REGULATION OF PHOSPHOLIPASE C-δ1 THROUGH DIRECT INTERACTIONS WITH THE SMALL GTPase RAL AND CALMODULIN

By

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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REGULATION OF PHOSPHOLIPASE C-δ1 THROUGH DIRECT INTERACTIONS WITH THE SMALL GTPase RAL AND CALMODULIN

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ABSTRACT

Second messengers generated from membrane lipids play a critical role in signaling and control diverse cellular processes. Despite being the most evolutionarily conserved of all the phospholipase C (PLC) isoforms, a family of enzymes responsible for hydrolysis of the membrane lipid phosphatidylinositol bisphosphate (PIP_2), the mechanism of PLC-δ1 activation is still poorly understood. Here we report a novel regulatory mechanism for PLC-δ1 activation that involves direct interaction of the small GTPase Ral and the universal calcium-signaling molecule calmodulin (CaM) with PLC-δ1. We have utilized the Yeast Two-Hybrid (Y2H) system to screen a human testis cDNA library with RalB as the bait to identify PLC-δ1 as a novel binding partner for this GTPase. The Y2H assay was used as an *in vivo* model system to confirm interaction of both RalA and RalB with PLC-δ1. *In vitro* binding experiments using the C-terminal Ral binding region in PLC-δ1 (PLC-$\delta1_{CT}$) demonstrated that endogenous Ral was precipitated from HeLa cell lysates. In reverse experiments, GST-RalA and GST-RalB bound endogenous PLC-δ1 from HeLa cell lysates. Ral interaction with the PLC-$\delta1_{CT}$ occurs in a calcium (Ca^{2+})-dependent manner and PLC-$\delta1_{CT}$ co-precipitates CaM with Ral at high Ca^{2+} concentrations. However, *in vitro* binding assays using PLC-$\delta1_{CT}$ and HeLa cell cytosol fraction, which contains CaM but not Ral, showed no binding to CaM. *In vitro* activity assays

using recombinant PLC-δ1 plus Ral led to an increased hydrolysis of the PLC substrate [^{3}H]-PIP_2 to [^{3}H]-inositol trisphosphate (IP_3). The addition of CaM resulted in inhibition while addition of Ral plus CaM led to reversal of Ral-mediated potentiation of PLC-δ1 enzyme activity. These results suggested that a CaM binding region exists in PLC-δ1. Using a database to search for CaM binding sequence patterns we identified a novel IQ type CaM binding motif within the catalytic region of PLC-δ1 that is not found in other PLC isoforms. *In vitro* binding assays using a synthetic peptide representing the sequence of the CaM binding target region in PLC-δ1 (PLC-IQ) confirmed that the peptide binds CaM in a typical IQ fashion, independent of Ca^{2+}. Enzyme assays in the presence of PLC-IQ exhibited inhibition of PLC-δ1 activity and a partial reversal of Ral-mediated potentiation of enzyme activity. These data suggest that the novel CaM binding IQ-motif sequence in PLC-δ1 may itself regulate enzyme activity.

In order to determine whether Ral is an effector of PLC-δ1 we performed binding assays using recombinant Ral protein preloaded with GTP or GDP or using Ral from cells treated with epidermal growth factor (EGF). Results indicated that Ral binding to PLC-δ1 occurs irrespective of the guanine nucleotide status. Binding experiments using various mutant Ral proteins overexpressed in mammalian cells showed that Ral interaction with PLC-δ1 requires the N-terminal 11 amino acids and mutation of residue 49 (Aspartic acid to Asparagine) in Ral significantly reduced the

binding. Measurements of enzyme activity using immunoprecipitated PLC-δ1 from cells overexpressing a constitutively active Ras mutant or various Ral mutants resulted in increased PIP_2 hydrolysis except in the case of N-terminal truncated Ral mutant. In summary, the results presented here establish a role for Ral and calmodulin in PLC-δ1 regulation and in calcium mediated pathways of the cell.

ACKNOWLEDGMENTS

I wish to thank Dr. Rajinder Bhullar for critical reading of the thesis, help in designing of experiments, stimulating scientific discussions, and most of all the trust placed in my judgment. The experience has been rewarding and thoroughly enjoyable.

Thanks to Drs. Gilbert Arthur, James Gilchrist and Archibald McNicol for participating on my advisory committee. The support has been immensely invaluable. Thank you to Dr. Parmjit S. Tappia for expert advice for PLC-δ1 enzyme assays. A special thank you to Dr. Elliott Scott, without whom the introduction to Dr. Bhullar would never have been possible.

I am most thankful to my wife, Kuljeet Kaur, for her patience, understanding and great support. This work is dedicated to my father Mewa Singh Sidhu and my father-in-law late Amar Singh Lakhyan who have been an inspiration for hard work and dedication. As well, my mother, mother-in-law, brother, brother-in-laws and sister-in-laws who have all played important roles in my life.

If we all worked on the assumption that what is accepted as true were really true, there would be little hope of advance. Orville Wright

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LIST OF ABBREVIATIONS

AEBSF – 4-[(2-aminoethyl)]-benzenesulfonyl fluoride

BSA – Bovine serum albumin

CaM – Calmodulin

DAG – Diacylglycerol

DMEM – Dulbecco's modified Eagle's Medium

DTT – Dithiothreitol

EDTA – Ethylene diaminetetraacetic acid

EGF – Epidermal Growth Factor

EGTA – Ethylene glycol-bis(β-aminoethyl ether)- N,N,N',N'-tetraacetic acid

FBS – Fetal Bovine Serum

GAP – GTPase activating protein

GEF – Guanine nucleotide exchange factor

GST – Glutathione S-transferase

HA – Hemagglutinin

IP_3 or $InsP_3$ - Inositol 1,4,5 trisphosphate

IPTG – Isopropyl-1-thio-β-D-galactopyranoside

MCS – Multiple cloning site

PBS – Phosphate buffered saline

PDK1 – PI3-kinase-dependent kinase

PH domain – Pleckstrin homology domain

PIP_2 – Phosphatidylinositol (4,5)bisphosphate

PKC – Protein kinase C

PLC – Phosphoinositide-specific phospholipase C

PLC-IQ – PLC-δ1 amino acids 473-492

PLC-$\delta 1_{CT}$ – PLC-δ1 amino acids 647-756

PLC-$\delta 1_{\Delta CT}$ – PLC-δ1 amino acids 1-646

PLD – Phospholipase D

PTK – Protein tyrosine kinase

PVDF – Polyvinyl difluoride

RalGDS – Ral Guanine nucleotide Dissociation Stimulator

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SH2 – Src homology 2

TIM-barrel – triosephosphate isomerase-like barrel

W7 – W7•HCl, N-aminohexyl-5-chloro-1-naphthalenesulfonamide, HCl

X-α-Gal – 5-bromo-4-chloro-3-indolyl-D-galactopyranoside

Chapter 1

INTRODUCTION

1.1. GTP-binding proteins in signal transduction

Cells of a living organism are continuously exposed to a vast array of physical and chemical signals. Receptors on the cell surface transmit the extracellular signals across the cell membrane stimulating the production of intracellular secondary messengers. Once evoked, second messengers initiate a cascade of reactions that ultimately lead to the production of a wide variety of cellular responses. Many different mechanisms have evolved to process the barrage of signals into physiological responses. Upon binding molecules, called ligands, some receptors, such as the acetylcholine class, function as channels that allow signals to be passed in the form of small ions. This movement of ions across the membrane results in changes in the electrical potential of the cell. The change in potential is then decoded by sensor proteins within the cell (http://web.indstate.edu/thcme/mwking/signal-transduction.html). Complex signal transduction mechanisms have evolved that couple the ligand-receptor interactions to events such as phosphorylation by tyrosine kinases and/or serine/threonine kinases. The protein phosphorylation can change enzyme activities and/or protein conformation eventually leading to alterations in cellular responses. There are three general classes of signal transducing receptors: (1) Receptors that penetrate the plasma membrane and have intrinsic enzymatic activity,

such as tyrosine kinases (cytokine, PDGF, insulin, EGF and FGF receptors), tyrosine phosphatases (macrophage and T cell CD45 [cluster determinant-45] protein), guanylate cyclases (natriuretic peptide receptors) and serine/threonine kinases (TGF-β and activin receptors); (2); Receptors that penetrate the plasma membrane and have intrinsic enzymatic activity (2) Receptors that are coupled to GTP-binding and hydrolyzing proteins (called G-proteins), such as the adrenergic receptors and certain hormone receptors (glucagon, angiotensin, vasopressin and bradykinin); (3) Intracellular receptors, such as steroid hormone receptors, that migrate from the cell cytoplasm to the nucleus upon ligand binding. There the ligand-receptor complex can directly activate gene transcription (http://web.indstate.edu/theme/mwking/signal-transduction.html). The vast array of research in signal transduction is continually being updated and resources have been compiled to maintain the database of information at sites such as the Alliance for Celluar Signaling (AfCS; http://www.signaling-gateway.org) and the Signal Transduction Knowledge Environment (STKE; http://stke.sciencemag.org).

A large superfamily of receptors containing seven membrane spanning regions, called *heterotrimeric G protein coupled receptors* (GPCRs), receive an extracellular signal in the form of hormones, neurotransmitters, chemokines, and autocrine and paracrine factors, and communicate the stimuli to G-proteins on the inner surface of cells (Neves *et al.*, 2002). The *heterotrimeric G-proteins*, which

consist of three subunits α, β, and γ form a network that regulates metabolic enzymes, ion channels, transporters and other cellular components that regulate many cellular processes (transcription, motility, contractility and secretion). These processes in turn regulate systemic functions such as embryonic development, gonadal development, learning and memory, and organismal homeostasis (Neves *et al.*, 2002).

During signaling, G proteins function as two distinct elements because the signal is communicated by either the α subunit or the $\beta\gamma$ complex. The $\beta\gamma$ complex cannot be dissociated under nondenaturing conditions, thus, within the cell the two subunits are constitutively associated. Presently, there are 20 known Gα (39-45 kDa), 6 Gβ (35-36 kDa), and 11 Gγ subunits (8 kDa) (McCudden *et al.*, 2005; Neves *et al.*, 2002; Stryer and Bourne, 1986). GDP-bound G protein α subunit has a high affinity for the β and γ complex and prevents binding to downstream effectors. The inactive state is maintained by a slow rate of dissociation of GDP ($k \sim 0.01$/min). Binding of an agonist to the receptor promotes the dissociation of GDP from the α subunit by a poorly understood mechanism. Since intracellular GTP is very high the transient GDP-unbound state of the G-protein quickly binds GTP resulting in a conformational change in the α subunit leading to the dissociation of α•GTP from the $\beta\gamma$ complex. Both of these can interact with cytoplasmic targets or effectors such as adenylyl cyclase, phospholipase C, cyclic nucleotide phosphodiesterase and ion channels (Berman and Gilman, 1998). The signal is terminated when GTP is hydrolyzed to

GDP (k_{cat} ~ 1-5/min) and dissociation from the effector protein occurs. The rate of hydrolysis determines the length of time Gα-GTP and Gβγ can interact with effectors (Zerangue and Jan, 1998). The family of proteins responsible for accelerating the intrinsic rate of GTP hydrolysis of Gα is called Regulators of G-protein Signaling or RGS proteins (Koelle *et al.*, 1996; Siderovski *et al.*, 1996; Watson *et al.*, 1996). The α•GDP subunit then reassociates with the βγ complex and is ready for a new cycle (Berman and Gilman, 1998).

The known Gα subunits are divided into 4 subfamilies $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12/13}$ based on sequence and functional homologies. These subunits have structural and functional homologies with other members of the GTP-binding protein superfamily (Neves *et al.*, 2002; Offermanns and Simon, 1996). The α subunit consists of two domains: the GTPase domain, which is homologous to other GTP-binding proteins, and a helical domain, which contributes to the stability of the nucleotide binding site (Offermanns and Simon, 1996).

The *Ras superfamily* of monomeric G-proteins, which contain more than 100 members, share a common structural design and molecular mechanism similar to that of Gα subunits of heterotrimeric G-proteins (Bourne *et al.*, 1991). The proteins of the Ras superfamily, which range between 20-30 kDa, participate in diverse cellular processes and are classified into four families (Ras-related, Rho, Rab/Ran and Sar1/Arf) based on sequence similarity (Colicelli, 2004) (Table I). The members of

the Ras subfamily share 30-50% sequence similarity with Ras-p21. The Ras-p21 (henceforth referred to as Ras) proteins (H-ras, K-ras and N-ras) received notoriety as being oncogenic gene products since they were found in nearly ~15-20% of all human tumours (Bos, 1988) with the highest incidence found in adenocarcinomas of the pancreas (90%), the colon (50%) and the lung (30%) and also in thyroid tumors (50%) and in myeloid leukemia (30%) (Bos, 1989).

Table 1: The Human small G protein superfamily [1]

Ras family		Rho family		Rab/Ran family[2]			SarA/Arf	
H-Ras	RasD1	Rac1	RhoC	Ran	RasEF	Rab5B	Arf1	Arl9
N-Ras	RasD2	Rac2	RhoB	RabL2A	Rab26	Rab22A	Arf3	Arl12
K-Ras2B	RasL10B	Rac3	RhoD	RabL2B	Rab37	Rab31	Arf4	Arf4L
E-Ras	RasL10A	Rac4	RhoF	RabL3	Rab2	Rab21	Arf2	Arl11
RalA	nKiRas1	RhoG	Rnd3	RabL5	Rab2B	Rab20	Arf5	Arf7
RalB	nKiRas2	RhoJ	Rnd2	Rab1A	Rab4A	Rab24	Arf6	339231
R-Ras1	Rerg	RhoQ	Rnd1	Rab1B	Rab4B	Rab6A	Trim23	DKZp761
R-Ras2	RasL11B	Cdc42	RhoBTB1	Rab35	Rab14	Rab6C	Arl1	Arf-Rp1
M-Ras	RasL11A	RhoU	RhoBTB2	Rab13	Rab11A	Rab6B	Arl2	Arf-Rp2
Rit1	RasL12	RhoV	RhoT1	Rab8A	Rab11B	Rab41	Arl3	Arl10A
Rit2	Gem	RhoH	RhoT2	Rab8B	Rab25	Rab34	Arl4A	Arl10B
Rap1A	R-Rad	RhoA		Rab10	Rab39	Rab36	Arl4B	Arl10C
Rap1B	Rem1			Rab12	Rab39B	Rab7L1	Arl5	344988
Rap2A	Rem2			Rab3A	Rab42	Rab29	Arl6	Sar1a
Rap2C	Rheb			Rab3C	Rab19	Rab32	Arl7	Sar1b
Rap2B	RebL1			Rab3B	Rab43	Rab38	Arl8	
Di-Ras1				Rab3D	Rab30	Rab23		
Di-Ras2				Rab40A	Rab33A	Rab28		
Arhi				Rab40B	Rab33B	RabL4		
				Rab40C	Rab18	Rab7		
				Rab15	Rab17	Rab7B		
				Rab44	Rab5A	Rab9B		
				Rab27A	Rab5C	Rab9A		
				Rab27B				

[1] Adapted from Colicelli, 2004 (Listed according to sequence homology)

[2] Ran proteins (Ran, RabL2A, RabL2B, RabL3, RabL5) were previously considered a separate family, however, based on sequence homology are presently considered a branch of the Rab family.

Chapter 2

LITERATURE REVIEW

2.1. Ras-related small G-proteins: Structural Considerations

The Ras-related small G-proteins share a common structural core, called the G domain, and significant sequence similarity. A great deal of information has been gained through the study of the three-dimensional structure of Ras (**Figure 1**). Comparisons of Ras with the multidomain bacterial protein EF-Tu, which is involved in protein synthesis, shows the guanine nucleotide binding domain of these two G proteins have a remarkable sequence conservation (Kjeldgaard *et al.*, 1996; Wittinghofer and Nassar, 1996). Structurally, Ras is composed of a hydrophobic core made of six strands of a β-sheet, connected by hydrophilic loops and α helices (Bourne *et al.*, 1991). An unusual feature of the GTP-binding domains of the G protein family is that the most highly conserved regions are the loop regions designated G1-G5 and not the β-sheet or α-helices (Paduch *et al*, 2001) (**Table 2**). The ***G1 region***, also called the ***P-loop*** (residues 10-17 in Ras), consists of the sequence motif $GX_4GK(S/T)$, where X is any amino acid. Hydrogens of amide groups from several residues and the ϵ-amino group of Lys^{16} in this region of Ras form bonds with the α- and β-phosphates of GTP or GDP. The three-dimensional structure of this region is identical in the GDP- and GTP-bound forms of Ras (Bourne *et al.*, 1991).

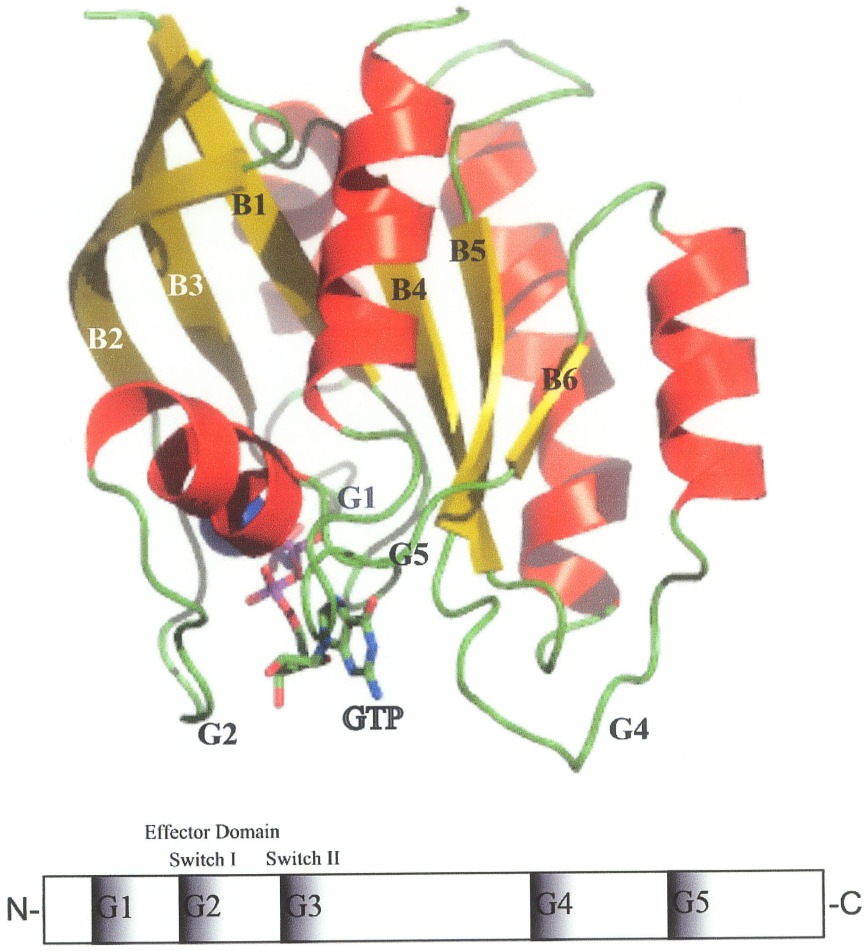


Figure 1: Structure of Small GTPases. (Top) Three-dimensional model of Ras (PDB 5P21) showing the conserved loop regions labeled G1, G2, G4 and G5. The G3 loop lies behind the guanine nucleotide and is not seen in this view. The β-strands are labeled B1-B6. (Bottom) A Schematic drawing of GTPases showing the layout of the consensus sequences (Table 2) for GTP/GDP binding and GTPase activities. The loop regions are indicated as G1-G5 and the G2/switch I (Effector domain) and G3 Switch II regions are also indicated.

Table 2: Sequence Alignment of G-protein conserved regions using CLUSTAL W (1.82) multiple sequence alignment

	G1 (P-Loop)	G2 (switch I) Effector loop	G3 (switch II)	G4	G5
	GXXXXGKS T	XTX	DXXG	NKXD TQ	EXSAX
Ral A (NP_005393.2)	GSGGVGKS	YEPTKAD	ILDTAGQE	VGNKSD	YVETSAK
Ral B (NP_002872.1)	GSGGVGKS	YEPTKAD	ILDTAGQE	VGNKSD	YVETSAK
H-Ras (P01112)	GAGGVGKS	YDPTIED	ILDTAGQE	VGNKCD	YIETSAK
Rab1A (AAV38336.1)	GDSGVGKS	YISTIGV	IWDTAGQE	VGNKCD	FLETSAK
Rac1 (P15154)	GDGAVGKT	YIPTVFD	LWDTAGQE	VGTKLD	QGLAMAK
Cdc42 (P25763)	GDGAVGKT	YVPTVFD	LFDTAGQE	VGTQID	TAEKLAR
Ran (NP_536351.1)	GAGESGKS	YVPSDQD	MFDVGGQR	FLNKQD	TTPEDAT
G_i, α-1 subunit (P04898)	GAGESGKS	YIPTQQD	MFDVGGQR	FLNKKD	NTYEEAA
Arf1 (P32889)	GLDAAGKT	YIPTIGF	VWDVGGQD	FANKQD	NWYIQAT
	* . **:	.:	: *..**	. .:	*

The ***G2 region*** or ***switch I*** (residues 32-40 in Ras) undergoes a conformational change upon GTP-binding mainly due to changes in the orientation of a critical threonine residue at position 35. The side-chain hydroxyls of Thr^{35} (as well as Ser^{17} from the G1 loop) and the oxygen atoms of the β- and γ-phosphates coordinate a magnesium (Mg^{2+}) ion, which is critical for GTP hydrolysis (Bourne *et al.*, 1991). The ***switch II*** or ***G3 region*** has the sequence motif Dx_2G (residues 53-62 in Ras) that is invariably present in all GTPases. This is structurally the most flexible element of the catalytic domain (Paduch *et al.*, 2001). The Asp residue at position 57 binds the catalytic Mg^{2+} through interaction with a water molecule. The amide proton of Gly^{60} forms a hydrogen bond with the γ-phosphate of GTP. Due to the inherent flexibility of the amino acid glycine a significant amount of rotation occurs at this position (Gly^{60}) when GDP is bound accounting for dramatically different conformation in this

loop region (Bourne *et al.*, 1991). The dynamics of switch I and II regions differs for the GTP- and GDP-bound forms (Paduch *et al.*, 2001).

