elegans phospholipase C, as a putative effector of Ras. *J.Biol.Chem.* 1998; **273**:6218-6222.

128. Song C, Satoh T, Edamatsu H *et al*. Differential roles of Ras and Rap1 in growth factor-dependent activation of phospholipase C epsilon. *Oncogene* 2002; **21**:8105-8113.

129. Lopez I, Mak EC, Ding J, Hamm HE, Lomasney JW. A novel bifunctional phospholipase c that is regulated by Galpha 12 and stimulates the Ras/mitogen-activated protein kinase pathway. *J.Biol.Chem.* 2001; **276**:2758-2765.

130. Kelley GG, Reks SE, Ondrako JM, Smrcka AV. Phospholipase C(epsilon): a novel Ras effector. *EMBO J.* 2001; **20**:743-754.

131. Wing MR, Houston D, Kelley GG, Der CJ, Siderovski DP, Harden TK. Activation of phospholipase C-epsilon by heterotrimeric G protein betagamma-subunits. *J.Biol.Chem.* 2001; **276**:48257-48261.

132. Ponting CP, Benjamin DR. A novel family of Ras-binding domains. *Trends Biochem.Sci.* 1996; **21**:422-425.

133. Song C, Hu CD, Masago M *et al*. Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras. *J.Biol.Chem.* 2001; **276**:2752-2757.

134. Wohlgemuth S, Kiel C, Kramer A, Serrano L, Wittinghofer F, Herrmann C. Recognizing and defining true Ras binding domains I: biochemical analysis. *J.Mol.Biol.* 2005; **348**:741-758.

135. Bunney TD, Harris R, Gandarillas NL *et al*. Structural and mechanistic insights into ras association domains of phospholipase C epsilon. *Mol.Cell* 2006; **21**:495-507.

136. Pacold ME, Suire S, Perisic O *et al*. Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. *Cell* 2000; **103**:931-943.

137. Kuriyama M, Harada N, Kuroda S *et al*. Identification of AF-6 and canoe as putative targets for Ras. *J.Biol.Chem.* 1996; **271**:607-610.

138. Quilliam LA, Rebhun JF, Castro AF. A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. *Prog.Nucleic Acid Res.Mol.Biol.* 2002; **71**:391-444.

139. Jin TG, Satoh T, Liao Y *et al*. Role of the CDC25 homology domain of phospholipase Cepsilon in amplification of Rap1-dependent signaling. *J.Biol.Chem.* 2001; **276**:30301-30307.

140. Kelley GG, Reks SE, Smrcka AV. Hormonal regulation of phospholipase Cepsilon through distinct and overlapping pathways involving G12 and Ras family G-proteins. *Biochem.J.* 2004; **378**:129-139.

141. Bunney TD, Katan M. Phospholipase C epsilon: linking second messengers and small GTPases. *Trends Cell Biol.* 2006; **16**:640-648.

142. Wing MR, Snyder JT, Sondek J, Harden TK. Direct activation of phospholipase C-epsilon by Rho. *J.Biol.Chem.* 2003; **278**:41253-41258.

143. Kozasa T, Jiang X, Hart MJ *et al*. p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science* 1998; **280**:2109-2111.

144. Hart MJ, Jiang X, Kozasa T *et al*. Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. *Science* 1998; **280**:2112-2114.

145. Fukuhara S, Chikumi H, Gutkind JS. Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho. *FEBS Lett.* 2000; **485**:183-188.

146. Kuner R, Swiercz JM, Zywietz A, Tappe A, Offermanns S. Characterization of the expression of PDZ-RhoGEF, LARG and G(alpha)12/G(alpha)13 proteins in the murine nervous system. *Eur.J.Neurosci.* 2002; **16**:2333-2341.

147. Hains MD, Wing MR, Maddileti S, Siderovski DP, Harden TK. Galpha12/13- and rho-dependent activation of phospholipase C-epsilon by lysophosphatidic acid and thrombin receptors. *Mol.Pharmacol.* 2006; **69**:2068-2075.

148. Niu J, Profirovic J, Pan H, Vaiskunaite R, Voyno-Yasenetskaya T. G Protein betagamma subunits stimulate p114RhoGEF, a guanine nucleotide exchange factor for RhoA and Rac1: regulation of cell shape and reactive oxygen species production. *Circ.Res.* 2003; **93**:848-856.