The ***G4 region***, which consists of four hydrophobic or apolar amino acids followed by (N/T)(K/Q)XD, corresponds to a hydrophobic β strand and a hydrophilic loop. Residues Asp^{119}, Asn^{116} and Lys^{117} along with residues 13 and 14 of the G1 loop of Ras contribute to stabilizing the guanine nucleotide (Bourne *et al.*, 1991). The ***G5 region*** (residues 144-146 of Ras) containing the sequence OO**EXSA**X (where O is a hydrophobic amino acid) is better conserved in the small GTPases and is thought to interact indirectly with guanine nucleotide (Bourne *et al.*, 1991).

Ras family proteins also contain signal sequences at the carboxyl-terminus that promote association with the plasma membrane. The C-terminal C*AA*X motif (where *A* is an aliphatic amino acid and X is methionine or serine) signals 3 post-translational modifications: addition of farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoid groups, *AA*X proteolysis, and carboxymethylation (Casey *et al.*, 1991; Hancock *et al.*, 1989). For some Ras proteins the membrane association signal sequence is a palmitate fatty acid (H-Ras, N-Ras, and K-Ras4A) or lysine-rich polybasic sequence (K-Ras-4B) (Reuther and Der, 2000). In contrast, the α subunit of heterotrimeric G-proteins is myristoylated at an N-terminal glycine residue (De Vries *et al.*, 2000).

2.2. Role of the magnesium ion

A magnesium (Mg^{2+}) ion is seen associated with all G-protein three-dimensional structures and is essential for proper GTP/GDP binding (Paduch *et al.*, 2001). The role of the Mg^{2+} ion is to coordinate the oxygen atoms of the β- and γ-phosphate groups on the guanine nucleotide. In the absence of bound, nucleotide G proteins have significantly decreased stability (Paduch *et al.*, 2001). In *in vitro* studies 100nM Mg^{2+} is sufficient to ensure nucleotide binding (Paduch *et al.*, 2001). Above micromolar concentrations of Mg^{2+} the dissociation rate constant (k_{off}) of Ras for GDP is decreased by four orders of magnitude (Paduch *et al.*, 2001). The presence of the γ-phosphate of GTP induces rigidity of the switch I/II due to interactions with the Mg^{2+} ion. Hydrolysis of GTP to GDP destabilizes the effector region (switch I) (Paduch *et al.*, 2001). The switch I/II, which are the most flexible regions, govern Mg^{2+} and γ-phosphate binding.

The G1 phosphate recognition loop and the G4 nucleotide recognition loop are conserved in all GTPases. However, the G2 Mg^{2+} coordination loop and G3 γ-phosphate binding loops are more diverse (Paduch *et al.*, 2001). The dissociation rate constant for Ras-GTP ($k_{off} \sim 10^{-5}$ s^{-1}) is greater than for GDP ($k_{off} \sim 10^{-4}$ s^{-1}). The Mg^{2+} ion is hexacoordinated to the β phosphate, γ phosphate, Ser (or Thr) from G1, Thr from G2 and to two water molecules (Paduch *et al.*, 2001).

2.3. The GTPase cycle

Guanine nucleotide exchange factors (GEFs) for monomeric G proteins act similarly to G protein coupled receptors (GPCRs) in that they promote the release of bound GDP from Ras for replacement with GTP (Bourne *et al.*, 1990). GEFs interact with Ras by formation of a beta-sheet interface and contacts in both switch I and II regions of Ras (Manser, 2002). In the GTP bound state Ras binds with high affinity to a set of cytoplasmic targets or effector proteins that initiate distinct downstream intracellular signaling cascades (Manser, 2002). The G2 region, also known as the effector-binding loop, is the site for the binding of effector molecules and GAP proteins (Paduch *et al.*, 2001). Three well established Ras effectors are the Raf kinases, phosphatidylinositol-3-OH kinases and Ral-specific GEFs. As is the case for the RGS proteins, which accelerate the GTPase activity of heterotrimeric G proteins, GTPase-activating proteins (GAPs) function similarly in the Ras family. That is, GAPs accelerate the slow intrinsic GTPase activity by as much as 100-fold (Trahey and McCormick, 1987; Gibbs *et al.*, 1988; Polakis and McCormick, 1993; Zerangue and Jan, 1998). GAPs help stabilize an important catalytic Gln residue in the switch II region of the GTPase and insert an Arg residue in the active site to stabilize the transition state of GTP hydrolysis reaction (Corbett and Alber, 2001). The core of the interface forms a continuous antiparallel β-sheet with residues provided by both the GTPase and the GAP proteins. The specificity of GAP binding to GTPases is determined outside the β-sheet core by additional interactions provided by other

surfaces (Corbett and Alber, 2001). The variation in specificity may be conferred by a large α-helical interaction domain with the variable regions of the small GTPases (Boguski and McCormick, 1993; Souchet *et al.*, 2000). Acceleration in the rate of GTP hydrolysis by GAPs leads to inactivation of the G protein and dissociation from effectors (Manser, 2002). Although all GAP proteins have not been isolated, based on BLAST searches from the available human and Drosophilia genomes, Bernards (2003) predicts that 0.5% of the total genes may encode proteins related to GAPs for Ras superfamily members. Some GAP proteins, such as the Rho family GAP p190, are promiscuous and can act on all Rho-like proteins whereas some GAPs are specific, for example Bcr is Rac specific and Grb-1, the p85 subunit of PI3-Kinase, is Ras-specific and does not act on any Rho/Rac-like protein (Boguski and McCormick, 1993). A family of proteins called guanine nucleotide dissociation inhibitors (GDI) exist for the Rho and Rab GTPases (Wu *et al.*, 1996; Olafsson, 1999). GDIs bind the GDP-bound form of these GTPases and maintain the complex in the inactive state within the cytosol (Wu *et al.*, 1996). The cycling of GTP binding proteins between the GDP-bound inactive and GTP-bound active conformations allows them to function as molecular switches (**Figure 2**) (Bourne *et al.*, 1990; Takai *et al.*, 2001).

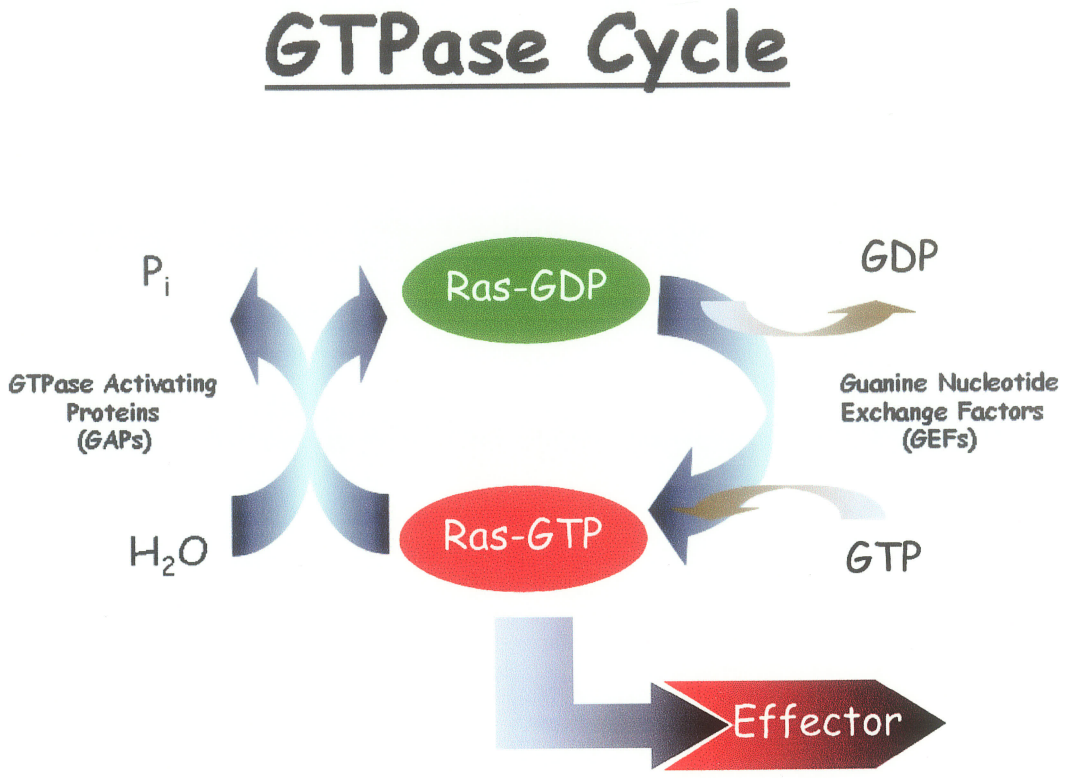


Figure 2: The GTPase cycle. In this example Ras is used, however, the generalized cycle can be applied for most of the small GTPases. See text for a detailed description of the cyle.

2.4. Ras effectors

In the GTP-bound conformation Ras binds and activates effector proteins. Three genuine Ras effectors are the Raf kinases (Raf1, B-Raf and A-raf), the p85-p110 phosphatidylinositol-3 kinase, and members of the RalGEF family (RalGDS, Rgl1, Rgl2, and Rlf) (Bos, 1998; Feig, 2003; Quilliam *et al.*, 2002). Binding to effectors occurs by direct interactions made between two anti-parallel strands formed by the two proteins (Wittinghofer and Nassar, 1996). As a general rule the basic

biochemical and structural findings for one member of this superfamily can usually be applied to all the others (Wittinghofer and Nassar, 1996).

The members of the RalGEF family are exchange factors for the Ras-related G-protein Ral and were identified on the basis of homology to yeast RasGEFs (Albright *et al.*, 1993). The RalGEFs were found to have *in vitro* GEF activity (removal of GDP and replacement with GTP) for the Ras-related RalA and RalB proteins (Wolthuis and Bos 1999; Bos 1998). The C-terminal region of RalGEFs contains a Ras binding domain (RBD) that binds Ras-GTP thereby allowing it to function as a downstream effector molecule. The likely role of Ras is to recruit RalGEFs to Ral at the plasma membrane (Matsubara *et al.*, 1999). Since RalGEF functions as a Ras effector, rapid activation of Ral occurs by stimulation of a variety of upstream GPCRs and tyrosine-kinase associated receptors. Most mitogens activate Ral at least five-fold, converting about 15-50% of Ral to its GTP-bound form (Wolthuis and Bos, 1999). However, Ras independent activation of Ral does occur, for instance in response to elevated levels of calcium (Ca^{2+}) (Hofer *et al.*, 1998; Wolthuis (a) *et al.*, 1998). Alternatively, Ras-independent Ral activation may occur through a Ca^{2+}-sensitive RalGEF, or more directly by Ca^{2+}/CaM, which binds to the C-terminus of Ral (Clough *et al.*, 2002; Wang *et al.*, 1997; Wolthuis and Bos, 1999). Nevertheless, although Ral is a downstream element in Ras-mediated signaling, Ral activation is controlled by both Ras-dependent and independent events.

2.5. Use of Ras Mutants

The classical Ras proteins have generated a lot of interest due to the high incidence of mutations of this gene in human cancers (Barbacid, 1989). Since the discovery of its involvement in human oncogenesis a great deal of progress has been made in the understanding of normal Ras protein function. The use of dominant inhibitory Ras mutants has contributed greatly to this understanding. Inhibitory Ras mutants were first identified by random mutagenesis studies (Sigal *et al.*, 1996). The most intensely studied of the early mutants was the dominant negative Ser to Asp mutation at position 17. Dominant-inhibitory mutants work by competing with normal Ras for binding to RasGEFs (Feig, 1999). The mutants cannot interact with downstream target proteins, so bind to GEFs and form "dead-end" complexes, thus, preventing the activation of endogenous Ras by RasGEFs. Hence, overexpression of wild-type Ras or Ras-specific GEFs can overcome the growth inhibitory effects of RasS17N. This Ser^{17} residue along with Thr^{35} is involved in binding of the Mg^{2+} (Feig, 1999). The RasQ61L mutant produces a similar biological response to RasS17N (Wittinghofer and Nassar, 1996).

A mutant Ras with a Gly to Val substitution at residue 12 (RasV12G) leads to a constitutively active Ras. Such a substitution has been known to be the cause of carcinogenesis due to the reduction in the intrinsic GTPase activity of Ras (Chung et al, 1993). Although the structure of the active site of this mutant Ras appears to be the same as the wild-type, molecular dynamic calculations suggest continuous changes

occur in the active site of the enzyme substrate complex thus rendering the GTPase unable to hydrolyze GTP to GDP. GAPs have no reaction-activating effect on this mutant and the intrinsic GTPase activity is only 15% that of the wild-type (Futatsugi and Tsuda, 2001)

Another very useful inhibitory mutant is a constitutively active mutant that lacks the C-terminal isoprenylation C*AA*X motif (RasV12ΔC*AA*X). This binds to and traps Ras effector proteins in the cytosol, where they are ineffectual (Feig, 1999). However, this mutant is not as effective as the Ras17N mutant in inhibiting normal Ras activity. This is possibly due to the fact that RasV12ΔC*AA*X also competes for RasGAP and thus increases the amount of endogenous Ras-GTP at the same time as it competes for effector proteins (Feig, 1999).

A powerful tool for studying the signaling events downstream of Ras has been the use of Ras effector domain mutants. The RasV12S35 and RasV12E38 are constitutively active mutants that bind only Raf and not RalGEF and PI-3 kinase, whereas RasV12G37 only binds RalGEF, and RasV12C40 only PI-3 kinase (Bos, 1998; Reuther and Der 2000; Bauer *et al.*, 1999). Although, the use of these and other effector domain mutants of Ras or Ras-related proteins can provide insight into GTPase function they should be used with caution. Introduction of mutations in the effector region can induce erroneous binding to effectors specific for other GTPases (Bauer *et al.*, 1999).

2.3. Structural basis for Ras interactions

Binding of Ras to the minimal Ras binding domains of its effectors, such as Raf-RBD (Ras binding domain) or the functionally equivalent RalGDS-RID (Ras-interacting domain) has been examined using X-ray crystallography (Huang *et al.*, 1997; Geyer *et al.*, 1997; Nassar *et al.*, 1996; Nassar *et al.*, 1995). Ras-associating (RA) domain sequences are extremely divergent but have conserved hydrophobic profiles (Ponting and Benjamin 1996). The RA-domains share a three-dimensional fold that is similar to that of ubiquitin (Huang *et al.*, 1997; Wohlgemuth et al., 2005). Crystal structures of Ras with RalGDS-RID, seen as a heterotetrameric complex, reveal that interactions occur between two antiparallel β-strands that include the switch I (effector) region of Ras interacting with one RID and the switch II region of Ras with another RID (**Figure 3**). Hence, effector domain mutations account for the differential selectivity for Ras binding partners. In addition, the Ras G60A mutation (analogous to RalG72A) abolishes the interaction with RalGDS likely due to perturbation of critical interaction in the switch II region with the RalGDS-RID (Huang *et al.*, 1998). Comparison of Ras with other Ras family members reveals that the three-dimensional structures are virtually identical despite having limited sequence identity (Nassar *et al.*, 1996). The specificity of Ras family protein interactions is determined mainly through electrostatic surface charge at critical regions on the proteins surface. Charge reversal by mutagenesis of Ras and Rap alters the

dissociation constants for Ras/Rap effectors Raf and RalGDS (**Table 3**) (Nassar *et al.*, 1996).

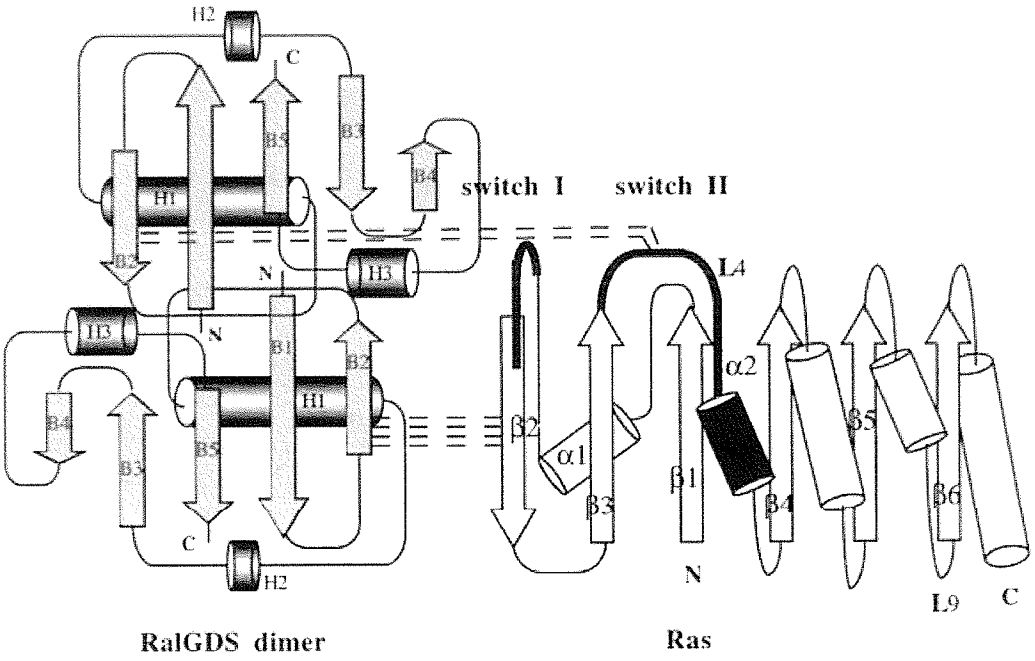


Figure 3: Topology Diagram of Ras interaction with RalGDS dimers. The β2 strand and the switch II region of Ras make contact with two individual RalGDS molecules (From Huang *et al.*, 1998)

The closely related Ral GTPase does not bind to the Ras binding domains of Ras effector proteins. The Ral binding domain of sec5 has been solved and is clearly different from that of the RBDs of Raf and PI-3 kinase. The Ral binding IPT domain of the Ral effector protein sec5 shares a fold similar to that of NF-κB (Mott *et al.*, 2003). Although the overall topology is different from RasBD, the intermolecular

antiparallel β-sheet formed between sec5 and Ral is similar to that of RBD-Ras binding complex (Fukai *et al.*, 2003).

Table 3 : Changes in K_D upon Charge reversal

	Raf-RBD	RalGDS-RBD
Ras	0.018	1
Rap1A	1.2	0.01
Rap(K31E)	0.06	0.15
Rap(E30D,K31E)	0.04	0.25

* *in μM* (Data from Nassar *et al.*, 1996)

2.6. Small GTPase Ral

The Ras superfamily includes classical Ras proteins (H-Ras, N-Ras, K-Ras) and the R-Ras (*Related to Ras*), Rap (*Ras-Proximal*), Ral (*Ras-like*), Rit (*Ras-like Protein in All Tissues*), E-Ras (*Embryonic Stem Cell-Expressed Ras*), Di-Ras (*Distinct Subgroup of Ras*), RasD (*Ras induced by Dexamethasone*), NKI-Ras (*NFκB Inhibitor-interacting Ras-like, also called κB-Ras*), Rem (*Rad and Gem-Related*), Rerg (*Ras-related and Estrogen regulated Growth Inhibitor*) and Rheb (*Ras Homolog Enriched in Brain*) proteins (Colicelli, 2004). The two Ral (RalA and RalB) proteins share about 85% sequence identity and >50% identity with Ras-p21 (H-Ras) (**Figure 4**) (Chardin and Tavitian, 1989; Chardin and Tavitian, 1986). The Ral proteins differ primarily in their C-terminal region (Chardin and Tavitian, 1986).

Figure 4: Ral A and B amino acid sequences. Conserved regions G1-G5 are shown boxed and in red text. The variable C-terminal domain is highlighted in blue while the isoprenylation C*AA*X motif shown in green.

2.7. Regulation of Ral Activation

Until recently it was generally accepted that Ras is found almost exclusively on the internal face of the plasma membrane while Ral is present at the plasma membrane and secretory vesicles (Cantor *et al.*, 1995; Mark *et al.*, 1996). Recent work demonstrates the presence of Ras on the Golgi apparatus (Bivona *et al.*, 2003) and perhaps other internal membranes, such as the endoplasmic reticulum (di Fiore, 2003). The presence of the distinct localization of Ras and Ral in cells and the Ral exchange factor activity of the Ras effector RalGDS indicates Ral activation is a downstream event of Ras signaling.

Ras-mediated activation of Ral occurs when Ras-GTP binds and delivers RalGEFs to Ral at cell membranes (Kishida *et al.*, 1997; Matsubara *et al.*, 1999). The binding of Ras-GTP to RalGDS removes the autoinhibitory domain action of RalGDS thus allowing guanine nucleotide exchange activity on Ral. Recent studies indicate

that Ral activation can be enhanced by a complementary mechanism involving another Ras effector, PI-3 kinase. When cells are stimulated with EGF, PI3-kinase binds PI3-kinase-dependent kinase (PDK1) to form a complex with RalGDS. This binding removes the autoinhibitory effect of RalGDS thereby activating Ral (Tian *et al.*, 2002). These studies indicate that Ral activation occurs as a consequence of the combined effect of Ras-induced relocalization of RalGDS to the membrane and enhancement of the catalytic activity of RalGDS by PDK1 (Feig, 2003).

Ras-independent pathways of Ral activation have also been demonstrated and could involve $[Ca^{2+}]_i$ elevation, diacylglycerol, Src-like kinases and PI-3 kinases (M'Rabet *et al.*, 1999; Hofer *et al.*, 1998 Biol; Wolthuis *et al.* (a), 1998). Recently, β-arrestin was shown to be activated when cells were exposed to the bacterial cell wall derived chemoattractant peptide formyl-Met-Leu-Phe (fMLP). RalGDS bound to β-arrestin in cytosol dissociates during fMLP receptor stimulation resulting in the Ras-independent Ral activation pathway required for cytoskeletal rearrangment (Bhattacharya *et al*, 2002). Another possible mechanism of Ral activation could involve the Rap small GTPase. In *Drosophila* the main activator of Ral is Rap not Ras and Rap has a greater affinity for Ral-GEFs than does Ras (Mirey *et al.*, 2003). Although Rap activation correlates with Ral activation in platelets stimulated with thrombin or treated with the calcium ionophore ionomycin (Wolthuis *et al* (b), 1998) it is still generally accepted that the main Ral activator in mammalian cells is the Ras-RalGEF pathway (Wolthuis et al (a), 1998). Another potential mechanism of Ral

activation has been proposed through the Ras-related protein TC21 (R-Ras2), which interacts directly with RalGEFs (Rosario *et al.*, 2001). However, not much is known about the mechanism of Ral activation by TC21.

Inactivation of Ral can be facilitated by GAP proteins, which increase the low intrinsic rate of GTP-hydrolysis of G proteins, thereby, turning "off" the GTPase signal. To date, no Ral-specific mammalian GAP gene has been isolated. However, GAP activity for Ral has been reported associated with a 34kDa protein found in platelet cytosol and particulate fractions (Bhullar and Seneviratne, 1996), a partially purified protein complex from cytosolic fractions of brain and testis (Emkey *et al.*, 1991) and the bacterial *Pseudomonas aeruginosa* toxin ExoS (Henriksson *et al.*, 2002).

2.8. Ral Effector pathways

Ral proteins possess pivotal roles in diverse cell functions such as the control of cell proliferation (Chien and White, 2003), migration (Gildea *et al.*, 2002), filapod formation (Sugihara *et al.*, 2002), differentiation (de Ruiter *et al.*, 2000), cytoskeletal organization (Bhattacharya *et al.*, 2002), vesicular transport (Moskalenko *et al.*, 2002), exocytosis (Polzin *et al.*, 2002) and receptor endocytosis (Jullien-Flores *et al.*, 2000; Nakashima *et al.*, 1999). A brief description of Ral's involvement in these pathways follows and is summarized in **Figure 5**.

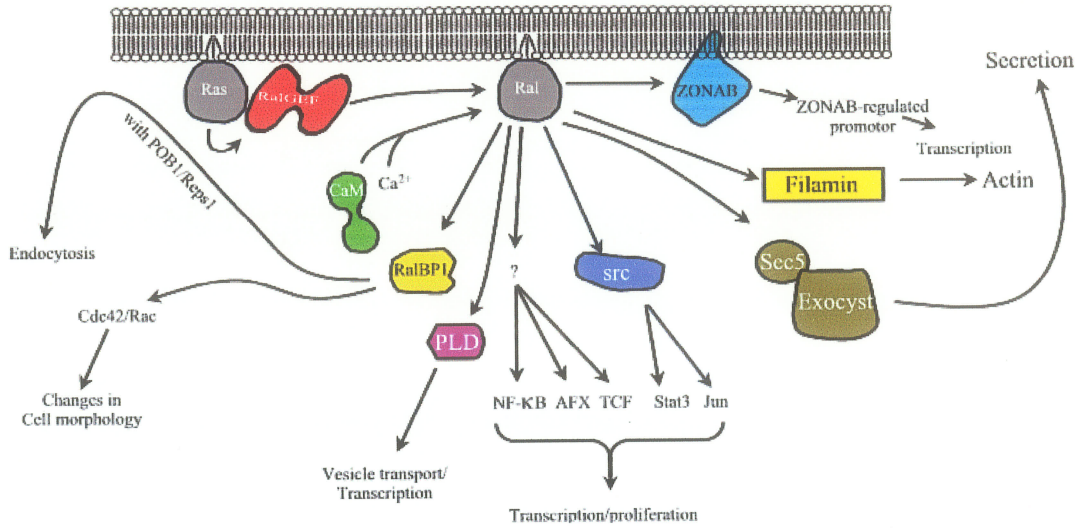


Figure 5: Summary of Ral effector pathways. Ral can be activated by Ras-dependent (Ras-RalGEF) or Ras-independent (e.g. Ca^{2+}/CaM) pathways. Ral controls several diverse cell functions such as endocytosis (through RalBP1), changes in cell morphology (via Cdc42/Rac and filamin), vesicle transport (phospholiase D (PLD)-mediated), transcription/proliferation (src, PLD and others) and secretion (through the exocyst complex).

2.8.1. Role in Vesicle Transport

The first Ral effector to be discovered was Ral binding protein 1 (RalBP1)/Ral interacting protein (also known as RLIP76 or RIP1) (Cantor *et al*., 1995; Jullien-Flores *et al*., 1995). RalBP1 regulates endocytosis of the EGF receptor, insulin receptor, and transferrin receptor via POB1 (Nakashima *et al*, 1999) and μ2, the medium chain of the AP2 complex (Jullien-Flores *et al*, 2000). RalBP1 associates with tyrosine phosphorylated POB1 or Reps1 to form a complex with EGF receptors (Wolthuis and Bos, 1999). The exact mechanism by which clathrin-coated vesicles are formed is unclear. However, it is known that the μ2 subunit promotes the

interaction of AP2 with integral membrane proteins and their recruitment into clathrin-coated pits. However, Ral-GTP binding is not required for RalBP1 association with POB1, Reps1 or AP2 (Feig, 2003). POB1 and Reps1 each contain a domain that is homologous to Eps15 called the Eps homology (EH) domain and is a protein known to be involved in regulating EGF receptor endocytosis. Thus, RalGEF-Ral signaling pathway may play a role in controlling receptor tyrosine kinase levels at the plasma membrane.

Ral has also been shown to participate in the regulated secretion of vesicles in polarized cells through the binding to sec5 and Exo84 of the exocyst complex (Moskalenko *et al.*, 2002). The exocyst is a multiprotein complex involved in the targeting of Golgi-derived vesicles to the basolateral membrane of polarized cells (Feig, 2003). The binding of Ral-GTP to the exocyst regulates exocytosis and filopod formation (Brymora *et al.*, 2001; Sugihara *et al*, 2002; Moskalenko *et al.*, 2002).

Phospholipase D (PLD) is an atypical Ral effector in that it binds constitutively to the amino terminal of Ral (Jiang *et al.*, 1995). Although Ral was shown to associate directly with PLD1 (Luo *et al.*, 1997), binding of Ral and ADP-ribosylation factor 1 (Arf1) or Arf6 to PLD synergistically enhances PLD activity (Kim *et al.,* 1998; Luo *et al.*, 1998; Xu *et al.*, 2003). Phosphatidic acid generated by PLD is required for the formation of vesicles from the Golgi and the transport of vesicles from the endoplasmic reticulum to Golgi (Feig, 2003; Wolthuis and Bos, 1999). Whether constitutive association of PLD with Ral functions in association

with the exocyst complex to influence vesicle secretion is not known. However, PLD is known to participate in receptor-mediated endocytosis possibly through its association with Ral (Shen *et al.*, 2001; Feig, 2003). That is, EGF receptor uptake is reduced when PLD activation is inhibited and receptor uptake is accelerated when PLD was overexpressed. In addition, Ral activation is a prerequisite for PLD activation by EGF (Lu *et al.*, 2000).

2.8.2. Role in Gene Expression

Ral signaling pathways modulate the activity of several transcription factors such as the Forkhead transcription factors FOXO and AFX classes (Essers *et al.*, 2004; De Ruiter *et al.*, 2001). The exchange factor Rlf has been shown to promote the phosphorylation and activation of c-Jun through the activation of the Jun N-terminal kinase (JNK) (De Ruiter *et al.*, 2000). In addition, Rlf phosphorylates and activates AFX, however, AFX activation may require cooperation with PI-3 kinase signaling (De Ruiter *et al.*, 2000). Recently, the activation of Ral by H_2O_2, resulting in JNK phosphorylation, was found to induce phosphorylation and activation of FOXO. The activation of the FOXO transcription factor is known to reduce cellular oxidative stress through increasing manganese superoxide dismutase (MnSOD) and catalase protein levels (Essers *et al.*, 2004).

It has also been observed that expression of constitutively activate RalA (GTPase defective) stimulates the tyrosine kinase receptor c-Src leading to activation

of the transcription factor Stat3 (Goi *et al.*, 1999). Further, Ral activation was necessary to promote c-Src and STAT activity by EGF stimulation (Goi *et al.*, 1999). Induction of RalGDS gene expression, by activation of the gp130 receptor, was seen to regulate Ral activation through the JAK/STAT3/RalGDS pathway suggesting cross-talk between JAK/STAT3 and the Ras/RalGDS/Ral pathways (Senga *et al.*, 2001). In other experiments RalB was seen to promote the activition of NF-κB, which in turn leads to the expression of cyclin D through an NF-κB site on its promotor (Henry *et al.*, 2000). Another example of cross-talk between Ral and other pathways was suggested by the synergistic requirement of RalGDS and Raf to activate the c-fos promoter (Okazaki *et al.*, 1997). Although the effector pathways through which Ral-GEF and Ral proteins affect gene transcription are not known Rlf, an exchange factor for Ral, mediated Ras-induced but Raf-independent c-fos promoter activation and induction of gene expression and cell proliferation (Wolthuis *et al.*, 1997). In addition, regulation of gene expression by Ras and Ral/RalGDS pathways, as well as Rlf activation, in ventricular myocytes has been shown to lead to gene expression resulting in cardiac hypertrophy (Fuller *et al.*, 1998; Post *et al.*, 2002; Kawai *et al.*, 2003).

Recently, using a modified Yeast two hybrid screen, which employs a membrane localized bait protein, the Y-box transcription factor ZONAB (Zo-1-associated nucleic acid-binding) protein was isolated as a RalA binding partner (Frankel *et al.*, 2004). The GTP-dependent binding of RalA to ZONAB primarily at

intracellular junctions was found to relieve the transcriptional repression of a ZONAB-regulated promoter in a cell density dependent manner (Frankel *et al.*, 2004).

2.8.3. Role in the Regulation of Cell morphology

The small GTPases Cdc42 and Rac have long been known to mediate filopod formation in mammalian cells. The Ral effector, RalBP1, which contains a GAP domain for Cdc42 and Rac GTPases, is an effector of Ral suggesting a possible link between Ral and filopod formation (Cantor *et al.*, 1995; Jullien-Flores *et al.*, 1995). It was not until Ral-GTP was found to bind Filamin and induce the formation of filopodia that a clear link between Ral and filopod formation was established (Ohta *et al.*, 1999). Ral has also been shown to mediate filopod formation induced by TNF-α and interleukin-1, as well as by the activation of Cdc42 alone (Sugihara *et al.*, 2002). Thus, the involvement of Ral in filopod formation implicates this small GTPase in the regulation of cell morphology.

Cell motility is a physiological function of cells that involves changes in cell morphology by rearrangements in the actin cytoskeleton. Ral activation has been implicated in cytoskeletal changes associated with cell stimulation by chemoattract peptides such as fMLP (Battacharya *et al.*, 2002). In addition, Ral was implicated in chemotactic migration of skeletal myoblasts and border cell migration during Drosophila melanogaster oogenesis, suggesting that Ral regulated cell motility is

conserved in invertebrates (Suzuki *et al.*, 2000). The involvement of Ral in changes in cell morphology has also been recognized by the finding that Ral-GTP binds to sec5 and Exo84 of the exocyst complex (Moskalenko *et al.*, 2002). In addition to its role in the delivery of vesicles the exocyst complex may also influence cell morphology through effects on the actin cytoskeleton (Sugihara *et al.*, 2002).

2.8.4. Role in cell proliferation and cancer

A great deal of evidence supports a role for the Ral-GEF/Ral pathway in promoting Ras-induced oncogenesis. In fact, recent evidence suggests that in human cells Ral-GEF/Ral signaling pathway, and not the previously held belief that Raf or PI3-K pathway, plays a more potent role in augmenting oncogenesis (Hamad *et al.*, 2002). There are also some indications that Ral mediated activation of PLD could also contribute to cell proliferation (Wolthuis and Bos, 1999). That is, overexpression of activated RalA in rat fibroblasts that have high levels of EGF receptor demonstrate enhanced oncogenic transformation (Lu *et al.*, 2000). Ral proteins also modulate tumor cell growth through the Ras related GTPase TC21, which has been shown to be mutated in breast cancer cells. In these cells, inhibition of Ral activity was shown to suppress tumor proliferation (Rosario *et al.*, 2001). Activation of PLD leads to the hydrolysis of PC to PA and cholate. PA is metabolically converted to DAG and to lyso-PA. PA activates mTOR, a kinase that regulates cell cycle progression and cell

growth (Foster and Xu, 2003). Since Ral activates PLD this may provide an alternate mechanism for promoting cell proliferation and cancer.

Until recently it was unknown whether RalA and RalB have overlapping, non-overlapping or partially over-lapping functions. Recent evidence suggests that RalA and RalB collaborate in the maintenance of oncogenic transformation by mediating both oncogenic proliferation and cell survival (Chien and White, 2003). Although RalA is dispensable for proliferation of human epithelial cells and tumor-derived cells it is required for proliferation of transformed cells that are "anchorage-independent". In contrast, RalB is required to prevent transformed cells from initiating programmed cell death (Chien and White, 2003). Oncogenic transformation requires both enhanced proliferative ability and suppression of apoptosis and Ral GTPases are crucial components of both of these pathways.

Chapter 3

PHOSPHOLIPASE C FAMILY

3.1. The Phospholipase C Family

Phosphoinositide-specific phospholipase C (PLC) family of enzymes are responsible for the hydrolysis of phosphatidylinositol 4,5-bisphosphate [$PtdInsP_2$, $PI(4,5)P_2$, PIP_2] that results in the generation of second messengers inositol 1,4,5 trisphosphate ($InsP_3$ or IP_3) and diacylglycerol (DAG) (**Figure 6**) (Williams, 1999). $InsP_3$ is a universal calcium (Ca^{2+}) mobilizing second messenger and DAG functions as an activator of protein kinase C (PKC). Presently, there are at least 13 known mammalian PLC isozymes that are divided into six classes (PLC-β1-4, PLC-γ1-2, PLC-δ1,3,4, PLC-ϵ, PLC-ζ and PLC-η1,2) (Rhee, 2001; Song *et al.*, 2001; Saunders *et al.*, 2002; Stewart *et al.*, 2005) (**Figure 7**).

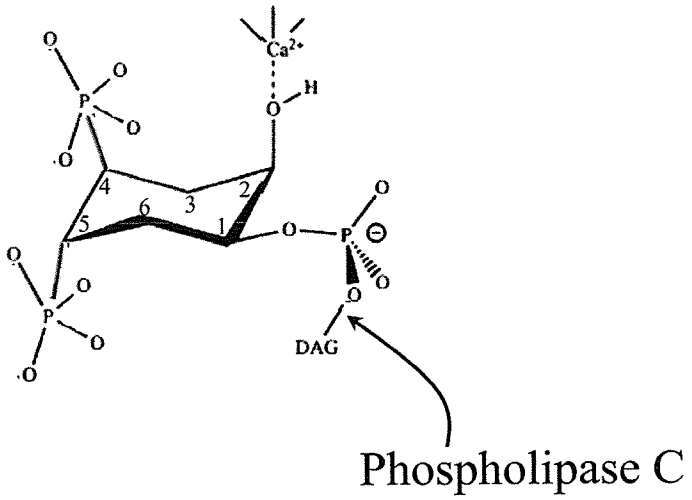


Figure 6: Phosphatidylinositol (4,5)-bisphosphate (PIP_2). Phospholipsase C family of enzymes generate the second messengers diacylglycerol and IP_3 by the hydrolysis of PIP_2 at the bond indicated by the arrow.

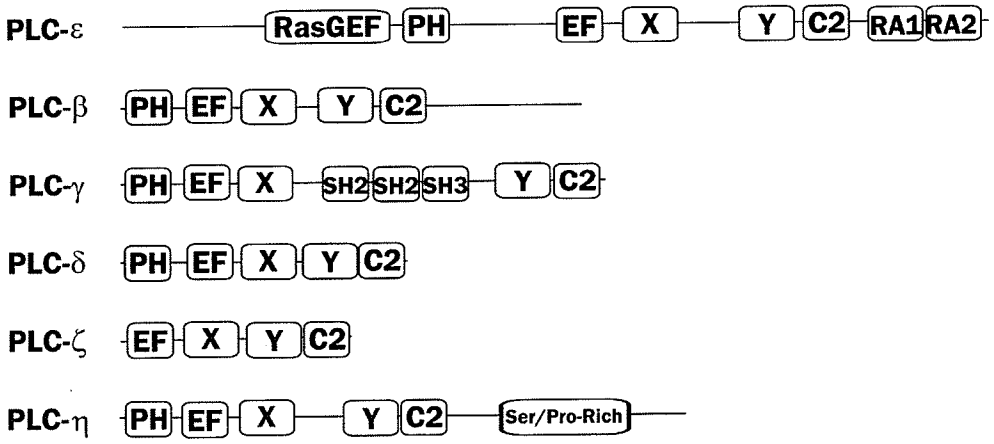


Figure 7: Phospholipase C family of enzymes. Proteins are arranged according to greatest sequence similarity and sizes are shown on a relative scale. Abbreviations used: PH = Pleckstrin Homology domain; EF = EF-hand domain; X = Catalytic X box; Y = Catalytic Y box; C2 = C2 domain; SH2 = Src Homology 2 domain; SH3 = Src Homology 3 domain; Ser/Pro-Rich = Serine/Proline rich region.

3.2. PLC Structure and catalytic mechanism

All the mammalian PLC enzymes are multisubunit proteins and share a common PLC-δ backbone. The N-terminal end contains a high affinity PIP_2 binding region called the pleckstrin homology (PH) domain that is required for membrane targeting (Katan and Allen, 1999). This is followed by four adjacent EF-hand domains, which are critical for catalytic activity (Essen *et al*, 1996). Located between the EF-hand and the C-terminal C2 domain is the catalytic core, composed of the X and Y domains. The catalytic X and Y domains have the greatest region of sequence similarity between all the PLC family members (Rhee *et al.*, 1989). X-ray crystallographic studies reveal that the X/Y domains converge in the three-dimensional structure to form two halves of a structural unit resembling a TIM (triosephosphate isomerase-like) barrel structure (Essen *et al.*, 1996). A disordered linker region containing several charged residues separate the X and Y domains. Cooperation of various regions in PLC-δ1 is highlighted in mutational studies showing that ablation of distal domains renders the enzyme inactive (Ellis *et al.*, 1993) **(Figure 8)**. The C2 domain has extensive interfaces with both the catalytic and EF-hand domains whereas the EF-hand domain does not make contacts with the catalytic domain (Essen *et al.*, 1996).

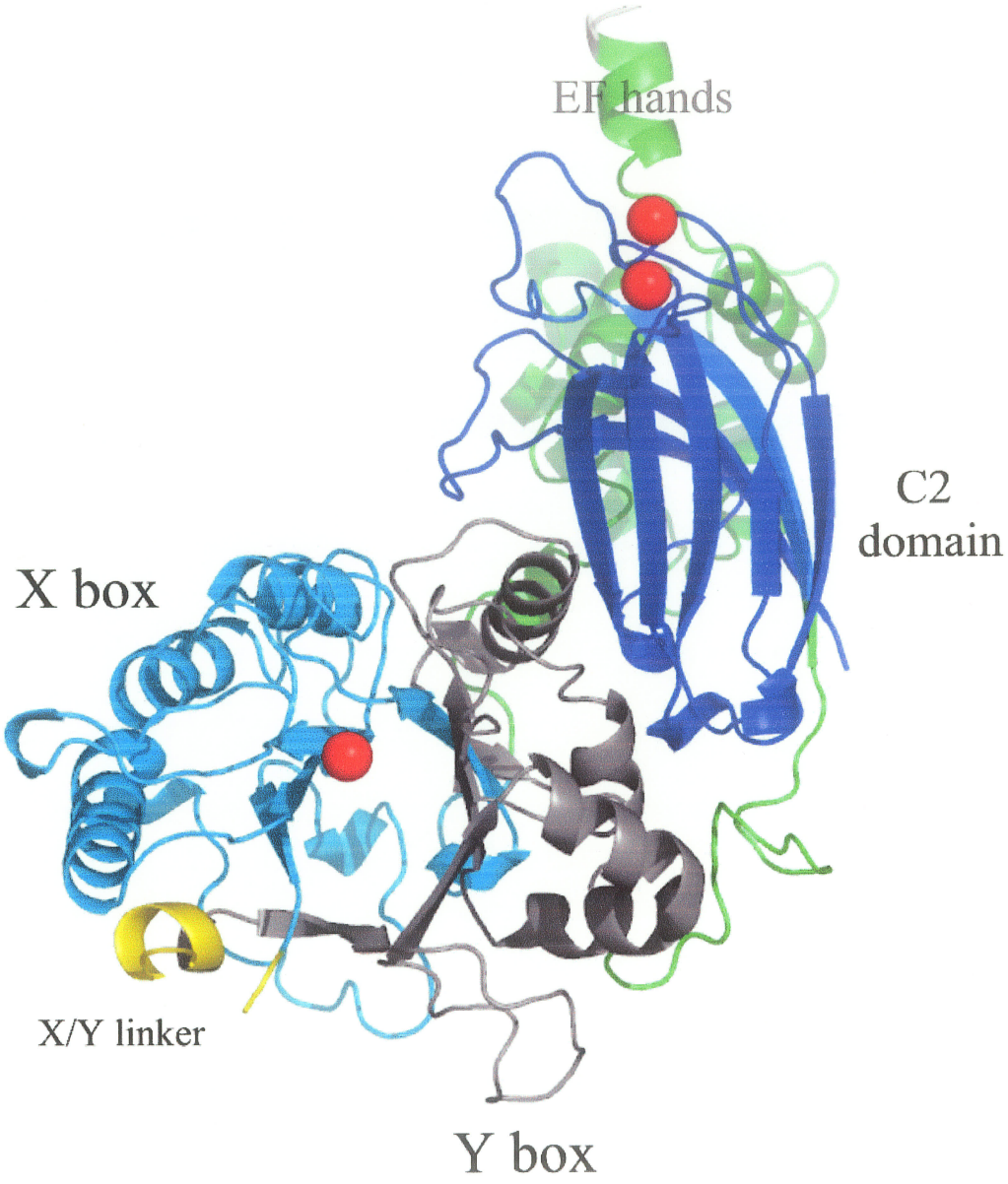


Figure 8: Three-dimensional structure of Phospholipase C-δ1. The available coordinates of crystallized PLC-δ1 (PDB 1DJX) show the modular organization of protein. The PH domain is missing from this structure but has been solved independently. The EF hand domain (**green**), X box (**aqua**), Y box (**grey**), partial X/Y linker (**yellow**), the C2 domain (**blue**) and calcium ions (**red**) are shown in the structure.