149. Bourguignon LY, Gilad E, Brightman A, Diedrich F, Singleton P. Hyaluronan-CD44 interaction with leukemia-associated RhoGEF and epidermal growth factor receptor promotes Rho/Ras co-activation, phospholipase C epsilon-Ca2+ signaling, and cytoskeleton modification in head and neck squamous cell carcinoma cells. *J.Biol.Chem.* 2006; **281**:14026-14040.

150. Schmidt M, Evellin S, Weernink PA *et al*. A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nat.Cell Biol.* 2001; **3**:1020-1024.

151. Vom DF, Sari AY, Sanders H *et al*. Inhibition of phospholipase C-epsilon by Gi-coupled receptors. *Cell Signal.* 2004; **16**:921-928.

152. Stope MB, Vom DF, Szatkowski D *et al*. Rap2B-dependent stimulation of phospholipase C-epsilon by epidermal growth factor receptor mediated by c-Src phosphorylation of RasGRP3. *Mol.Cell Biol.* 2004; **24**:4664-4676.

153. Kariya K, Kim BY, Gao X, Sternberg PW, Kataoka T. Phospholipase Cepsilon regulates ovulation in Caenorhabditis elegans. *Dev.Biol.* 2004; **274**:201-210.

154. Bai Y, Edamatsu H, Maeda S *et al*. Crucial role of phospholipase Cepsilon in chemical carcinogen-induced skin tumor development. *Cancer Res.* 2004; **64**:8808-8810.

155. Tadano M, Edamatsu H, Minamisawa S *et al*. Congenital semilunar valvulogenesis defect in mice deficient in phospholipase C epsilon. *Mol.Cell Biol.* 2005; **25**:2191-2199.

156. Wang H, Oestreich EA, Maekawa N *et al*. Phospholipase C epsilon modulates beta-adrenergic receptor-dependent cardiac contraction and inhibits cardiac hypertrophy. *Circ.Res.* 2005; **97**:1305-1313.

157. Hinkes B, Wiggins RC, Gbadegesin R *et al*. Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. *Nat.Genet.* 2006; **38**:1397-1405.

158. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* 1990; **348**:125-132.

159. Branden CI, Eklund H. Structure and mechanism of liver alcohol dehydrogenase, lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. *Experientia Suppl* 1980; **36**:40-84.

160. Sprang SR. G protein mechanisms: insights from structural analysis. *Annu.Rev.Biochem.* 1997; **66**:639-678.

161. Vetter IR, Wittinghofer A. The guanine nucleotide-binding switch in three dimensions. *Science* 2001; **294**:1299-1304.

162. Sondek J, Lambright DG, Noel JP, Hamm HE, Sigler PB. GTPase mechanism of Gproteins from the 1.7-A crystal structure of transducin alpha-GDP-AIF-4. *Nature* 1994; **372**:276-279.

163. Li G, Zhang XC. GTP hydrolysis mechanism of Ras-like GTPases. *J.Mol.Biol.* 2004; **340**:921-932.

164. Schweins T, Geyer M, Scheffzek K, Warshel A, Kalbitzer HR, Wittinghofer A. Substrate-assisted catalysis as a mechanism for GTP hydrolysis of p21ras and other GTP-binding proteins. *Nat.Struct.Biol.* 1995; **2**:36-44.

165. Schweins T, Scheffzek K, Assheuer R, Wittinghofer A. The role of the metal ion in the p21ras catalysed GTP-hydrolysis: Mn2+ versus Mg2+. *J.Mol.Biol.* 1997; **266**:847-856.

166. Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. *Physiol Rev.* 2001; **81**:153-208.

167. Hall A. The cellular functions of small GTP-binding proteins. *Science* 1990; **249**:635-640.

168. Valencia A, Chardin P, Wittinghofer A, Sander C. The ras protein family: evolutionary tree and role of conserved amino acids. *Biochemistry* 1991; **30**:4637-4648.

169. Shih TY, Williams DR, Weeks MO, Maryak JM, Vass WC, Scolnick EM. Comparison of the genomic organization of Kirsten and Harvey sarcoma viruses. *J.Virol.* 1978; **27**:45-55.

170. Der CJ, Stanbridge EJ. A tumor-specific membrane phosphoprotein marker in human cell hybrids. *Cell* 1981; **26**:429-438.

171. Hall A, Marshall CJ, Spurr NK, Weiss RA. Identification of transforming gene in two human sarcoma cell lines as a new member of the ras gene family located on chromosome 1. *Nature* 1983; **303**:396-400.

172. Glomset JA, Farnsworth CC. Role of protein modification reactions in programming interactions between ras-related GTPases and cell membranes. *Annu.Rev.Cell Biol.* 1994; **10**:181-205.

173. Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. *Annu.Rev.Biochem.* 1996; **65**:241-269.

174. Magee T, Marshall C. New insights into the interaction of Ras with the plasma membrane. *Cell* 1999; **98**:9-12.

175. Hancock JF, Paterson H, Marshall CJ. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* 1990; **63**:133-139.

176. Boguski MS, McCormick F. Proteins regulating Ras and its relatives. *Nature* 1993; **366**:643-654.

177. Overbeck AF, Brtva TR, Cox AD *et al*. Guanine nucleotide exchange factors: activators of Ras superfamily proteins. *Mol.Reprod.Dev.* 1995; **42**:468-476.

178. Boriack-Sjodin PA, Margarit SM, Bar-Sagi D, Kuriyan J. The structural basis of the activation of Ras by Sos. *Nature* 1998; **394**:337-343.

179. Gamblin SJ, Smerdon SJ. GTPase-activating proteins and their complexes. *Curr.Opin.Struct.Biol.* 1998; **8**:195-201.

180. Scheffzek K, Ahmadian MR, Kabsch W *et al*. The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* 1997; **277**:333-338.

181. Herrmann C. Ras-effector interactions: after one decade. *Curr.Opin.Struct.Biol.* 2003; **13**:122-129.

182. Rodriguez-Viciana P, Warne PH, Dhand R *et al*. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* 1994; **370**:527-532.

183. Feramisco JR, Gross M, Kamata T, Rosenberg M, Sweet RW. Microinjection of the oncogene form of the human H-ras (T-24) protein results in rapid proliferation of quiescent cells. *Cell* 1984; **38**:109-117.

184. Noda M, Ko M, Ogura A *et al*. Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. *Nature* 1985; **318**:73-75.

185. Bar-Sagi D, Feramisco JR. Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. *Cell* 1985; **42**:841-848.

186. Ulrich E, Kauffmann-Zeh A, Hueber AO *et al*. Gene structure, cDNA sequence, and expression of murine Bak, a proapoptotic Bcl-2 family member. *Genomics* 1997; **44**:195-200.

187. Evellin S, Nolte J, Tysack K *et al*. Stimulation of phospholipase C-epsilon by the M3 muscarinic acetylcholine receptor mediated by cyclic AMP and the GTPase Rap2B. *J.Biol.Chem.* 2002; **277**:16805-16813.

188. Nisimoto Y, Freeman JL, Motalebi SA, Hirshberg M, Lambeth JD. Rac binding to p67(phox). Structural basis for interactions of the Rac1 effector region and insert region with components of the respiratory burst oxidase. *J.Biol.Chem.* 1997; **272**:18834-18841.

189. McCallum SJ, Wu WJ, Cerione RA. Identification of a putative effector for Cdc42Hs with high sequence similarity to the RasGAP-related protein IQGAP1 and a Cdc42Hs binding partner with similarity to IQGAP2. *J.Biol.Chem.* 1996; **271**:21732-21737.

190. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 1992; **70**:389-399.

191. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 1992; **70**:401-410.

192. Kozma R, Ahmed S, Best A, Lim L. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol.Cell Biol.* 1995; **15**:1942-1952.

193. Adamson P, Marshall CJ, Hall A, Tilbrook PA. Post-translational modifications of p21rho proteins. *J.Biol.Chem.* 1992; **267**:20033-20038.

194. Adamson P, Paterson HF, Hall A. Intracellular localization of the P21rho proteins. *J.Cell Biol.* 1992; **119**:617-627.

195. Rossman KL, Sondek J. Larger than Dbl: new structural insights into RhoA activation. *Trends Biochem.Sci.* 2005; **30**:163-165.

196. Schmidt A, Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev.* 2002; **16**:1587-1609.

197. Rossman KL, Der CJ, Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat.Rev.Mol.Cell Biol.* 2005; **6**:167-180.

198. Yamauchi J, Miyamoto Y, Tanoue A, Shooter EM, Chan JR. Ras activation of a Rac1 exchange factor, Tiam1, mediates neurotrophin-3-induced Schwann cell migration. *Proc.Natl.Acad.Sci.U.S.A* 2005; **102**:14889-14894.

199. Moon SY, Zheng Y. Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol.* 2003; **13**:13-22.

200. Ridley AJ, Self AJ, Kasmi F *et al*. rho family GTPase activating proteins p190, bcr and rhoGAP show distinct specificities in vitro and in vivo. *EMBO J.* 1993; **12**:5151-5160.