3.2.1. The PH domain

The N-terminal region of PLC-δ1 contains a PH domain, which is a module consisting of about 120 amino acids, and was first described in the platelet protein pleckstrin (Tyers *et al.*, 1988). The structures of PH domains in several proteins have been solved and show remarkable conservation in three-dimensional shape despite having limited sequence similarity (Ferguson *et al.*, 1995). The domain consists of seven antiparallel β-strands arranged in a barrel-like structure. The two halves of the barrel are formed by two sheets of three and four strands that is closed at the bottom by a long C-terminal α-helical tail (**Figure 9**) (Rebecchi and Scarlata, 1998). The PH domain of PLC-δ1 is necessary and sufficient for membrane association through interactions with phosphoinositides such as $PI(3,4,5)P_3$, $PI(4,5)P_2$, $PI(3,4)P_2$, PI(4)P and PI (Rebecchi and Scarlata, 1998; Williams *et al.*, 1999). Although, the PH domain can strongly bind $PI(3,4,5)P_3$ *in vitro*, the catalytic site in PLC-δ1 cannot accommodate the 3-position phosphomonoester (Rebecchi and Scarlata, 1998; Garcia *et al.*, 1995; Varnai *et al.*, 2002). In addition, the binding of $I(1,4,5)P_3$ (K_ds ~ 0.2μM) to the PH domain of PLC-δ1 is fivefold stronger than $PI(4,5)P_2$ (K_ds ~ 1.2-5μM) (Rebecchi and Scarlata, 1998). Measurements of the cellular levels of $PI(4,5)P_2$ suggest that, although the lipid is rare, there is sufficient levels to bind up all the intracellular PLC-δ1 (Rebecchi and Scarlata, 1998). PH domains in PLC-γ and PLC-β bind and are preferentially recruited to membranes by $PI(3,4,5)P_3$ and PI(3)P, respectively but not by $PI(4,5)P_2$ (Rhee, 2001).

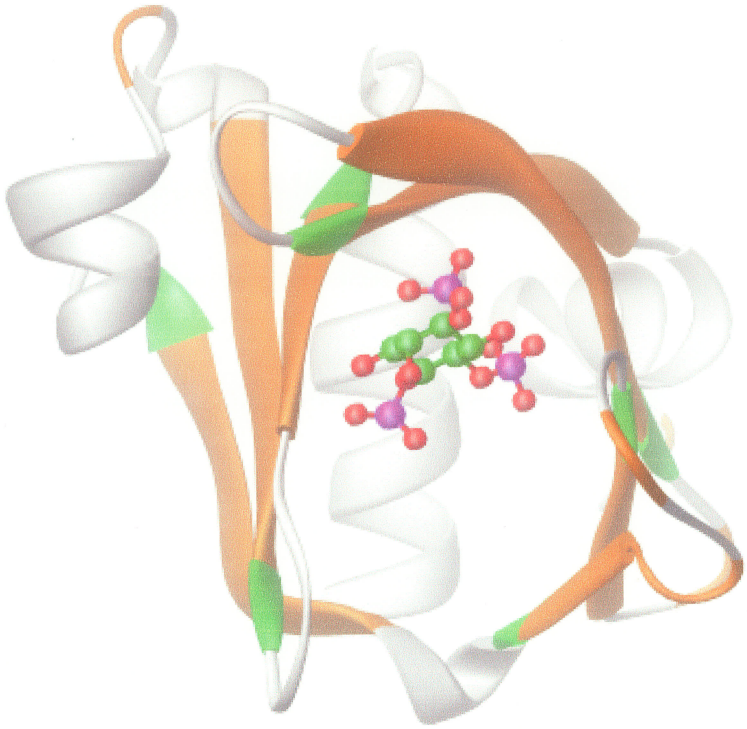


Figure 9: Top view of Pleckstrin Homology (PH) domain. The α-helices and loops (grey) and the β-strands (brown) are shown in complex with an IP_3 molecule (PDB 1MAI).

In unstimulated cells PLC-δ1 is associated with the plasma membrane through interactions of the PH domain with PIP_2 (Paterson *et al.*, 1995). Activation of endogenous PLC-δ1 by ionophores or agonist-induced receptor stimulation results in a transient translocation of PLC-δ1 from the plasma membrane to the cytosol. This is consistent with the reduction in membrane PIP_2 and increase in the IP_3 reaction product. Thus, it is likely that a dynamic equilibrium exists between membrane bound and soluble PLC-δ1. Removal of the PH domain from PLC-δ1 or specific amino acid substitutions in several key polar and basic residues (Lys^{30}, Lys^{32}, Arg^{37}, Arg^{38}, Arg^{40}

and Lys^{57}) abolishes high affinity $I(1,4,5)P_3$ binding, but the catalytic site remains functional (**Figure 10**) (Yagisawa *et al.*, 1998; Paterson *et al.*, 1995). Thus, the main role of the PH domain in PLC-δ1 is to provide a membrane tether point directing the enzyme to a substrate enriched membrane surface (Rebecchi and Scarlata, 1998). A single report of a polymorphism in the PH domain of PLC-δ1 (Arg^{105} to His) has been detected in an Alzheimer's disease patient (Shimohama *et al.*, 1998). The analogous rat PLC-δ1 protein demonstrated that the missense resulted in a 40% reduction in enzymatic activity *in vitro* (Shimohama *et al.*, 1998).

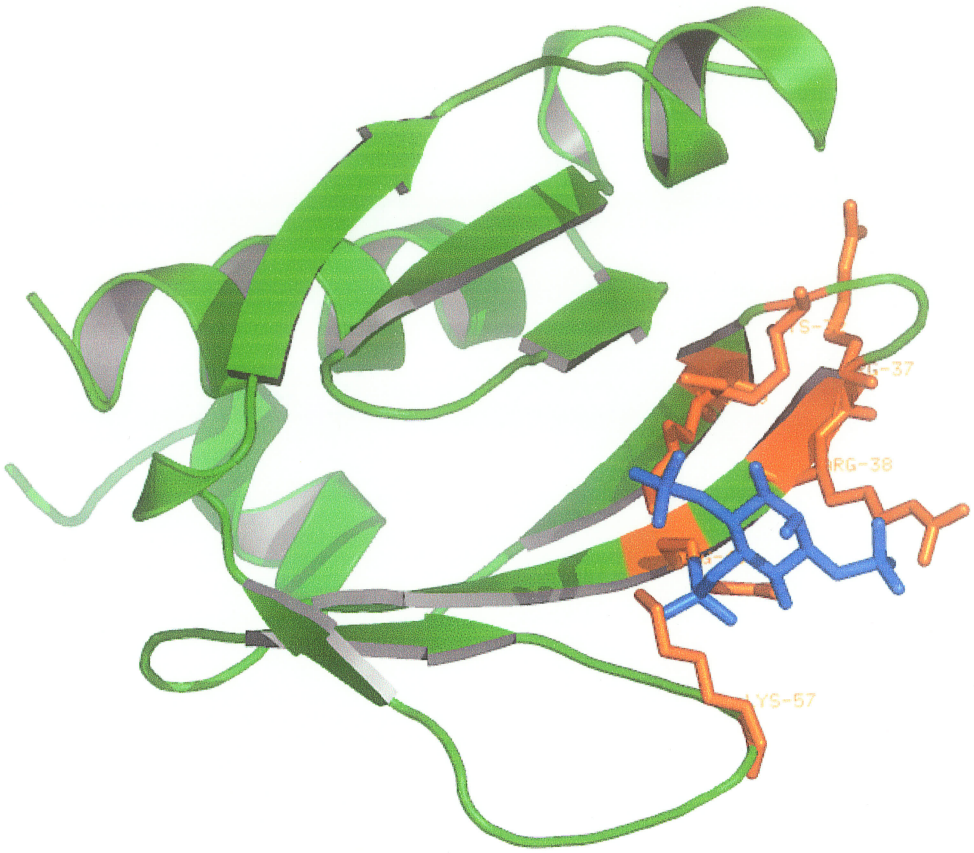


Figure 10: PH domain interactions with IP_3. Residues making contact with IP_3 (**blue**) (Lys^{30}, Lys^{32}, Arg^{37}, Arg^{38}, Arg^{40} and Lys^{57}) are shown in **brown** (PDB 1MAI).

3.2.2. EF-Hand domain

The EF hand domain is a region of the enzyme that forms a flexible tether for the PH domain with the rest of the protein (Williams, 1999). It consists of four helix-loop-helix motifs arranged in two lobes (each containing two EF-hands). The EF-hand domain structure resembles that of calmodulin and is discussed in more detail later. However, unlike calmodulin, there is no direct evidence showing that the EF-

hand domains of PLC-δ1 bind Ca^{2+}. The stoichiometry of Ca^{2+} binding is inconsistent with Ca^{2+} binding to all four EF-hands (Nakashima *et al.*, 1995). The first pair of EF-hands conform to the consensus Ca^{2+} binding motif while the second pair of EF hands were not recognized until the three-dimensional structure was determined (Ellis *et al*, 1993) (**Figure 8**). Although, calcium binding to the EF-hands is not observed in X-ray crystal structures it is conceivable that Ca^{2+} ligands may be present in EF-hands 1 and 2 (Grobler *et al.*, 1996; Essen *et al.*, 1997). That is, a Ca^{2+} dependent increase in the binding of PtdIns(4,5)P_2 to the PH domain was observed in the presence of the EF-hand domain alone (Yamamoto *et al.*, 1999). However, whether binding of Ca^{2+} ions to the EF-hand domain requires interactions with the PH domain was not tested. In addition, mutation of conserved acidic amino acids (Asp^{153}, Asp^{157} or Glu^{164}) did not affect PLC-δ1 activity and Ca^{2+} dependency suggesting that the EF-hand domain may not play a role in PLC-δ1 Ca^{2+}-sensitivity. Nevertheless, the EF hand domain is still essential for PLC-δ1 catalytic activity (Ellis *et al.*, 1993; Nakashima *et al.*, 1995).

3.2.3. PLC Catalytic Site and Mechanism of Phosphoinositide Hydrolysis

The catalytic domain (residues 299-606 in PLC-δ1) contains the region of highest sequence similarity among the various mammalian phospholipases (Rhee *et al.*, 1989). This domain resembles a closed, but distorted TIM-barrel, and is formed by the X (299-445) and Y boxes (489-606). The region between the conserved X and Y box is the X/Y linker region and is variable among the PLC family members. In

PLC-δ1 (amino acids 446-488) this flexible linker region consists of a stretch of charged residues (Williams, 1999). The analogous region in PLC-γ consists also of a split PH domain, one SH3 and two SH2 domains (Rhee, 2001).

All PLC family members require Ca^{2+} for catalytic function. The PLC-ζ isoform is the most sensitive to Ca^{2+} concentrations of all the PLC family members, about 100 times more sensitive than PLC-δ1 (Kouchi *et al.*, 2004). However, PLC-δ1 catalytic activity is about 50 times greater than PLC-ζ (Kouchi *et al.*, 2004). The active site, which is located at the C-terminal end of β-barrel, contains two catalytic histidines (His^{311} and His^{356}). The affinity of the active site for PIP_2 (K_d>0.1mM) is much lower compared with the PH domain. PLC enzymes can hydrolyze PI, PI(4)P and PIP_2 with a preference for PIP_2, but not 3-polyphosphoinositides. Also, there is a high stereospecificity for the D-*myo*-inositol configuration of the headgroup but not for the configuration of the C-2 position of the diacylglycerol moiety (Rebecchi and Pentyala, 2000). Although glycerophosphorylinositol phosphates can be hydrolyzed, the presence of at least short lipid side chains is required for efficient catalysis (Ellis *et al.*, 1998). Phospholipase C functions in a processive mode of catalysis, such that there can be several cycles of PIP_2 hydrolysis (Ellis *et al.*, 1998).

The structure of PLC-δ1 revealed that there is a cluster of hydrophobic residues (Leu^{320}, Phe^{360} and Trp^{555}) at the rim of the active site opening (hydrophobic ridge) that provide a membrane interaction site which allows penetration of the catalytic domain into the phospholipid membrane (Rhee, 2001). Mutational analysis

revealed that residues Ser^{522}, Lys^{438} and Arg^{549} in PLC-δ1 are important determinants for preferential hydrolysis of polyphosphoinositides, whereas replacement of Lys^{440} selectively affects only hydrolysis of PIP_2 (Ellis *et al.*, 1998). Substitutions at residues Asn^{312}, Glu^{390}, Glu^{341} and Asp^{343} lower the Ca^{2+} sensitivity requiring higher Ca^{2+} concentrations for PIP_2 hydrolysis, suggesting these residues may interact with Ca^{2+} (**Figure 11**) (Ellis *et al.*, 1998). Also, point mutations of His^{311} and His^{356} result in a 20,000 and 6,000-fold reduction in catalytic activity, respectively (Ellis *et al.*, 1998). The 2-hydroxyl group of the inositol ring is essential and helps coordinate the Ca^{2+} ion. The 1-phosphoryl group forms hydrogren bonds with the side chain nitrogen atoms of His^{311} and His^{356}. The 3-hydroxyl group of IP_3 forms hydrogen bonds with Glu^{341} and Arg^{549} at the bottom of the active site and as a result there is insufficient space for the active site to accommodate a 3-phosphoryl group. Substrate specificity is determined largely by the Arg^{549} which, along with Lys^{438}, form a salt bridge between the 4-phosphoryl group (Essen *et al.*, 1996). Lastly, Tyr^{551} is almost parallel with the sugar ring of IP_3 and forms Van der Waals interactions (hydrophobic) (Essen *et al.*, 1996).

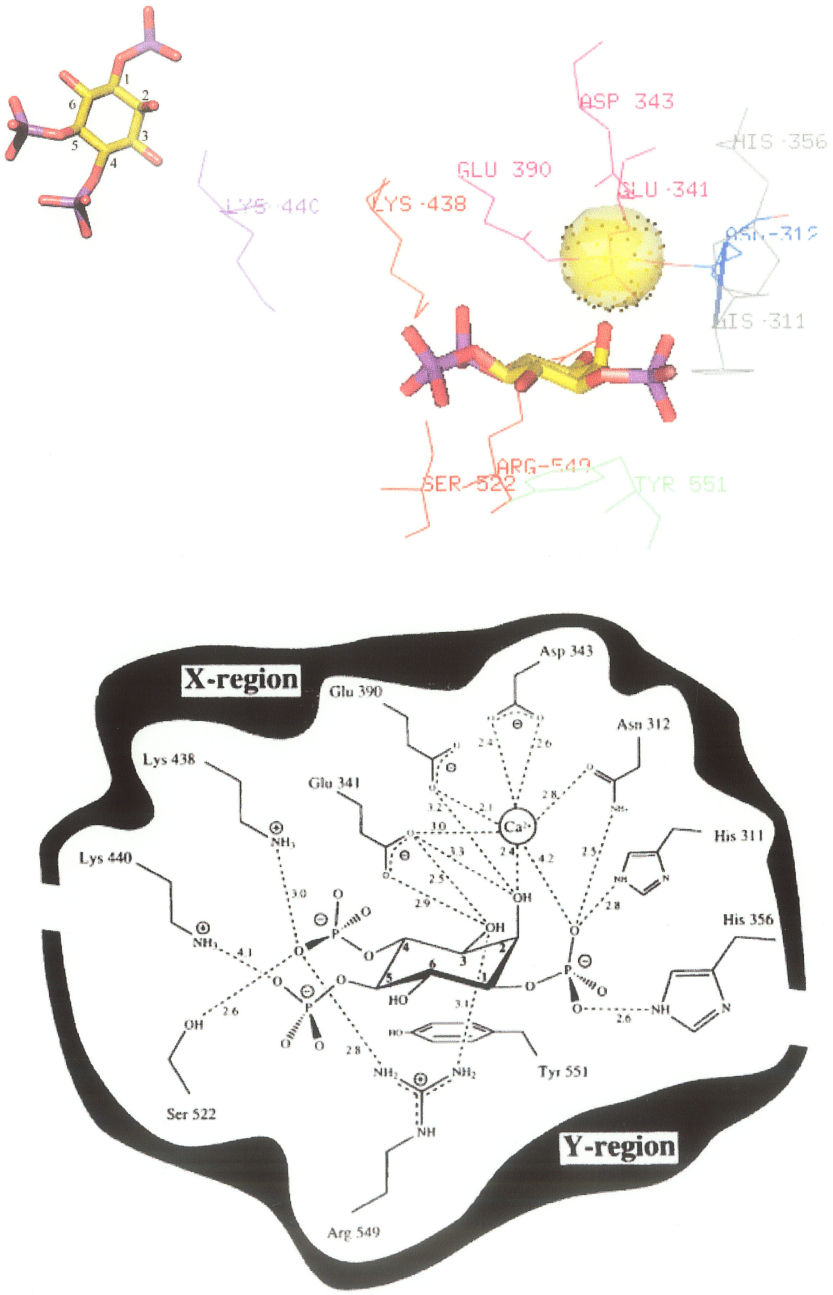


Figure 11: PLC-δ1 catalytic site. The IP_3 (top) and in the catalytic site (middle) are shown. The essential residues in the catalytic site and the calcium ion (yellow) are shown as described in the text. Bottom panel shows the residues involved in catalysis and stabilization of the inositol sugar ring. The contact sites on the IP_3 and Ca^{2+} ion are described in detail in the text (bottom panel from Essen *et al.*, 1996).

Hydrolysis of phosphoinositides occurs by a sequential two-step mechanism that involves general acid-base catalysis with the formation of a cyclic inositol phosphate intermediate (Essen *et al.*, 1996). In the first step a cyclic phosphodiester is formed by nucleophilic attack of the 1-phosphoryl group by an axial 2-hydroxyl group (**Figure 12**). The Ca^{2+} ligand may be involved in orienting the 2-hydroxyl group to facilitate the coordination of the deprotonation step. Although, His^{311} is the likely candidate to be the general base in the reaction it may be too distant to hydrogen bond with the 2-hydroxyl group. Thus, Glu^{341} and Glu^{390} are also potential candidates to be the general base in the reaction. His^{356} is likely to act as the general-acid catalyst for the protonation of the diacylglycerol leaving group. In the second step of the reaction there is hydrolysis of the 1,2-cyclic-inositolphospate intermediate. In this step His^{356} promotes the nucleophilic attack of the water by acting as a general-base catalyst. The general-base of the first step now becomes the acid catalyst in the reaction. From structural analysis of PLC-δ1 it has been proposed that the Ca^{2+} ion and His^{311} are likely required to stabilize the pentavalent phosphoryl transition state (Essen *et al.*, 1996).

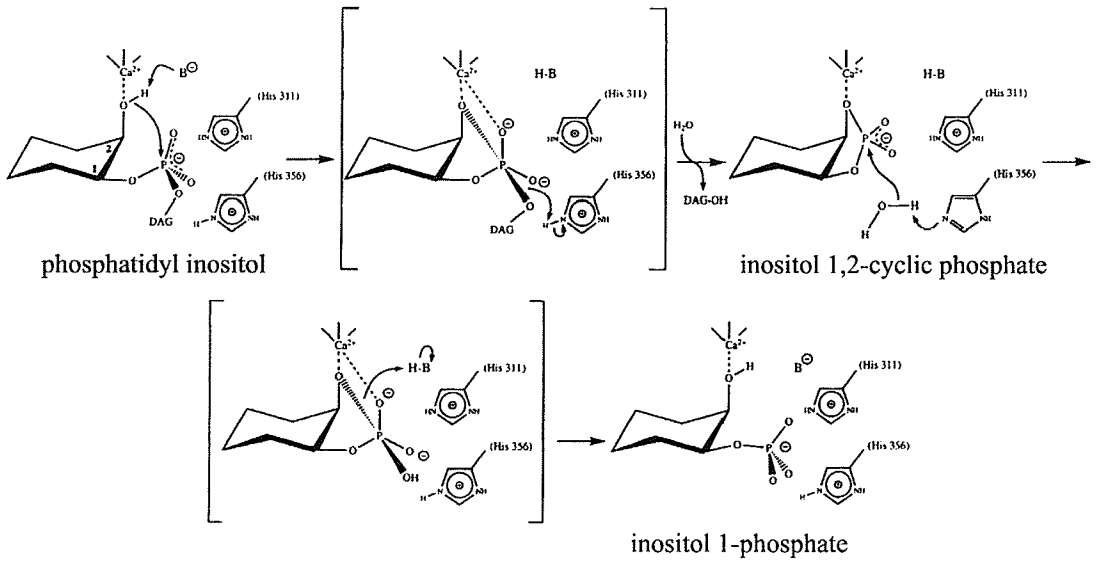


Figure 12: Proposed reaction mechanism of 1-position phosphoinositides. See text for a detailed description of the reaction mechanism (From Essen et al., 1996)

3.2.4. C2 domain

The C2 domains are modules of about 120 amino acids that often bind Ca^{2+} and mediate Ca^{2+}-dependent interaction of proteins with lipids (Rhee, 2001). The crystallographic structure reveals the C2 domain in PLC-δ1 is an eight-stranded antiparallel β-sandwich with four of the strands constituting the core domain (Singer *et al.*, 1997). Two loops extend from the end of the core and contain residues (Asn^{645}, Asp^{653}, Asp^{706} and Asp^{708} in PLC-δ1) that are likely involved in the coordination of the Ca^{2+} ion (**Figure 13**) (Essen *et al.*, 1996; Grobler *et al.*, 1996; Singer *et al.*, 1997). The Ca^{2+} ions were shown to enhance enzyme activity likely by promoting the

formation of an enzyme-Ca^{2+}-phosphatidylserine complex bridge (Singer *et al.*, 1997). This is an effort by the enzyme to increase the affinity for the substrate within vesicles and membranes (Lomasney *et al.*, 1999; Rhee, 2001). The C2 domains of PLC-β isozymes do not exhibit any affinity for membranes in the presence of Ca^{2+} but interact with the α subunits of G_q proteins for membrane localization (Rhee, 2001).