201. Keep NH, Barnes M, Barsukov I *et al*. A modulator of rho family G proteins, rhoGDI, binds these G proteins via an immunoglobulin-like domain and a flexible N-terminal arm. *Structure.* 1997; **5**:623-633.

202. Hoffman GR, Nassar N, Cerione RA. Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. *Cell* 2000; **100**:345-356.

203. Gosser YQ, Nomanbhoy TK, Aghazadeh B *et al*. C-terminal binding domain of Rho GDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases. *Nature* 1997; **387**:814-819.

204. Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J.Cell Biol.* 1994; **126**:801-810.

205. Brown AM, O'Sullivan AJ, Gomperts BD. Induction of exocytosis from permeabilized mast cells by the guanosine triphosphatases Rac and Cdc42. *Mol.Biol.Cell* 1998; **9**:1053-1063.

206. Glise B, Noselli S. Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in Drosophila morphogenesis. *Genes Dev.* 1997; **11**:1738-1747.

207. Hall A. Rho GTPases and the actin cytoskeleton. *Science* 1998; **279**:509-514.

208. Burbelo PD, Drechsel D, Hall A. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J.Biol.Chem.* 1995; **270**:29071-29074.

209. Hoffman GR, Cerione RA. Flipping the switch: the structural basis for signaling through the CRIB motif. *Cell* 2000; **102**:403-406.

210. Flynn P, Mellor H, Palmer R, Panayotou G, Parker PJ. Multiple interactions of PRK1 with RhoA. Functional assignment of the Hr1 repeat motif. *J.Biol.Chem.* 1998; **273**:2698-2705.

211. Bishop AL, Hall A. Rho GTPases and their effector proteins. *Biochem.J.* 2000; **348 Pt 2**:241-255.

212. Martinez O, Goud B. Rab proteins. *Biochim.Biophys.Acta* 1998; **1404**:101-112.

213. Novick P, Zerial M. The diversity of Rab proteins in vesicle transport. *Curr.Opin.Cell Biol.* 1997; **9**:496-504.

214. Weis K. Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. *Cell* 2003; **112**:441-451.

215. Li HY, Cao K, Zheng Y. Ran in the spindle checkpoint: a new function for a versatile GTPase. *Trends Cell Biol.* 2003; **13**:553-557.

216. Goldberg J. Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. *Cell* 1998; **95**:237-248.

217. SUTHERLAND EW, RALL TW. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J.Biol.Chem.* 1958; **232**:1077-1091.

218. Rodbell M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* 1980; **284**:17-22.

219. Northup JK, Sternweis PC, Smigel MD, Schleifer LS, Ross EM, Gilman AG. Purification of the regulatory component of adenylate cyclase. *Proc.Natl.Acad.Sci.U.S.A* 1980; **77**:6516-6520.

220. Wedegaertner PB, Wilson PT, Bourne HR. Lipid modifications of trimeric G proteins. *J.Biol.Chem.* 1995; **270**:503-506.

221. Coleman WB, Throneburg DB, Grisham JW, Smith GJ. Overexpression of c-K-ras, c-N-ras and transforming growth factor beta co-segregate with tumorigenicity in morphologically transformed C3H 10T1/2 cell lines. *Carcinogenesis* 1994; **15**:1005-1012.

222. Berstein G, Blank JL, Jhon DY, Exton JH, Rhee SG, Ross EM. Phospholipase C-beta 1 is a GTPase-activating protein for Gq/11, its physiologic regulator. *Cell* 1992; **70**:411-418.

223. Siderovski DP, Hessel A, Chung S, Mak TW, Tyers M. A new family of regulators of G-protein-coupled receptors? *Curr.Biol.* 1996; **6**:211-212.

224. Koelle MR, Horvitz HR. EGL-10 regulates G protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins. *Cell* 1996; **84**:115-125.

225. Dohlman HG, Song J, Ma D, Courchesne WE, Thorner J. Sst2, a negative regulator of pheromone signaling in the yeast Saccharomyces cerevisiae: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit). *Mol.Cell Biol.* 1996; **16**:5194-5209.

226. Fukami K. Structure, regulation, and function of phospholipase C isozymes. *J.Biochem.(Tokyo)* 2002; **131**:293-299.

227. Jiang H, Lyubarsky A, Dodd R *et al*. Phospholipase C beta 4 is involved in modulating the visual response in mice. *Proc.Natl.Acad.Sci.U.S.A* 1996; **93**:14598-14601.

228. Fukami K, Nakao K, Inoue T *et al*. Requirement of phospholipase Cdelta4 for the zona pellucida-induced acrosome reaction. *Science* 2001; **292**:920-923.