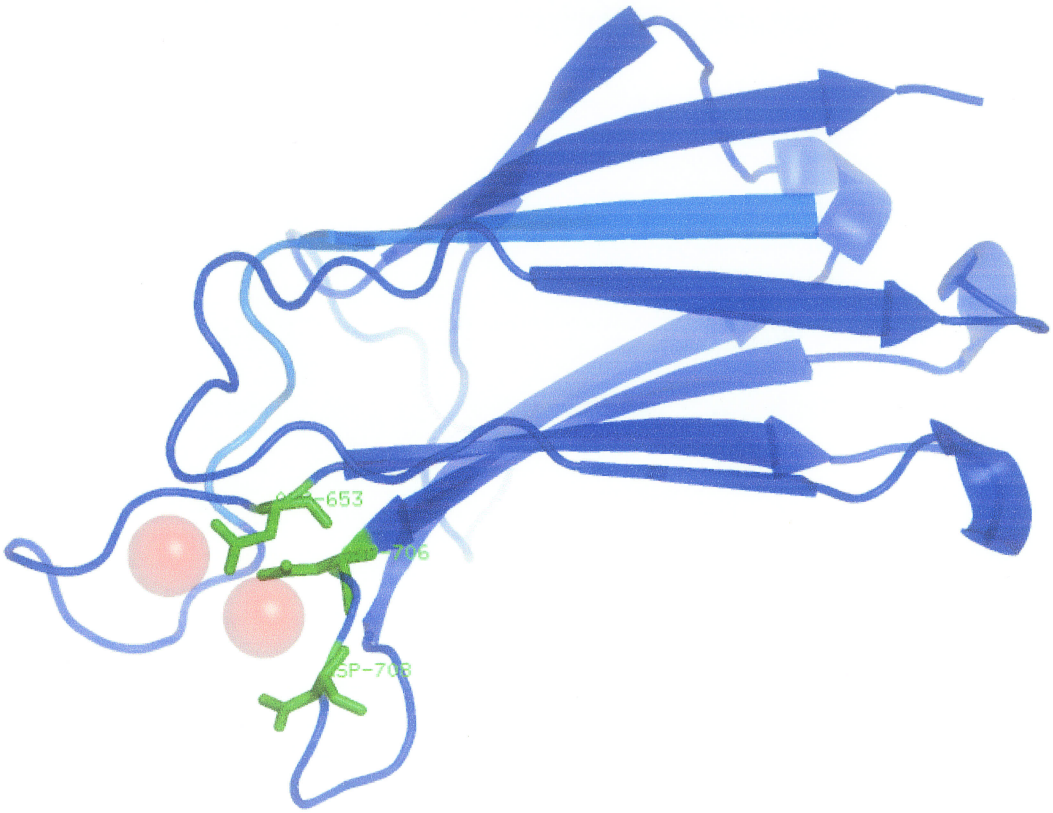


Figure 13: C2 domain of PLC-δ1. The C2 domain core structure containing eight-stranded antiparallel β-sandwich is shown with Asp^{653}, Asp^{706} and Asp^{708} in **green** next to Ca^{2+} ions (**red**) (PDB 1DJX)

3.3. Regulation of PLC family members

Regulation of PLC-β, -γ and -ϵ isoforms can be partly explained by the specific regions incorporated on top of the PLC-δ backbone structure (**Figure 7**). For example, PLC-β isoforms contain a long C-terminal extension that is required for interaction with α subunits of the Gq/11 subfamily of heterotrimeric G proteins or the G$\beta\gamma$ dimers (Park *et al.*, 1993; Wu *et al.*, 1993). In contrast, binding of growth factors (PDGF, EGF and FGF) to their respective receptors results in the stimulation of the intrinsic protein tyrosine kinase (PTK) activity. The PTK activity phosphorylates and activates PLC-γ by binding to the Src homology 2 (SH2) or SH3 domains (Rhee, 2001). Nonreceptor PTKs can also phosphorylate and activate PLC-γ (Rhee, 2001). PLC-ϵ, contains a RasGEF domain at the N-terminus and two Ras-associating (RA) domains at the C-terminus necessary for activation by Ras-p21 (Song *et al.*, 2001). The small GTPase Rho was also shown to activate PLC-ϵ, but in a mechanism independent of the RA domains (Wing *et al.*, 2003). The sperm-specific PLC-ζ isoform was found to trigger fertilization-like Ca^{2+} oscillations in eggs (Saunders *et al.*, 2002). *In vitro* measurements demonstrated that, although enzymatic activity is lower, PLC-ζ has a 100-fold greater sensitivity to Ca^{2+} indicating that this isoform is active at Ca^{2+} concentrations equivalent to those in cells at the resting state (Kouchi *et al.*, 2004). Recently, two groups independently cloned novel PLC-η isoforms that are

activated in a calcium dependent fashion (Saunders *et al.*, 2002; Hwang *et al.*, 2005). All of the PLC-η isoforms have a longer X/Y linker region and at least two have additional Ser/Pro rich C-terminal tail (Saunders *et al.*, 2002; Hwang *et al.*, 2005).

It has been proposed that the role of PLC-δ1 is to amplify Ca^{2+}-mediated signal transduction pathways initiated by the PLC-β, -γ and -ϵ isoforms (Rebecchi and Pentyala, 2000). The mechanism by which PLC-δ isoforms are coupled to membrane receptors is poorly understood. However, previous work suggests regulation of PLC-δ1 by two distinct mechanisms. The first mechanism requires the high molecular weight GTP-binding protein G_h (also known as transglutaminase II), which was shown to stimulate PLC-δ1 activity (Feng *et al.*, 1996). The second route involves a protein similar to Rho-GAP that was also shown to stimulate PLC-δ1 activity (Homma and Emory, 1995). Nevertheless, the mechanism of PLC-δ1 activation is poorly understood but is believed to involve increases in intracellular calcium concentrations.

Chapter 4

CALMODULIN

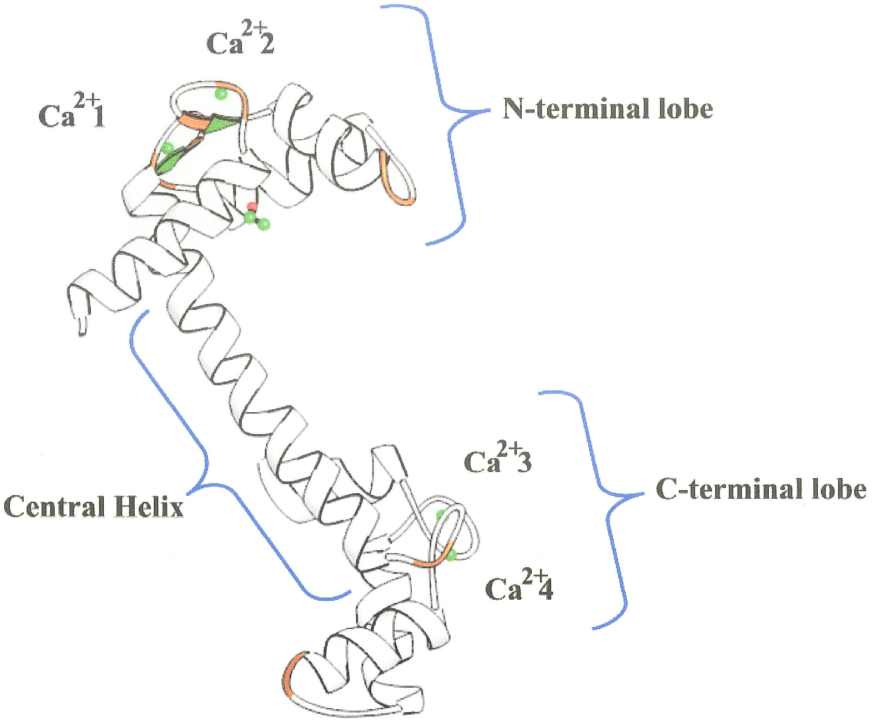
4.1 Calmodulin

Calmodulin is a ubiquitiously expressed calcium sensor protein involved in a multitude of signal transduction pathways. The calcium ion concentrations in cells are typically maintained as low as 10^{-7}M (100nM) at rest and can increase to about 10^{-6}M in activated cells (1000nM) (Berridge *et al.*, 2000; Clapham, 1995). Calcium signals in cells can be relayed rapidly and specifically in response to stimuli. Ral has been found to be an important partner in Ca^{2+} and Ca^{2+}-CaM mediated signal transduction pathways (Wang *et al.*, 1997; Clough *et al*, 2002). We have shown that RalA and -B bind CaM in a Ca^{2+}-dependent and -independent manner and that CaM is required for the activation of this GTPase (Clough *et al.*, 2002).

4.2 Calmodulin Structure and Dynamics

Calmodulin is an acidic 149 amino acid residue protein that is conserved throughout evolution. Binding of Ca^{2+} to CaM imparts a characteristic dumbbell-shape, with similar loops connected by a solvent exposed central α-helix (Babu *et al.*, 1988). Each lobe contains three α-helices and two Ca^{2+} binding EF-hand loops, with a short antiparallel β-sheet between adjacent EF-hand loops (Chattopadhyaya *et al.*, 1992) (**Figure 14**). In the presence of a CaM-binding peptide, such as smooth muscle myosin light chain kinase (MLCK), the Ca^{2+}-bound CaM forms a compact globular

shape. The central helix in the native form unwinds and expands engulfing the helical target peptide, comparable to an articulated jaw (Meador *et al.*, 1992) (**Figure 15**). The single long central helix (residues 65-92) in the peptide-free structure divides into two helices by the expansion of residues 73-77 in the peptide-bound Ca^{2+}-CaM form (Meador *et al.*, 1992). In MLCK the CaM binding sequence is involved in autoinhibition, which is relieved upon binding of Ca^{2+}-CaM. Basic residues in the target peptide make salt bridges with CaM involving all nine of CaM's Met residues. The high symmetry formed by the two lobes of the peptide-bound CaM structure provides a large interaction surface in CaM, which could double the ways in which CaM could bind target proteins. Thus, explaining why CaM forms complexes with so many CaM binding proteins with little sequence identity (Meador *et al.*, 1992).

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1   MADQLTEEQI AEFKEAFSLF DKDGDGTITT KELGTVMRSL GQNPTEAELQ

51  DMINEVDADG NGTIDFPEFL TMMARKMKDT DSEEEIREAF RVFDKDGNGY

101 ISAAELRHVM TNIGEKLTDE EVDEMIREAD IDGDGQVNYE EFVQMMTAK
```

Figure 14: Stucture of Ca^{2+} bound Calmodulin. The four EF-hand domains of Ca^{2+}-saturated are divided into two lobes separated by long linker α-helix. The four Ca^{2+} ions (**green**) are labeled. PDB coordinates 1CLL. The sequence of human CaM (below) is shown with α-helices in boxes. The central helix unwinds at the residues indicated in **blue** to form two distinct α-helices.

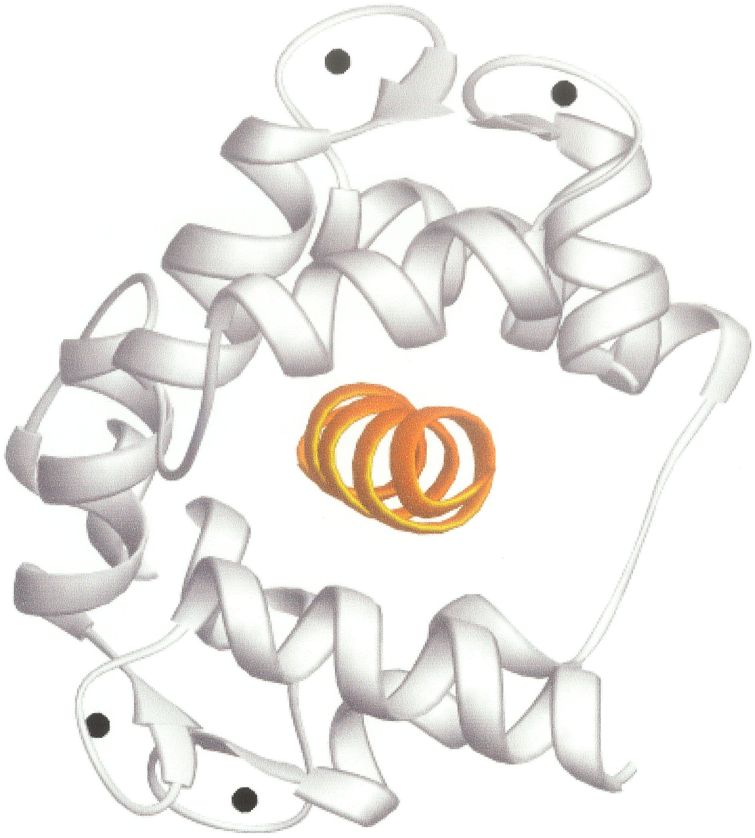


Figure 15: Ca^{2+}-CaM bound to target peptide. The 20 amino acid amphipathic α-helical CaM-binding peptide from MLCK is shown bound to Ca^{2+}-saturated CaM (PDB 1CDL). **Black** dots represent Ca^{2+} ions.

In the absence of Ca^{2+}, the Apo-CaM form seen by nuclear magnetic resonance (NMR), displays a distinct tertiary structure characterized by an extremely flexible midpoint (Jurado *et al.*, 1999). In the structure shown in **Figure 16** residues 76-81 of the linker region are loosened creating two separated helices similar to peptide bound Ca^{2+}-CaM. The two globular domains each contain four α-helices, which run approximately parallel/antiparallel to each other, giving a relatively

compact structure (Yamniuk and Vogel, 2004). Calcium binding to CaM occurs sequentially, where the C-terminal lobe binds two Ca^{2+} ions with high affinity (sites III and IV), followed by binding of Ca^{2+} at the N-terminal lobes (sites I and II) with a lower affinity. Upon binding of Ca^{2+} the two globular domains rotate outward increasing the distance between them, thus, changing the overall conformation of apo-CaM from a globular ellipsoid to the dumbbell-shaped Ca^{2+}-CaM (Yamniuk and Vogel, 2004). The two domains of CaM contain four Met residues in each domain that are buried in Apo-CaM (Yamniuk and Vogel, 2004). Binding of Ca^{2+} exposes the Met-rich hydrophobic binding patches allowing access to the anchor residues of the target peptide.

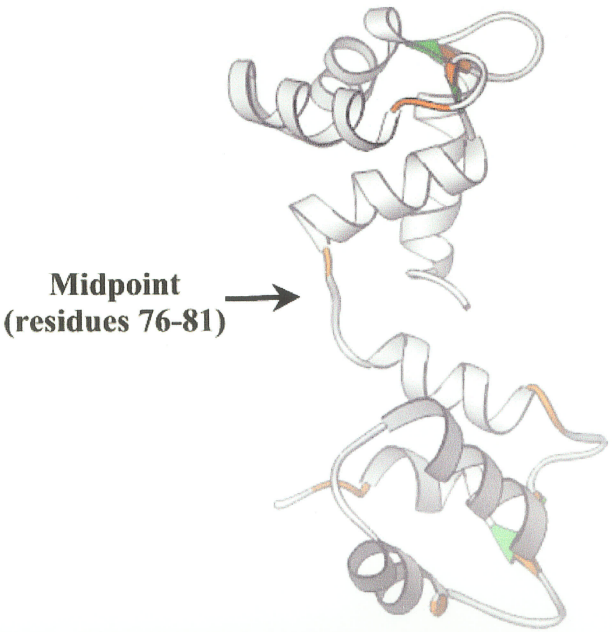


Figure 16: Apo-Calmodulin. Ca^{2+}-free Calmodulin (PDB 1DMO) forms a distinct structure from Ca^{2+}-bound CaM due to the unwinding of amino acids 76-81 to form two separate α-helices.

4.3 Diversity of Calmodulin binding motifs

Calcium dependent CaM binding sequences in target proteins share little sequence identity but many have some common characteristics. Typically CaM binding sequences vary in length between 16-30 amino acids and have a propensity to form amphipathic α-helices with a hydrophobic and basic face (Yamniuk and Vogel, 2004). CaM binding motifs typically fall into one of four groups. Three of these groups (1-10, 1-14, and 1-16 motifs) are distinguished by the spacing of the bulky hydrophobic and basic amino acids that are flanked by aromatic residues at either end. The 1-10 motifs are distinguished by two bulky hydrophobic residues spaced by 8 amino acid residues ([FILVW]xxxxxxxx[FILVW]). Some have additional anchoring residues in the middle (1-5-10 or 1-4-10 motifs). The 1-14 motifs are characterized by two bulky hydrophobic residues spaced by 12 amino acid residues. Additional anchoring residues are often also found in these motifs (1-5-8-14 or 1-8-14). The 1-16 motif is unique in that, aside from the bulky hydrophobic residues being separated by 14 amino acids, the peptide has an atypical binding orientation (http://calcium.uhnres.utoronto.ca). All three groups of CaM binding motifs bind CaM in the presence of Ca^{2+}. Another CaM binding motif, called the IQ motif ([IVL]Qxxx[RK]xxxxx[RK] where x is any amino acid) binds CaM largely in the absence of Ca^{2+} (Bahler and Rhoads, 2002). IQ motifs also have amphipathic

character with a hydrophobic and basic face, thus, some IQ motif-containing proteins can interact with both apo-CaM and Ca^{2+}-CaM (Bahler and Rhoads, 2002).

4.4 Calmodulin mediated Regulation of proteins

Calmodulin can regulate target proteins through different mechanisms. The CaM-dependent serine/threonine protein kinases, such as CaM kinase I/II/IV, CaM kinase kinase, myosin light chain kinase, and the phosphatase calcineurin are activated by displacement of an autoinhibitor domain (AID) (Wilmanns *et al.*, 2000). The AID in these enzymes acts like a pseudosubstrate and is adjacent to, or contains, the CaM binding site. CaM binding to the target protein induces a conformational change of the inhibitory domain and allows full enzyme activity (Hoeflich and Ikura, 2002). Adenylyl cyclase (AC), a membrane-bound enzyme that catalyzes the conversion of ATP to cyclic AMP, involves an alternate mechanism of activation by CaM binding. The CaM binding site in AC is solvent exposed and undergoes rearrangement of key switches to create an active site in the enzyme (Hoeflich and Ikura, 2002). A third mechanism of CaM action is the induction of dimers. Small conductance Ca^{2+}-CaM-activated potassium (SK) channels are Ca^{2+}-gated membrane channels that bind constitutively to CaM. An increase in intracellular Ca^{2+} induces a conformational change in the CaM-target complex leading to the formation of CaM-SK channel dimers (Schumacher *et al.*, 2001). This is the first observation of a 2:2 CaM-target complex formation (Hoeflich and Ikura, 2002). Although yet to be discovered, given

the considerable structural change in CaM and the diversity of CaM binding targets, a 1:2 CaM:target binding ratio also seems to be a plausible scenario.

4.5 Calcium-Calmodulin-mediated regulation of the Ral small GTPase

Ral can be activated by Ras-dependent and independent events (Bos, 1998). Ras-independent activation of Ral can occur by elevated intracellular Ca^{2+} concentrations (Urano *et al.*, 1996; Wolthuis *et al.*, 1998a; Bos 1998; Hofer *et al.*, 1998; M'Rabet *et al.*, 1999). The importance of RalA in the Ca^{2+}-dependent intracellular signaling pathways (Wang and Roufogalis, 1997) was established when cells treated with the Ca^{2+} ionophore ionomycin had elevated levels of active GTP-bound Ral. Activation of PLC leads to the production of IP_3, which binds to and opens endoplasmic reticulum resident Ca^{2+} channels. Hofer *et al.* (1998) observed that lysophosphatidic acid or epidermal growth factor-mediated activation was partially blocked by pretreatment with the phospholipase C inhibitor, U73122. This suggests that the release of Ca^{2+} from intra-organelle stores presents a Ras-independent mechanism of Ral activation (Hofer *et al.*, 1998). In addition, increases in intracellular Ca^{2+} by release from intracellular stores using thapsigargin could also activate Ral while depletion of Ca^{2+} using the chelator BAPTA-AM inhibited thrombin-mediated activation of Ral in platelets (Wolthuis *et al.*, 1998b). In *in vitro* experiments, Ca^{2+} was found to stimulate the binding of GTP and reduce the binding of GDP to Ral in a dose-dependent manner (Park, 2001) suggesting that it may act like

a GEF. Taken together, RalA activation could be mediated by a common signaling event that involves Ca^{2+} (Wolthuis *et al.*, 1998b).

Calcium may also activate RalA through the binding of calmodulin to a region at the C terminus of RalA. In previous work RalA, but not RalB, was shown to contain a novel C-terminally located CaM binding region that does not fit into any of the typical CaM binding target sequence groups. In the sequence ten of 18 residues are basic and hydrophobic residues are at positions 1, 8, 9 and 12 (Wang *et al.*, 1997). We showed that both RalA and RalB bind CaM and that CaM is necessary for thrombin-mediated activation in platelets (Clough *et al.*, 2002). In *in vitro* experiments using purified erythrocyte membranes and recombinant RalA, CaM was shown to bind RalA in a Ca^{2+}-dependent manner (Wang *et al.*, 1997). Also, exogenous Ca^{2+}-CaM enhanced GTP loading on Ral from purified erythrocyte membranes by three-fold (Wang and Roufogalis, 1999; Wang *et al.*, 1997). A role for CaM in the regulation of Ral has also been suggested in experiments showing the dissociation of RalA from synaptic vesicle membranes by exogenous CaM in a Ca^{2+}-dependent manner (Park *et al.*, 1999). A similar regulatory mechanism was seen for K-RasB (Sidhu *et al.*, 2003) and the Ras-related Rab3 small GTPase (Park *et al.*, 1997). Interestingly, although the GTPase activity of RalA was also reported to be stimulated by Ca^{2+}-CaM, the Ca^{2+}-CaM-mediated dissociation of RalA from membranes was independent of nucleotide status (Park *et al.*, 1999). Such studies

highlight the importance of Ca^{2+}/CaM in Ral activation, however, the exact role of Ca^{2+} and CaM in the regulation of the small GTPase Ral requires further research.

Chapter 5

RESEARCH OBJECTIVES AND EXPERIMENTAL APPROACH

In order to characterize new signaling pathways for Ral, we employed the yeast two-hybrid system to identify novel binding partners for this GTPase. Using this method we identified Phospholipase C-δ1 as a new Ral interacting protein. *The hypotheses of the present research are:*

(1) That binding of Ral to phospholipase C-δ1 regulates the hydrolysis of phosphoinositol (4,5)-bisphosphate by phospholipase C-δ1.

(2) That calmodulin either directly or through Ral binding regulates phospholipase C-δ1 activity.

Thus, the overall objective of this work was to determine the mechanism of phospholipase C-δ1 regulation by Ral and the Ral-binding protein calmodulin.

Specific Objectives

A. Establish the properties of Ral binding to PLC-δ1 and its effect on enzymatic activity.
    a. Characterization and expression of the isolated clone as PLC-δ1.
    b. To confirm the binding of both RalA and RalB to PLC-δ1 using the Yeast two-hybrid assay as an *in vivo* model system.

c. To determine whether the binding of Ral to PLC-δ1 is direct using purified recombinant proteins in an *in vitro* system.

d. To determine whether Ral binding to PLC-δ1 is altered by changes in Ca^{2+} concentrations within the physiological range.

e. To determine whether mutations in Ral affect binding to PLC-δ1.

f. To determine the effect of Ral on PLC-δ1 activity using an *in vitro* approach.

B. To establish whether the Ral binding protein calmodulin can bind and influence PLC-δ1 activity directly.

a. To determine whether CaM influences enzymatic activity of PLC-δ1 in the presence or absence of Ral in order to establish the role of the Ral-CaM-PLC-δ1 complex.

b. To establish the CaM binding region in PLC-δ1 using sequence analysis.

c. To determine the Ca^{2+}-dependence of CaM binding to PLC-δ1 and the synthetic peptide of the newly identified CaM binding sequence.

d. To determine whether the synthetic CaM binding peptide can be used as an inhibitor of PLC-δ1 enzyme activity.

e. To determine whether Ral-CaM-PLC-δ1 can be isolated as a complex from cell lysates.

C. To establish whether the Ral signaling pathway influences PLC-δ1 activity in mammalian cells.

   a. Various Ral mutants and a constitutively active Ras mutant is used to determine whether activation of the Ral signaling pathway regulates PLC-δ1 from mammalian cells.

Chapter 6

MATERIALS AND METHODS

4.1. Chemicals and Plasmids –Bovine brain Calmodulin and W7•HCl were purchased from EMD Biosciences Calbiochem. Sepharose 4B coupled Calmodulin, cyanogen bromide (CNBr)-activated Sepharose beads, ECL plus Western Blotting Detection System (Cat. #RPN2132), Hybond PVDF Transfer membrane (Cat. #RPN303F) and pGEX-4T3 vector was purchased from Amersham Biosciences. Lipofectamine 2000 and cell culture media were obtained from Invitrogen. The Matchmaker Yeast two-hybrid system 3, all accompanying reagents including the human testes cDNA library were obtained from BD Biosciences Clontech. Antibodies for HA, RalA, RalB, Ras and PLC-β1 were obtained from BD Biosciences Transduction while anti-calmodulin was purchased from Upstate Biotechnology. Phospholipase C-δ1 antibodies were from Santa Cruz Biotechnology (goat polyclonal Clone C-20, Cat. #sc-7521), BD Biosciences Transduction (mouse monoclonal Cat. #610357), and Upstate Biotechnology (mouse monoclonal Clone S-11-2, Cat. #05-343). Phosphatidylinositol-4,5-bisphosphate, [inositol-2-^{3}H(N)] was from Perkin Elmer NEN (Cat. #NET895) while unlabeled L-α-Phosphatidylinositol-4,5-bisphosphate was from Sigma (Cat. #P9763). Scintisafe scintillation fluid was purchased from Fisher Scientific. The predicted PLC-δ1 CaM-binding IQ motif peptide (NH_2-VRSQVQHKPKEDKLKLVPELS-COOH) was custom synthesized by

the University of Calgary Peptide Synthesis Service. Calcium Calibration Buffer kit (Cat. #C3009) was purchased from Molecular Probes. Protein assays were performed according to the Bradford method, using the Bio-Rad kit (Bradford, 1976). Full-length Rat PLC-δ1 in the pGEX-2T and pMT2 vectors were generously provided by Dr. M. Katan (The Institute of Cancer Research, London, U.K.) while the pDCR-[RasV12] vector and pRK5-[RalB] mutant vectors [RalB(V23), RalB(N28), RalB(V23, D49), RalB(V23, ΔN11), RalB(V23, ΔCAAX)] were kindly provided by Dr. M. White (U. of Texas Southwestern Medical Center, Dallas, Texas, U.S.A.). Restriction enzymes and molecular biology reagents were from New England Biolabs, Invitrogen or Promega. All other reagents were from Sigma unless otherwise indicated.

4.2. ***Yeast Two-hybrid and Library Screening*** - BD Biosciences Clontech's yeast Matchmaker Gal4 two-hybrid system 3 was used according to manufacturer's instructions. Human testes cDNA library in pACT2 vector was screened for potential protein-protein interactions with pGBKT7-[RalB] as bait (Clough et al, 2002). The yeast AH109 cells grow optimally at 30°C, are auxotrophic for tryptophan (Trp) and leucine (Leu) and contain the *HIS3*, *ADE2* and *LacZ* reporter genes which are under the control of separate promoters but activated by the GAL4 transcription factor. Since the pGBKT7 and pACT2 plasmids induce the synthesis of Trp and Leu, positive transformants can be selected on –Trp and –Leu synthetic dropout (SD) medium agar

plates, respectively. The yeast cells are transformed, by either simultaneous or sequential procedures, with the pGBKT7 and pACT2 plasmids containing GAL4 transcription factor binding domain (BD) and activation domain (AD), respectively. Binding of the bait (BD-RalB) and target (AD-library clone) fusion proteins activates the promoters controlled by the GAL4 transcription factor. This allows growth on high stringency SD media agar plates (lacking histidine, adenine, leucine and tryptophan). In addition, activation of the *LacZ* gene results in the expression of α-galactosidase and is monitored by digestion of X-α-Gal incorporated on the appropriate SD agar plates, which produces blue coloration of colonies. Positive colonies are restreaked on selective media (lacking histidine, adenine, leucine and tryptophan) two more times on fresh SD agar plates lacking all four amino acids. As positive controls pCL1, which contains the complete GAL4 transcription factor, was transformed into AH109 cells. Clones containing positive interactions were tested for protein expression by Western blotting of yeast cell lysates using anti-HA antibody. In addition, DNA from the clones was isolated and sequenced. Sequencing was performed by the Manitoba Institute of Cell Biology DNA sequencing facility (University of Manitoba) or the Centre for Applied Genomics (Hospital for Sick Children, Toronto).