229. Mizuguchi M, Yamada M, Kim SU, Rhee SG. Phospholipase C isozymes in neurons and glial cells in culture: an immunocytochemical and immunochemical study. *Brain Res.* 1991; **548**:35-40.

230. Wing MR, Bourdon DM, Harden TK. PLC-epsilon: a shared effector protein in Ras-, Rho-, and G alpha beta gamma-mediated signaling. *Mol.Interv.* 2003; **3**:273-280.

231. Seifert JP, Wing MR, Snyder JT, Gershburg S, Sondek J, Harden TK. RhoA activates purified phospholipase C-epsilon by a guanine nucleotide-dependent mechanism. *J.Biol.Chem.* 2004; **279**:47992-47997.

232. Illenberger D, Stephan I, Gierschik P, Schwald F. Stimulation of phospholipase C-beta 2 by Rho GTPases. *Methods Enzymol.* 2000; **325**:167-177.

233. Kapust RB, Tozser J, Fox JD *et al*. Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. *Protein Eng* 2001; **14**:993-1000.

234. Illenberger D, Walliser C, Strobel J *et al*. Rac2 regulation of phospholipase C-beta 2 activity and mode of membrane interactions in intact cells. *J.Biol.Chem.* 2003; **278**:8645-8652.

235. Baek KJ, Kang S, Damron D, Im M. Phospholipase Cdelta1 is a guanine nucleotide exchanging factor for transglutaminase II (Galpha h) and promotes alpha 1B-adrenoreceptor-mediated GTP binding and intracellular calcium release. *J.Biol.Chem.* 2001; **276**:5591-5597.

236. Yoda A, Oda S, Shikano T *et al*. Ca2+ oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation. *Dev.Biol.* 2004; **268**:245-257.

237. Waldo GL, Paterson A, Boyer JL, Nicholas RA, Harden TK. Molecular cloning, expression and regulatory activity of G alpha 11- and beta gamma-subunit-stimulated phospholipase C-beta from avian erythrocytes. *Biochem.J.* 1996; **316 (Pt 2)**:559-568.

238. Hepler JR, Kozasa T, Smrcka AV *et al*. Purification from Sf9 cells and characterization of recombinant Gq alpha and G11 alpha. Activation of purified phospholipase C isozymes by G alpha subunits. *J.Biol.Chem.* 1993; **268**:14367-14375.

239. Morris AJ, Waldo GL, Downes CP, Harden TK. A receptor and G-protein-regulated polyphosphoinositide-specific phospholipase C from turkey erythrocytes. II. P2Y-purinergic receptor and G-protein-mediated regulation of the purified enzyme reconstituted with turkey erythrocyte ghosts. *J.Biol.Chem.* 1990; **265**:13508-13514.

240. Ren XD, Bokoch GM, Traynor-Kaplan A, Jenkins GH, Anderson RA, Schwartz MA. Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. *Mol.Biol.Cell* 1996; **7**:435-442.

241. Chatah NE, Abrams CS. G-protein-coupled receptor activation induces the membrane translocation and activation of phosphatidylinositol-4-phosphate 5-kinase I alpha by a Rac- and Rho-dependent pathway. *J.Biol.Chem.* 2001; **276**:34059-34065.

242. Houssa B, de Widt J, Kranenburg O, Moolenaar WH, van Blitterswijk WJ. Diacylglycerol kinase theta binds to and is negatively regulated by active RhoA. *J.Biol.Chem.* 1999; **274**:6820-6822.

243. Brown HA, Lazarowski ER, Boucher RC, Harden TK. Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5'-nucleotide receptor in human airway epithelial cells. *Mol.Pharmacol.* 1991; **40**:648-655.

244. Seifert JP, Snyder JT, Sondek J, Harden TK. Direct activation of purified phospholipase C epsilon by RhoA studied in reconstituted phospholipid vesicles. *Methods Enzymol.* 2006; **406**:260-271.

245. Bokoch GM, Vlahos CJ, Wang Y, Knaus UG, Traynor-Kaplan AE. Rac GTPase interacts specifically with phosphatidylinositol 3-kinase. *Biochem.J.* 1996; **315 (Pt 3)**:775-779.

246. Keely PJ, Westwick JK, Whitehead IP, Der CJ, Parise LV. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* 1997; **390**:632-636.

247. Sankaran B, Osterhout J, Wu D, Smrcka AV. Identification of a structural element in phospholipase C beta2 that interacts with G protein betagamma subunits. *J.Biol.Chem.* 1998; **273**:7148-7154.