4.3. Plasmid Constructs - The isolated clone in the pACT2 vector was restricted with *EcoRI* and *Xho1* and the resulting 700bp fragment was purified following agarose gel

electrophoresis. The insert was ligated into pGEX-4T3 using T4 Quick DNA ligation kit (New England Biolabs) at the same restriction sites for the expression of GST tagged protein in *E. coli*. This construct encodes amino acids 647-756 of PLC-δ1 (called PLC-$\delta 1_{CT}$). A trunctated PLC-δ1 lacking amino acids 647-756 was created from full-length PLC-δ1 in pGEX-2T vector by restriction with *EcoRI* to remove the C-terminal 327bp. An *EcoRI* site at the 3' end of PLC-δ1 insert, but still within the MCS, was utilized and the DNA was closed by re-ligation with T4 Quick DNA ligase.

4.4. Isolation of GST fusion proteins - Plasmids encoding GST fusion proteins were expressed in DH5-α strain of *E. coli* cells using 0.5mM IPTG as described previously (Jilkina and Bhullar, 1996). Following stimulation of protein expression for 2-4 hours at room temperature cells were pelleted by centrifugation at 6,000 × *g*. Cells were resuspended in NETT buffer (20mM Tris-HCl pH 7.5, 1mM EDTA, 100mM NaCl, 1% Triton X-100, 1mM PMSF and 10mg/ml lysozyme) and homogenized with 5 strokes in a glass homogenizer. To ensure cell disruption homogenates were subjected to 2-3 pulses of 30-45 seconds using an ultrasonic cell disrupter. Bacterial cell lysates containing recombinant proteins were centrifuged at 12,000 × *g* for 30 min to remove insoluble materials and the clarified lysates were frozen at -80°C in aliquots with the addition of 20% glycerol. To purify GST-fusion proteins, bacterial cell lysate aliquots were incubated with 250-750μl of glutathione agarose beads (prepared in 1:1 volume of NT buffer [20mM Tris-HCl pH 8.0, 100mM NaCl]) for 30min at 4°C and

GST fusion proteins obtained by washing beads to remove unbound proteins 3× with NETT and 2× with NT. Purity of the final protein preparation was assessed using SDS-PAGE.

4.5. Cell Culture - HeLa cells were maintained in DMEM supplemented with 10% FBS (v/v), 1.5g/L $NaHCO_3$ and 100U penicillin/streptomycin solution at 37°C in 5% CO_2 and 95% air (v/v) on 24-well, 35mm or 100mm plates.

4.6. Transfections - Mammalian expression vectors were transfected into HeLa cells, cultured overnight in the absence of antibiotics, using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. For transfection, either 1μg or 4μg of DNA plus Lipofectamine was added to HeLa cells (~70% confluent) in 24-well or 35mm plates, respectively. Proteins were expressed overnight and treated or lysed in the appropriate buffers.

4.7. Preparation of membrane and cytosolic fractions - To prepare the cytosol and particulate fractions, pelleted cells were resuspended in fractionation buffer (20mM HEPES, pH 7.4, 200mM KCl, 1mM $MgCl_2$ and 1mM PMSF) and lysed by sonication. The lysate was centrifuged at 100,000 × *g* for 2 hr at 4°C. The pellet representing particulate proteins was solubilized for 1 hr at 4°C in above buffer with the addition of 20% glycerol and 0.55% Triton X-100. The solubilized proteins were collected by

centrifugation at 50,000 × *g* for 30 min. The soluble cytosol fraction, which contains CaM but not Ral (Jilkina and Bhullar, 1996), was used in some binding experiments.

4.8. In vitro binding assays - Guanine nucleotide loading was performed according to the method of Cantor *et al.* (1995). Briefly, 1mM GTPγS or GDPβS was incubated with GST-Ral for 20min at 37°C in 20mM Tris-HCl (pH 7.5), 1mM dithiothreitol (DTT), 2mM EDTA, 25mM NaCl and 40μg BSA per mL. Reactions were stopped by the addition of 10mM $MgCl_2$ (final concentration). Beads were washed with 1ml of 20mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 20mM NaCl and 0.5% NP-40 buffer.

When necessary, GST was removed from GST-Ral fusion protein by thrombin cleavage (2 Units/ml) overnight at room temperature. The recombinant protein was recovered in the supernatant following centrifugation (Jilkina and Bhullar, 1996). Protein was used immediately or aliquoted and stored at –80°C. For binding experiments equal amounts of GST-fusion proteins (~50μg) were used. To test binding of Ral or CaM to PLC-δ1, recombinant Ral (10μM) or purified CaM (10μM) or HeLa cell lysate (500μg) was incubated with GST or GST-PLC-$\delta1_{CT}$ for 2 hr at 4°C in binding buffer (50mM Tris-HCl, pH 7.5, 200mM NaCl, 2mM $MgCl_2$, 0.1% NP-40, 10% glycerol, plus protease inhibitor cocktail). To investigate the binding of PLC-$\delta1_{CT}$ to CaM directly, HeLa cell cytosol fractions (500μg) were incubated with GST-PLC-$\delta1_{CT}$ in fractionation buffer overnight at 4°C. To establish binding of

endogenous PLC-δ1 to Ral, GST-RalA or –RalB was incubated with HeLa cell lysates (500μg) for 2 hr at 4°C in binding buffer.

CaM binding assays were performed using 50μl of Calmodulin Sepharose 4B beads or Sepharose 4B beads blocked with Tris buffer as described previously (Clough *et al.*, 2002). To confirm the dependence of Ca^{2+} for CaM binding to PLC-δ1, reactions were performed by incubating purified CaM with GST-PLC-δ1 overnight at 4°C diluted in commercially available Ca^{2+} calibration buffers exactly as described previously (McCullar *et al.*, 2003). For competition assays, PLC-IQ peptide was resuspended in water and pre-incubated with GST-PLC-δ1 for 1 hr at 4°C. For binding assays with PLC-IQ peptide as bait, the peptide (2.5mg/ml) was covalently coupled to cyanogen bromide (CNBr)-activated Sepharose beads according to the manufacturer's instructions and used in binding studies. At the end of the binding reactions, unbound proteins were washed with incubation buffer (including the appropriate Ca^{2+} and/or EGTA concentrations). Laemmli's sample buffer (Laemmli, 1970) was added to the recovered beads. Samples were heated at 100°C for 3 minutes, centrifuged briefly and separated on 11% or 13% SDS-PAGE gels. Proteins were transferred to PVDF membrane and Western blotting was performed using appropriate antibodies. The antigen-antibody complex was visualized using ECL plus Western detection reagent.

4.9. Calcium concentration determinations & Statistical Analysis - Calcium concentrations were determined by measuring total calcium ions using atomic absorption spectrophotometry. Free calcium concentrations were calculated, using the MAXCHELATOR software (http://www.stanford.edu/~patton/maxc.html) and adjusted with calcium concentrations obtained from atomic absorption data. Statistical comparisons were performed using Students T-test with either Excel (Microsoft Corporation) or SigmaStat (Systat) software.

4.10. PLC-δ1 enzyme assay - Phospholipase C-δ1 enzyme assay was performed by a modification of the method described previously (Wahl *et al.*, 1992; Ziegelhoffer *et al.*, 2001). Briefly, phosphatidylinositol-4,5-bisphosphate, [inositol-2-^{3}H(N)] (Perkin Elmer NEN) was mixed with unlabeled PIP_2, dried by passing N_2 gas (henceforth called [^{3}H]-PIP_2) and then resuspended in 132mM sodium cholate overnight. Just before the start of enzyme assays the substrate was diluted to bring the sodium cholate concentration to 116mM. GST-PLC-δ1 was expressed in *E. coli* for 2 hr at room temperature in the presence of 0.5mM IPTG. GST-PLC-δ1 was purified and resuspended in reaction buffer (30 mM HEPES, pH 6.8, 100mM NaCl, 70mM KCl plus 2mM EGTA, 1.8mM Ca^{2+} giving a free Ca^{2+} concentration of 10μM). Protein assays were performed on an aliquot of the purified GST-PLC-δ1 protein and GST-PLC-δ1 protein was divided equally (40μl, ~100μM) into test tubes. Prior to the start

of the enzyme assay, Ral, PLC-IQ peptide, CaM, or W-7 was pre-incubated with GST-PLC-δ1 for 1 hr at room temperature. Concentrations of Ral, PLC-IQ, CaM and W-7 used were derived from preliminary experiments (Ral, PLC-IQ and CaM) or from K_D values suggested by manufacturers for established drug targets (W-7). Reactions were carried out by the addition of 20μM [^{3}H]-PIP_2 (20-30 dpm/pmol) at 37°C for 2.5 min. Reactions were terminated by the addition of 5% trichloroacetic acid solution. Soluble [^{3}H]-IP_3 was obtained in the supernatant following centrifugation at 12,000 × *g* for 5 min. Radioactivity was measured in an aliquot of the supernatant by liquid scintillation counting in 10ml of Scintisafe scintillation fluid. In order to measure PLC-δ1 activity *in vivo*, control or cells overexpressing various Ral mutants were lysed in buffer (30mM HEPES, pH 6.8, 150mM NaCl, 10mM EDTA and 1% sodium cholate) for 2 hr at 4°C. Immunoprecipitation using 5μg mouse monoclonal anti-PLC-δ1 (Upstate Biotechnology, clone S-11-2) was performed overnight at 4°C and enzyme reactions were carried out on the immunoprecipitates as described above. Statistical comparisons were made to control PLC-δ1 activity in the absence of any other treatments, which was performed at the same time as other experimental conditions.

Chapter 7

RESULTS

In order to characterize new signaling pathways for Ral, we employed the yeast two-hybrid system to identify novel binding partners for this GTPase. Using the small GTPase RalB in a screen of the human testis cDNA library we detected several novel interacting clones. These were isolated and identified by DNA sequencing. One clone revealed a match to the C-terminal region (amino acids 647-756) of phospholipase C-delta1 (PLC-$\delta 1_{CT}$) (see **Appendix I**). The yeast two-hybrid experiments were repeated with both RalA and RalB to confirm interaction with PLC-$\delta 1$. The positive colonies were re-streaked three times in high stringency media to ensure the propagation of the correct phenotype (**Figure 17**). Protein expression from the isolated clone was confirmed in the yeast using antibodies for -HA and PLC-$\delta 1$. To confirm that the isolated clone coded for PLC-$\delta 1$, the insert containing the PLC-$\delta 1_{CT}$ was isolated from the yeast plasmid and inserted in-frame into the GST expression vector pGEX-4T3 (**Appendix I**). Expression of GST-PLC-$\delta 1_{CT}$ in *Escherichia coli* was confirmed using two different antibodies for PLC-$\delta 1$ (**Figure 18**). The bands recognized by PLC-$\delta 1$ antibodies corresponded to the expected molecular mass for GST-PLC-$\delta 1_{CT}$ and had mobility on SDS-PAGE similar to that observed for GST-PLC-$\delta 1_{CT}$ protein.

Figure 17: Yeast Two-hybrid assay of isolated clone with RalA and RalB (**A**) cDNA Library clones in pACT2 vectors were screened for potential protein-protein interactions using pGBKT7-[RalB] as bait. Positive clones (blue colonies) were selectively grown, their DNA isolated and sequenced. One of the isolated binding-partners encodes the C-terminal portion of PLC-δ1 (amino acids 647-756). To confirm this interaction the isolated pACT2-PLC-$\delta 1_{CT}$ clone was re-transfected into AH109 cells with pGBKT7-[RalA] or -[RalB] and spread onto high stringency SD agar plates containing X-α-Gal. The positive control pCL1 and the appropriate negative controls were performed to validate the results.

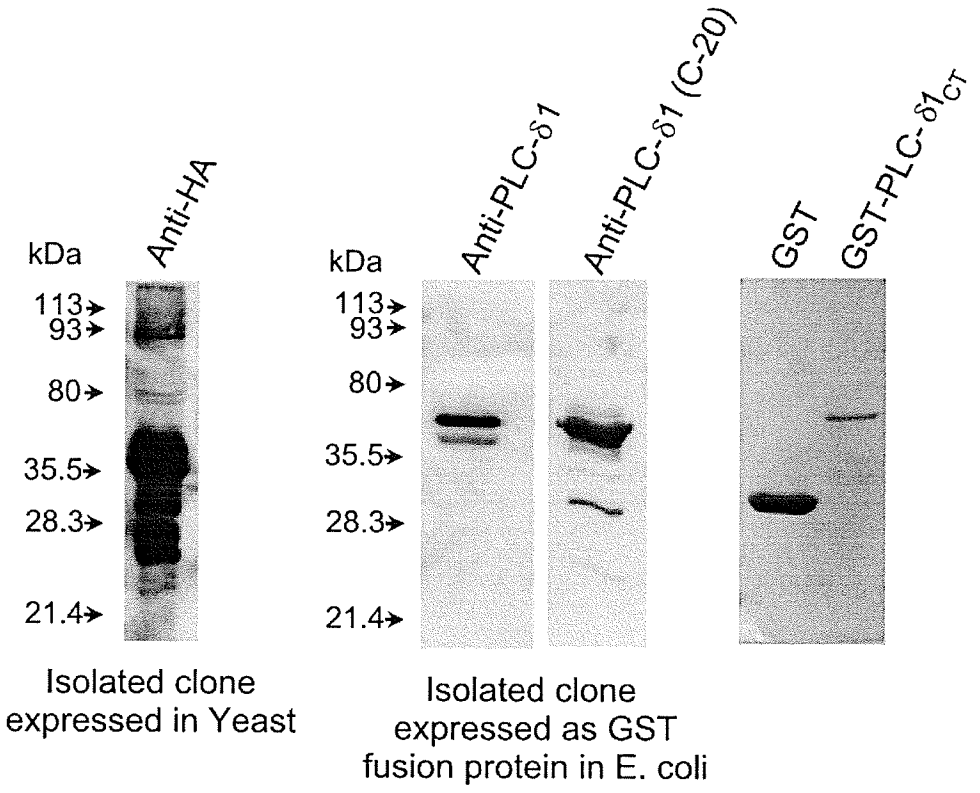


Figure 18: Characterization of isolated Yeast-two hybrid library clone. To characterize the isolated clone and confirm protein expression, the PLC-$\delta 1_{CT}$ cDNA was isolated and inserted into the GST expression vector pGEX-4T3. Expression of GST-PLC-$\delta 1_{CT}$ in *Escherichia coli* was confirmed using PLC-δ1 antibodies from two sources (BD Biosciences Transduction and Santa Cruz Biotechnology clone C-20). Right panel shows coomassie blue stained gel of GST and GST-PLC-$\delta 1_{CT}$ isolated and purified from *E. coli* cells.

To determine whether Ral binding to PLC-δ1 occurs only in the C-terminal region of PLC-δ1, a truncated version of PLC-δ1 lacking the C-terminal region (amino acids 647-756, PLC-$\delta 1_{\Delta CT}$) was created by restriction of the full-length PLC-δ1 clone with EcoR1. The full-length PLC-δ1 and PLC-$\delta 1_{\Delta CT}$ were tested by restriction enzyme analysis (**Figure 19**) and sequencing (**Appendix I**). Both

constructs were tested for expression in *E. coli*, however, only the full-length protein was expressed (**Figure 20**). Anti-PLC-δ1 did not recognize a band representing PLC-$\delta 1_{\Delta CT}$. Thus, we cannot not rule out the possibility that a second Ral binding site exists in the PLC-δ1. The clone isolated from the Yeast-two hybrid screen was the C-terminal portion of human PLC-δ1 while the full-length clone used in our experiments was of rat origin. Comparison of the rat and human PLC-δ1 sequence reveals that the protein in the two species is 91.5% identical and 96.8% homologous (**Figure 21**). Thus, the high degree of homology between the proteins from rat or human species validates use of either protein in this study.

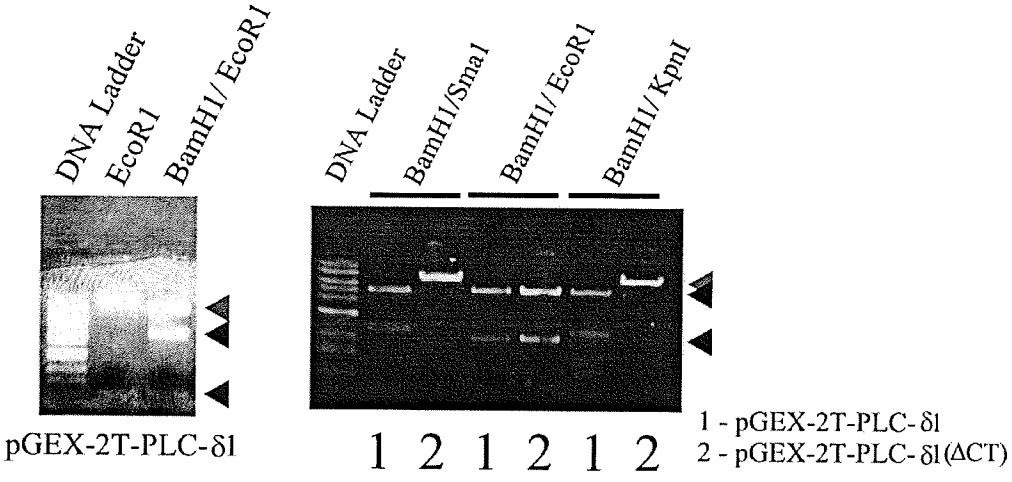


Figure 19: Restriction enzyme analysis of full-length PLC-δ1 and truncated PLC-δ1 (PLC-$\delta 1_{\Delta CT}$). Left panel: Enzyme restriction with EcoR1 alone removes PLC-$\delta 1_{CT}$ bottom (band), while double restriction with BamH1/EcoR1 removes the first 1943bp (center band) and PLC-$\delta 1_{CT}$. Top band indicates pGEX-2T vector alone or with PLC-$\delta 1_{647\text{-}756}$ and is distinguished by mobility. Right panel: The top band indicates single enzyme cut pGEX-2T-PLC-$\delta 1_{CT}$. The second band indicates pGEX-2T vector with PLC-δ1 or PLC-$\delta 1_{CT}$ excised. Restriction sites for SmaI and KpnI lie at the end of PLC-δ1 or within the C-terminal region of PLC-δ1, respectively. Double restriction with BamH1/EcoR1 removes PLC-$\delta 1_{647\text{-}756}$ confirming the successful construction of PLC-$\delta 1_{CT}$.

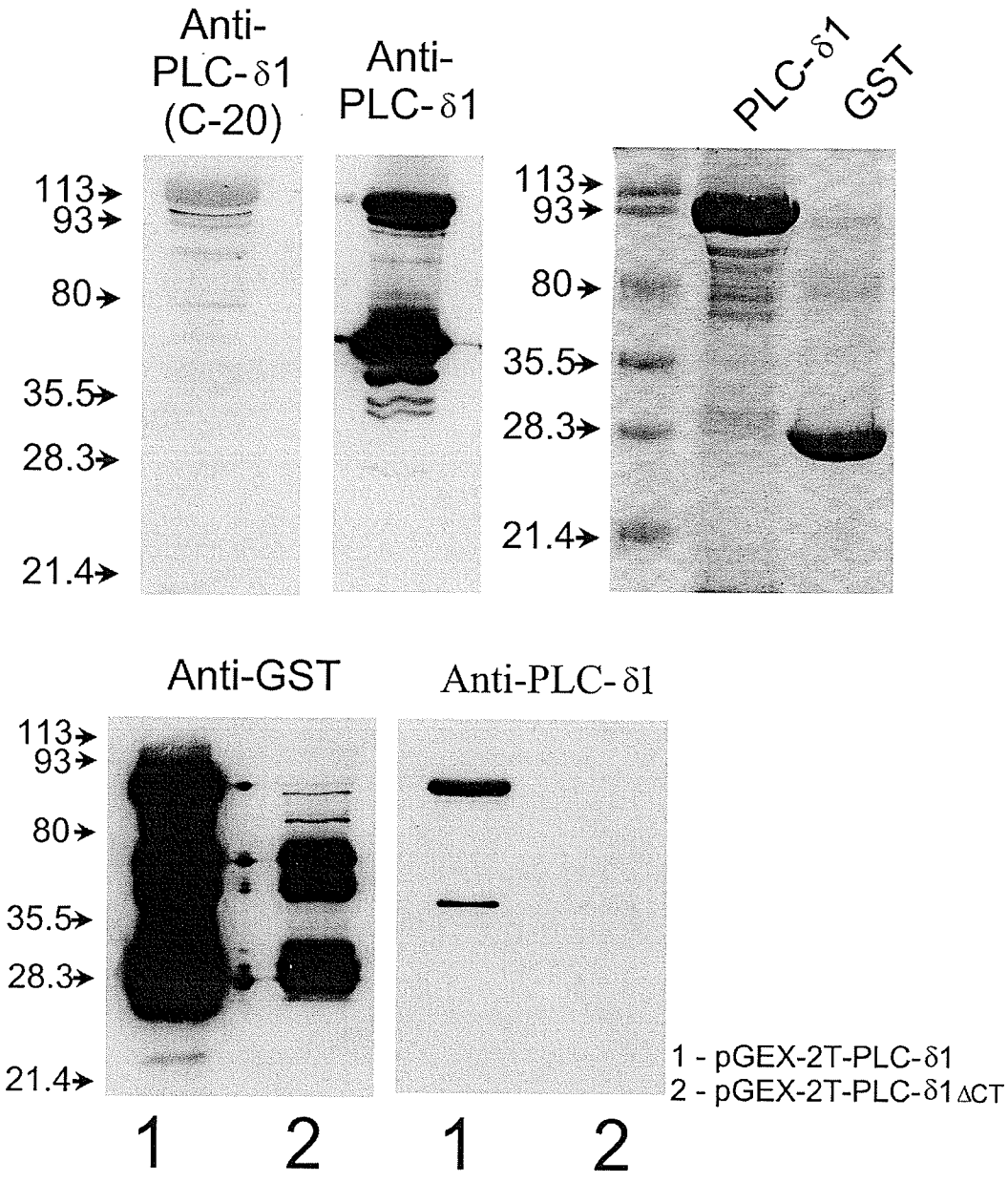


Figure 20: Expression of full-length PLC-δ1 and PLC-δ1$_{\Delta CT}$. Western blot of full-length PLC-δ1 using two different PLC-δ1 antibodies (top left) and coomassie blue stained gel of full-length PLC-δ1 compared to GST (top right). Bottom panel shows Western blot of full-length and PLC-δ1$_{\Delta CT}$ using GST and PLC-δ1 antibodies (bottom).

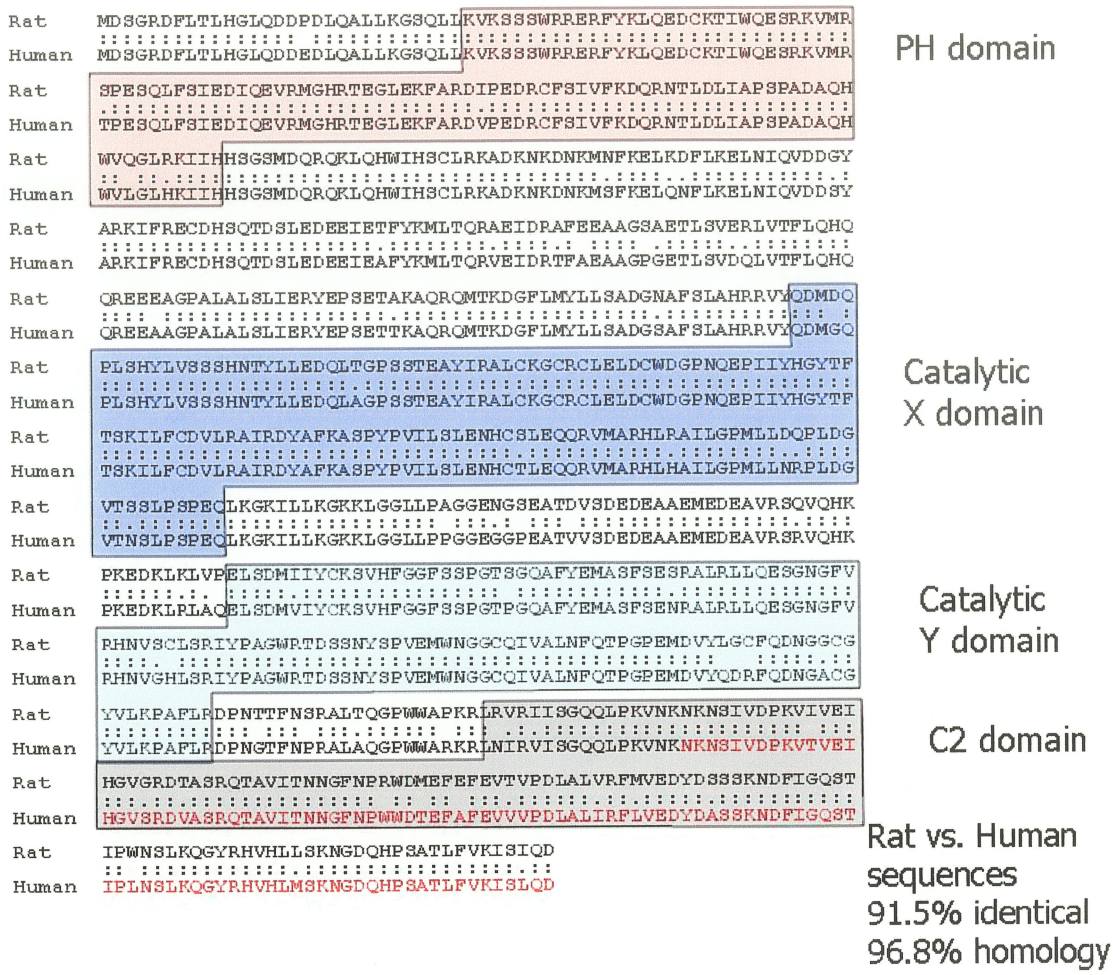


Figure 21: Comparison of the rat and human PLC-δ1 sequences. The red text indicates the sequence of the clone isolated from the yeast two-hybrid library screen (PLC-δ1$_{CT}$). The double dots (:) indicate conserved residues while the single dot (.) represents conservative amino acid changes.

To further confirm the interaction, GST-PLC-δ1$_{CT}$ was incubated with lysates from HeLa cells. RalA but not Ras-p21 (**Figure 22A**) was found to interact as determined using Western blotting. The addition of EGTA to the binding buffer resulted in a slightly higher interaction between PLC-δ1$_{CT}$ and RalA. We also

employed the reverse approach in which GST-RalA or GST-RalB was incubated with HeLa cell lysates. Western blotting using antibody to PLC-δ1 revealed binding does indeed occur to RalA and RalB but not GST (**Figure 22B**). Results using purified recombinant RalA incubated with full-length GST-PLC-δ1 and GST-PLC-$\delta 1_{CT}$ provided similar results suggesting that Ral interacts directly with PLC-δ1 (**Figure 22C**). The binding with full-length GST-PLC-δ1 was greater than with GST-PLC-$\delta 1_{CT}$. In all cases no binding was seen to GST protein alone. There was no cross-reactivity of RalA antibody with GST- or GST-PLC-δ1 proteins (**Figure 22C**).

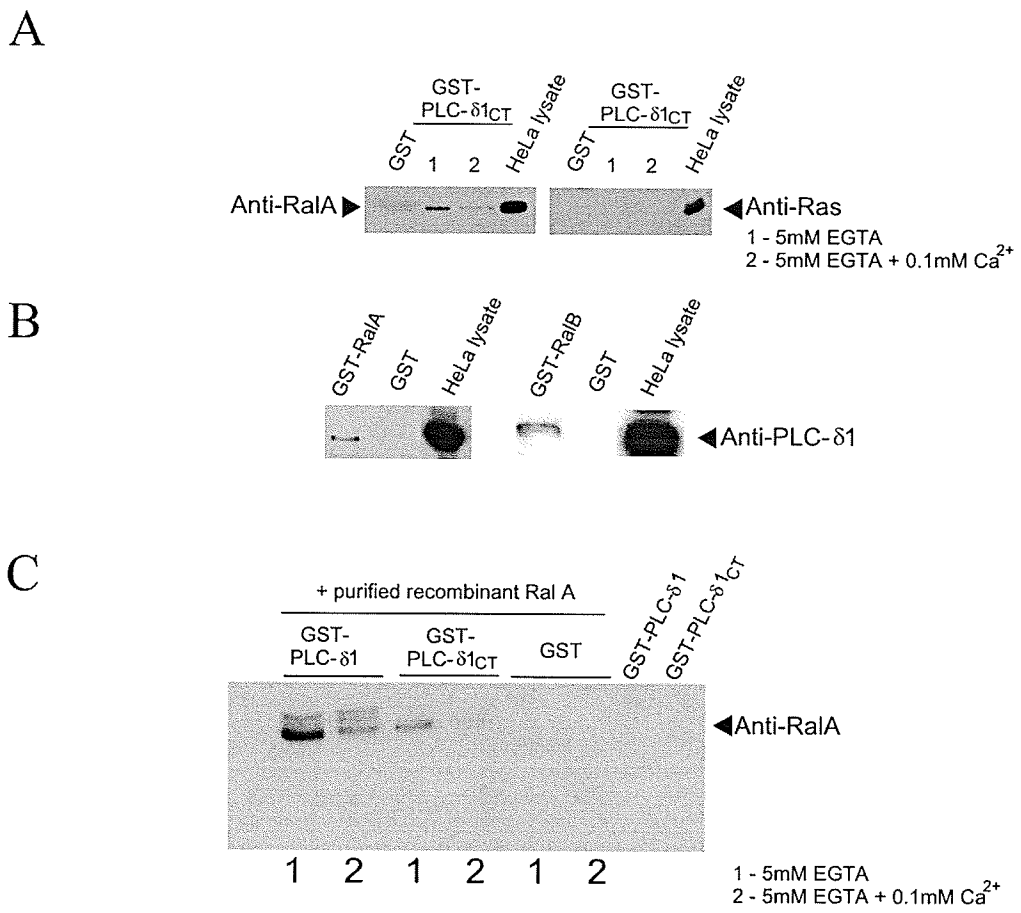


Figure 22: Full-length PLC-δ1 or PLC-$\delta 1_{CT}$ selectively bind recombinant RalA and RalB directly or from HeLa cell lysates. (A) GST or GST-PLC-$\delta 1_{CT}$ fusion proteins were incubated with lysates from HeLa cells in the presence of 5mM EGTA or 5mM EGTA + 0.1mM Ca^{2+} for 2 hr at 4°C. Western blotting was performed using antibodies against RalA and Ras-p21. (B) The reverse experiments, using GST-RalA and -RalB, were also performed to show binding to full-length PLC-δ1. In all experiments GST protein was used as control. (C) To establish a direct interaction, purified recombinant RalA was incubated with full-length GST-PLC-δ1 or GST-PLC-$\delta 1_{CT}$ in the presence of 5mM EGTA or 5mM EGTA + 0.1mM Ca^{2+}. RalA antibody did not show cross-reactivity with the fusion proteins. All binding experiments (A-C) were performed in buffer containing 50mM Tris-HCl, pH 7.5, 200mM NaCl, 2mM $MgCl_2$, 0.1% NP-40, 10% glycerol, plus protease inhibitor cocktail for 2 hr at 4°C.

We and others have previously shown that Ral is an important partner in calcium and Ca^{2+}/calmodulin mediated signal transduction pathways (Clough *et al.*, 2002; Hofer *et al.*, 1998; Wang *et al.*, 1997). Also, PLC-δ1 is highly sensitive to calcium concentrations and can be activated at concentrations as low as 10μM (Kim *et al.*, 1999; Allen *et al.*, 1997). Thus, we sought to determine the dependence of Ral binding to PLC-δ1 on calcium. GST-PLC-$\delta 1_{CT}$ was incubated with HeLa cell lysates containing various exogenous calcium concentrations. Binding of Ral to GST-PLC-$\delta 1_{CT}$ occurred at low levels of calcium (0.22nM) and increased proportionally with higher free calcium concentrations (up to 17.0μM), as determined quantitatively (**Figure 23**). Interestingly, in the presence of EGTA, Ral binding to PLC-δ1 was not abolished and may be analogous with that seen with Apo-CaM (Ca^{2+}-free CaM) binding to target proteins. Ral is known to interact with CaM (Clough *et al.*, 2002; Wang *et al.*, 1997). Therefore, we wished to determine whether CaM can bind PLC-δ1 as a complex with Ral. Reprobing the same blot with CaM antibody showed a nearly equal binding of CaM and Ral to GST-PLC-$\delta 1_{CT}$ at 17.0μM free Ca^{2+} (**Figure 23**). The binding of PLC-δ1 to CaM could occur either directly or through Ral. This possibility was tested using GST-PLC-$\delta 1_{CT}$ incubated with HeLa cell cytosolic fractions (contains CaM but no Ral protein) (**Figure 23**). However, no binding of GST-PLC-$\delta 1_{CT}$ to CaM was seen suggesting that the CaM present in the complex was due to its association with Ral.

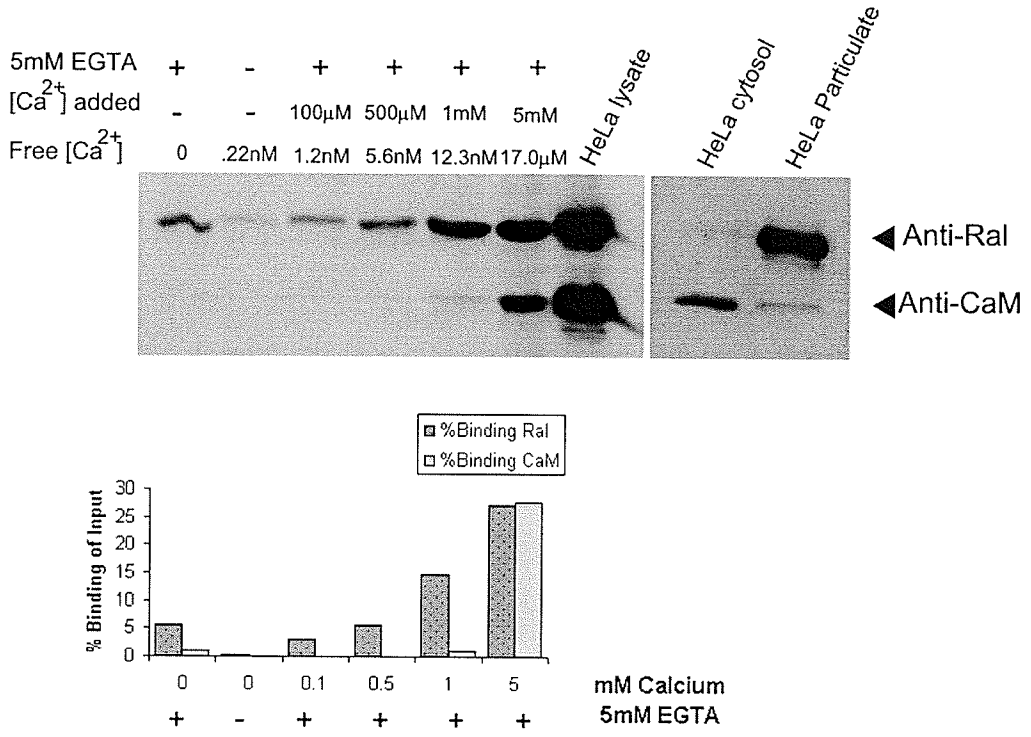


Figure 23: Ral binds PLC-δ1 in a calcium dependent manner. GST-PLC-$\delta 1_{CT}$ was incubated with HeLa cell lysates containing varying amounts of calcium and/or EGTA. Western blotting with antibodies against Ral and CaM was used to demonstrate binding to PLC-$\delta 1_{CT}$. The top panel (Western blot) and the bottom panel of quantified values (expressed as percent of Ral or CaM from total lysate) are from a single experiment but are representative of repeated experiments. Total calcium was measured using atomic absorption spectrophotometry and free calcium was calculated using MAXCHELATOR.

We next wished to determine whether Ral or CaM affect PLC-δ1 activity. At a constant calcium concentration (10μM), pre-incubation with 260μM Ral increased PLC-δ1 activity by >20% (**Figure 24**). In contrast, pre-incubation with 20μM CaM resulted in a 25% reduction in PLC-δ1 activity suggesting that CaM may also bind to PLC-δ1 directly in a region other than through the binding of CaM to Ral. When both

Ral and CaM were pre-incubated with PLC-δ1 full activity was restored. Addition of the calmodulin antagonist W-7 to the reaction mixture fully reversed the inhibition of PLC-δ1 by CaM. However, W-7 alone also inhibited PLC-δ1 activity suggesting W-7 also binds to PLC-δ1 directly (**Figure 25**). Thus, binding of the CaM antagonist W-7 to PLC-δ1, likely at the CaM-like EF-hand domain, suggests that these inhibitors are not limited to CaM but can also interact with CaM-like structures in other proteins.

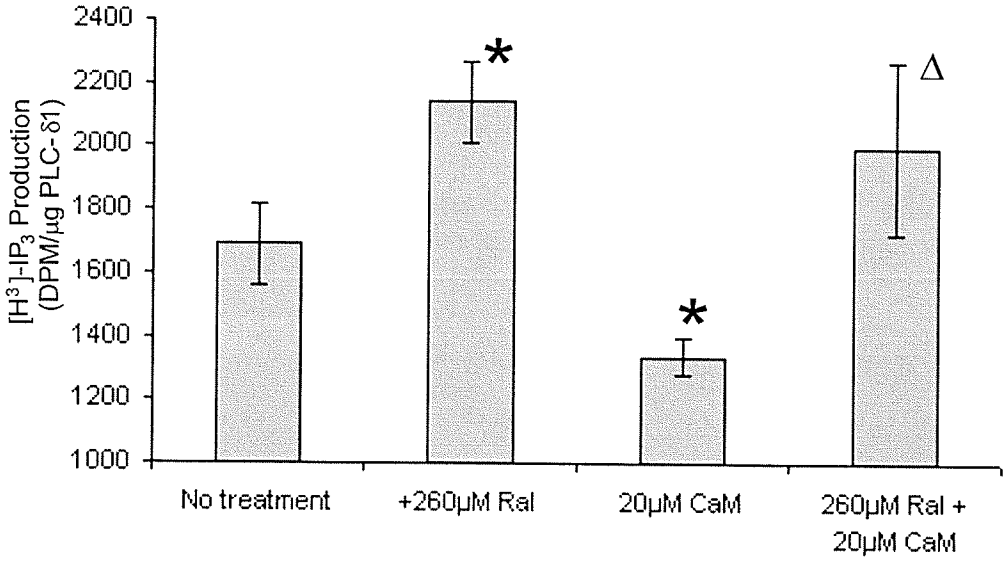


Figure 24: Ral potentiates PLC-δ1 activity. PLC-δ1 activity was measured at 37°C following pre-incubation with 260μM Ral and/or 20μM CaM for 1 hr at room temperature. *P values <0.05 were considered significantly different from *PLC-δ1 activity alone or $^{\Delta}$PLC-δ1 activity in the presence of 20μM CaM ($n \geq 3$).

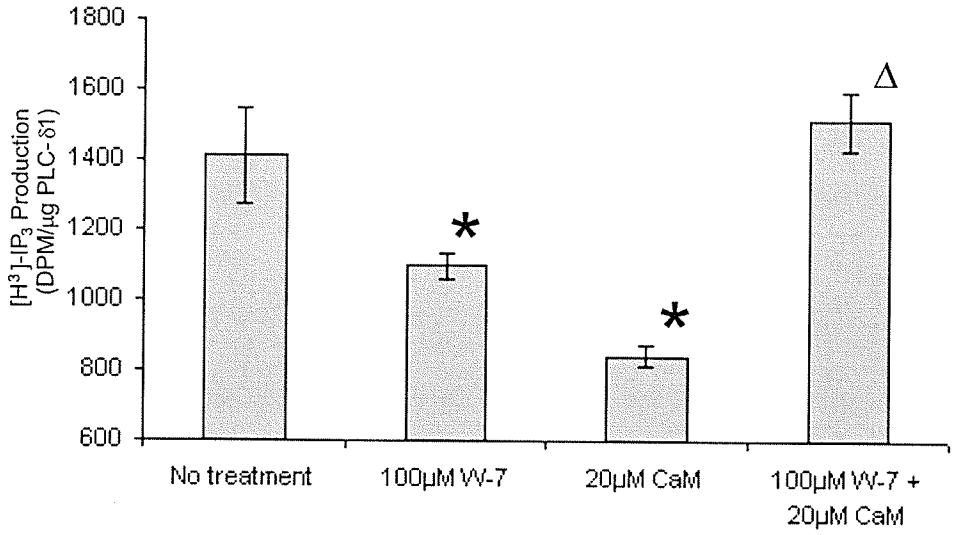


Figure 25: The CaM antagonist W-7 reverses CaM-mediated inhibition of PLC-δ1 activity. To determine whether the CaM inhibition of PLC-δ1 activity can be reversed using a CaM antagonist, activity was measured following pre-incubation with or without 100μM W-7. *P values <0.05 were considered significantly different from *PLC-δ1 activity alone or $^{\Delta}$PLC-δ1 activity in the presence of 20μM CaM (n≥3).

Since CaM alone inhibited PLC-δ1 activity we next screened the phospholipase C-δ1 sequence for potential CaM binding domains using the CaM target database (http://calcium.oci.utoronto.ca/). The database revealed a region with CaM binding potential (amino acids 473-492) located in the loop region separating the catalytic X (296-430) and Y domains (492-609), which converge to form the catalytic α/β barrel (Rhee *et al.*, 2001).

The binding of CaM to target proteins can be calcium dependent and/or independent (Yamniuk and Vogel, 2004). Typically CaM binding sequences vary in length between 16-30 amino acids and have a propensity to form amphipathic α-

helices with a hydrophobic and a basic face (James *et al.*, 1995; Rhoads and Friedberg, 1997). The putative CaM binding motif identified by us predicts the sequence in the X/Y linker region of PLC-δ1 to be an IQ type (VQXXX[K/R]XXXX[K/R] where X is any amino acid) CaM binding motif (**Figure 26** and **Table 4**). Calcium free-CaM (apo-CaM) forms a distinct structure and typically binds proteins that contain the IQ motif (Bahler and Rhoads, 2002). IQ motifs also have an amphipathic character with hydrophobic and basic faces and thus can interact with both apo-CaM and Ca^{2+}/CaM (Yamniuk and Vogel, 2004). Analysis of the putative CaM binding motif using ANTHEPROT software suggests the CaM binding region in PLC-δ1 is hydrophilic and solvent exposed (**Figure 27**).

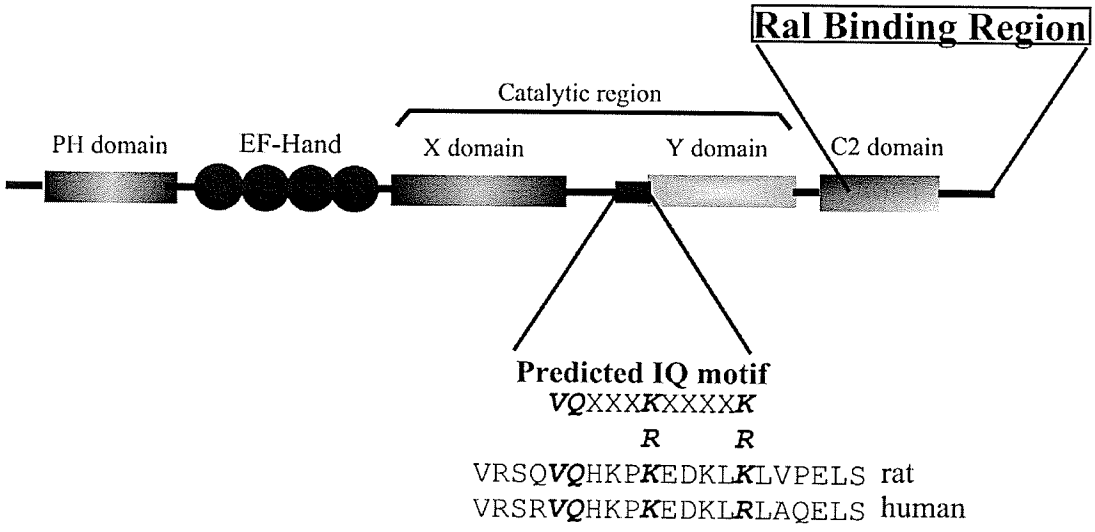


Figure 26: PLC-δ1 contains a novel CaM binding IQ motif in the X/Y linker region. (A) A search for putative CaM binding regions in PLC-δ1 revealed an IQ motif located in the X/Y linker region. The IQ motif and conserved (**bold**) residues are indicated in relation to the Ral binding region in PLC-δ1.

Table 4: Properties of the Putative Calmodulin binding IQ peptide in PLC-δ1 VRSQVQHKPKEDKLKLVPEL[3]

Average Residue Weight :	117.694
Average Hydropathy :	-1.04
Average Hydrophobic Moment :	0.554
Average Propensity for Alpha-Helix Formation :	1.08
Percent Hydrophobic Residue ACFGHILMVWY :	35
Percent Hydrophilic Residue DEKNPQRST :	65
Net +ve Charge :	2
Number of Basic Residues :	5
Number of Acidic Residues :	3

[3] (calcium.oci.utoronto.ca/)

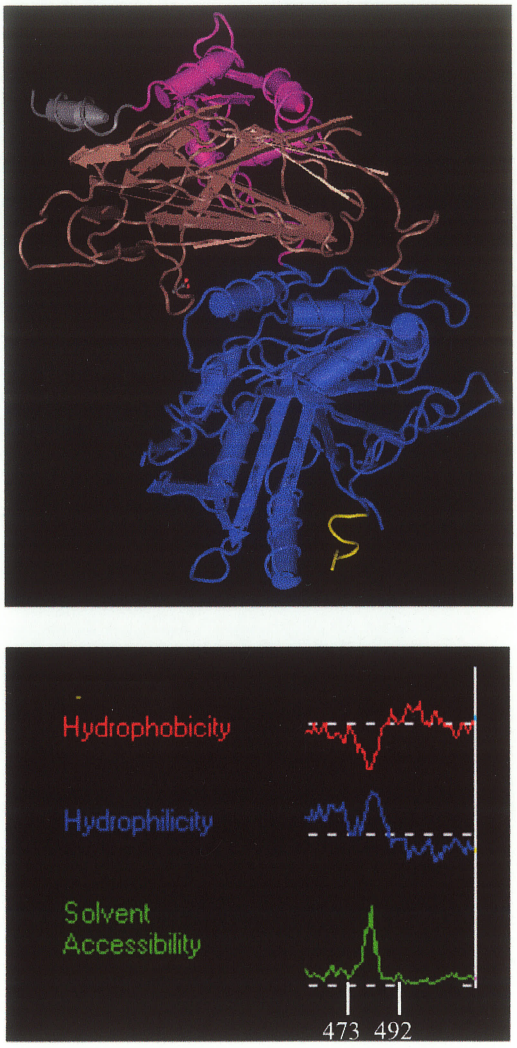


Figure 27: Structure of PLC-δ1 showing the solvent accessibility of the IQ motif (top panel). Residues 484-492 of the IQ motif (yellow) lie in the linker region (residues 446-488) within the catalytic region (blue). The C2 and EF-hand domains are shown in brown and gray, respectively. The region between the catalytic and EF-hand domains is also shown (purple). Analysis of the IQ motif (473-492), which is in close proximity to the catalytic domain, using ANTHEPROT V5.0, suggests the region is solvent accessible (bottom panel). This provides a potential mechanism for the regulation of the catalytic activity of PLC-δ1 by the IQ motif. The available data for the X/Y linker region indicates a disordered structure, thus, residues 446-483 are not visible. Structures of PLC-δ1 (PDB 1DJX) were generated using Cn3D 4.1.

Since we showed that PLC-$\delta 1_{CT}$ does not bind CaM, we next wished to determine whether full-length PLC-δ1 can bind CaM. Incubating GST-PLC-δ1 with purified CaM at various Ca^{2+} concentrations showed CaM does bind full-length PLC-δ1 irrespective of the Ca^{2+} concentration, indicating a Ca^{2+} independent binding (**Figure 28A**). When CaM was incubated with GST-PLC-δ1 in the presence of a peptide corresponding to the potential IQ-CaM-binding motif (PLC-IQ) we found inhibition of CaM binding with increasing concentrations of PLC-IQ only when low Ca^{2+} concentrations were used (**Figure 28B**). We then used the PLC-IQ peptide coupled to CNBr-activated Sepharose beads in binding experiments with lysates from HeLa cells containing 0, 12.3nM and 17.0μM free Ca^{2+} concentrations (**Figure 28C**). PLC-IQ co-precipitated PLC-δ1 independently of Ca^{2+} concentrations. However, binding of Ral and CaM to PLC-IQ was inversely related to Ca^{2+} concentrations. Binding of Ral, CaM and PLC-δ1 was inhibited when PLC-IQ was added in competition experiments (**Figure 28C**). The X/Y linker region is an unstructured region that has little sequence conservation between the various PLC family members (Williams, 1999). We performed sequence alignments to determine whether other members of the PLC family contain an IQ motif in the loop region but were unable to find any region of similarity. PLC-β is known to bind CaM but at an N-terminal region (McCullar *et al.*, 2003). In binding experiments using the PLC-IQ peptide with HeLa cell lysates we were unable to detect any PLC-β1 suggesting that PLC-δ1 is unique in its ability to bind CaM via an IQ-type CaM binding motif.

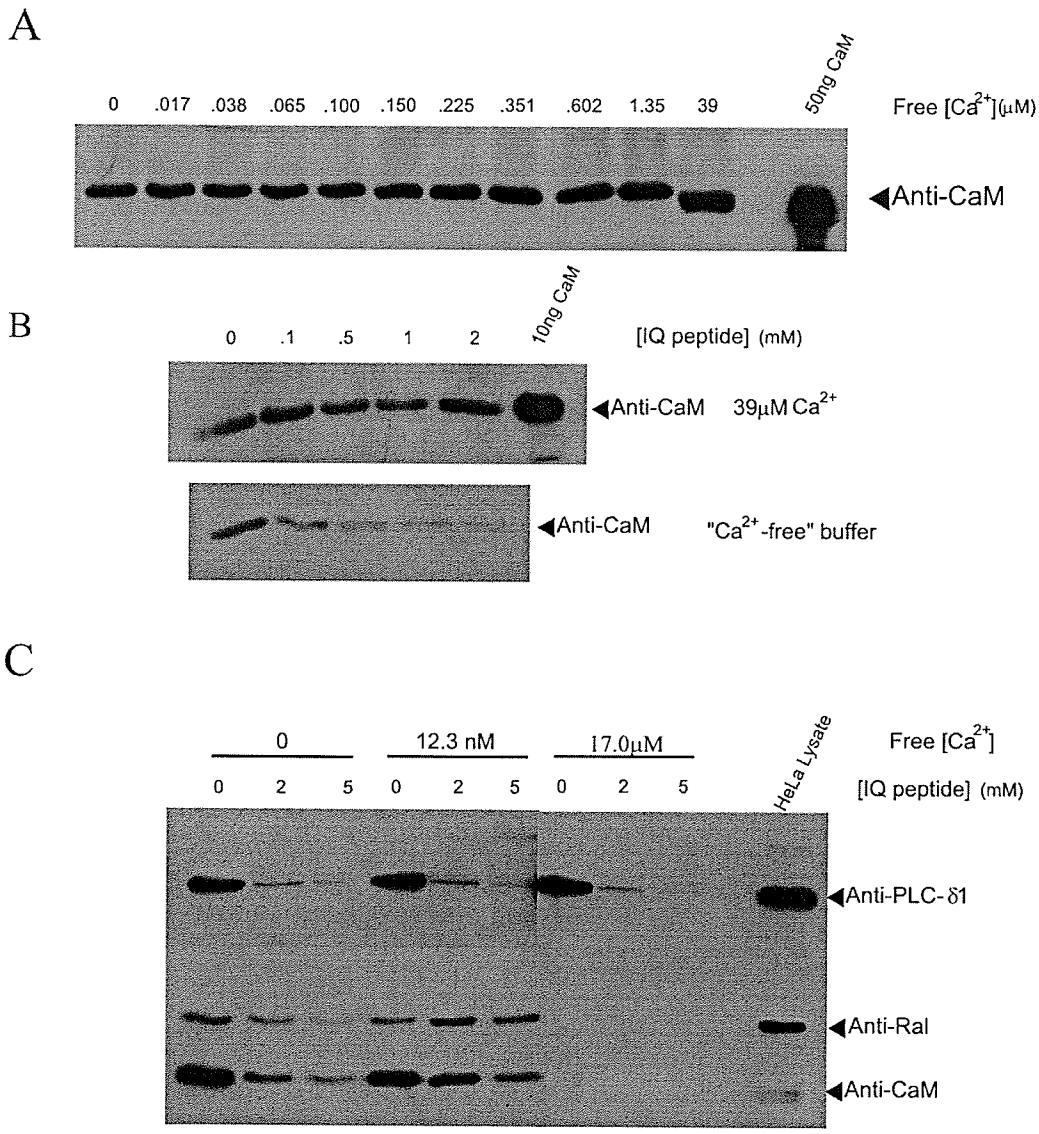


Figure 28: CaM binds PLC-δ1 in a Ca^{2+} independent manner and PLC-IQ co-precipitates Ral, CaM and PLC-δ1 from cell lysates. (**A**) Binding of GST-PLC-δ1 to purified CaM was performed using Ca^{2+}-calibration buffers ranging from zero to 39μM free-Ca^{2+} as described previously (35). (**B**) To establish whether the CaM binding to PLC-δ1 could be inhibited with the peptide, PLC-IQ was pre-incubated with GST-PLC-δ1 for 1 hr prior to incubation with purified CaM overnight at 4°C in the presence or absence of Ca^{2+}. (**C**) To determine whether the putative IQ peptide co-precipitates Ral, CaM and PLC-δ1, HeLa cell lysates were incubated with the peptide coupled to CNBr-activated Sepharose beads at 0, 12.3nM and 17μM Ca^{2+} plus 0, 2 or 5mM PLC-IQ peptide. Proteins were separated by SDS-PAGE and Western blotting was performed using antibodies against Ral, CaM and PLC-δ1.

PLC-δ1 itself contains a calmodulin-like structure, the EF-hand domains (**Figure 27**). Previous work suggests that PLC-δ1 activity is inhibited in the presence of CaM binding peptides and CaM (Richard *et al.*, 1997). Therefore, we examined whether the PLC-IQ peptide could also inhibit PLC-δ1 enzyme activity. We found that incubation of PLC-δ1 with 10μM and 100μM PLC-IQ peptide caused a 22% and 53% reduction in PLC-δ1 activity, respectively (**Figure 29**). Incubation with 100μM PLC-IQ peptide and 10μM CaM simultaneously reduced PLC-δ1 activity by 55%. In contrast, pre-incubation of PLC-δ1 with Ral partially reversed the inhibition caused by the IQ peptide alone (**Figure 30**). To test the possibility that the reversal of IQ-mediated inhibition by Ral could be due to direct interaction with IQ peptide we incubated IQ-Sepharose beads with purified recombinant Ral protein. However, we were unable to detect any binding of Ral to the IQ peptide. Thus, binding of Ral to PLC-δ1 partially reverses the IQ-mediated inhibition of PLC-δ1 activity.

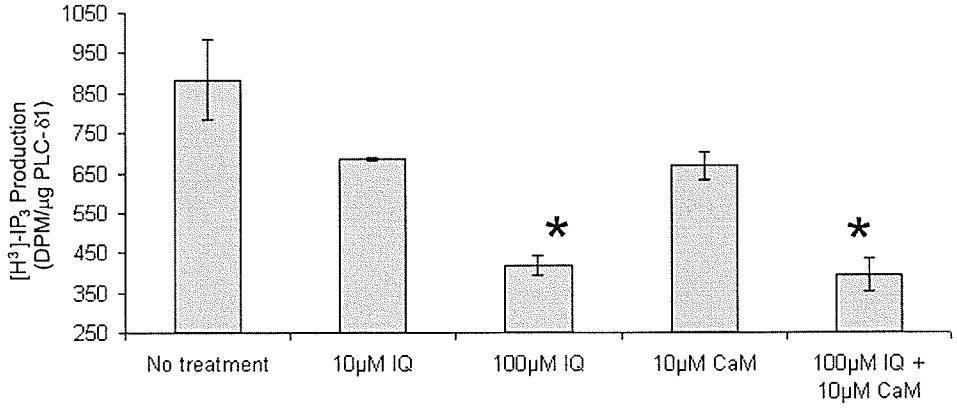


Figure 29: Peptide corresponding to the IQ motif in PLC-δ1 inhibits PLC-δ1 activity. Pre-incubation of PLC-δ1 with the IQ peptide resulted in a decrease in PLC enzyme activity, which was not reversed by the addition of CaM (A). *P values <0.05 were considered significantly different from *PLC-δ1 activity alone (n≥3).

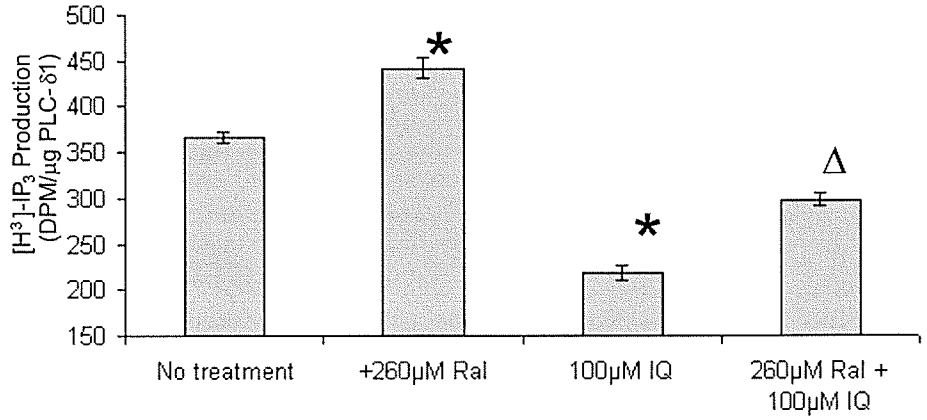


Figure 30: Ral reverses IQ peptide mediated PLC-δ1 inhibition. Addition of 260μM Ral caused a reversal of IQ dependent inhibition of PLC-δ1 activity (B). *P values <0.05 were considered significantly different from *PLC-δ1 activity alone or $^{\Delta}$PLC-δ1 activity in the presence of 100μM IQ (n≥3).

Ral recognizes various effector molecules depending on its nucleotide (GTP or GDP) status (Bauer *et al*, 1999). Incubation of recombinant Ral loaded with GTPγS

or GDPβS demonstrated that PLC-δ1 binding is not guanine nucleotide dependent (**Figure 31A**). Activation of serum-starved HeLa cells with EGF, a known activator of Ral (Goi *et al.*, 2000; our unpublished data), followed by incubation of the cell lysates with GST-PLC-$\delta 1_{CT}$ or full-length GST-PLC-δ1 showed no preference for GTP- or GDP-bound Ral (**Figure 31B**). We next wished to determine the binding preference of PLC-δ1 for various Ral mutants. GST-PLC-$\delta 1_{CT}$ was incubated with lysates from HeLa cells overexpressing various Ral mutants. The constitutively-active RalB(V23), dominant-negative RalB(N28) and RalB lacking the C-terminal isoprenylated moiety (ΔC*AA*X) all bound PLC-δ1 (**Figure 32**). Quantification revealed a significant decrease in PLC-δ1 binding to the effector domain mutant RalB(V23,D49) while RalB lacking the first 11 amino acids, RalB(ΔN11), showed no binding (**Figure 32**). In all experiments no binding to GST was observed.

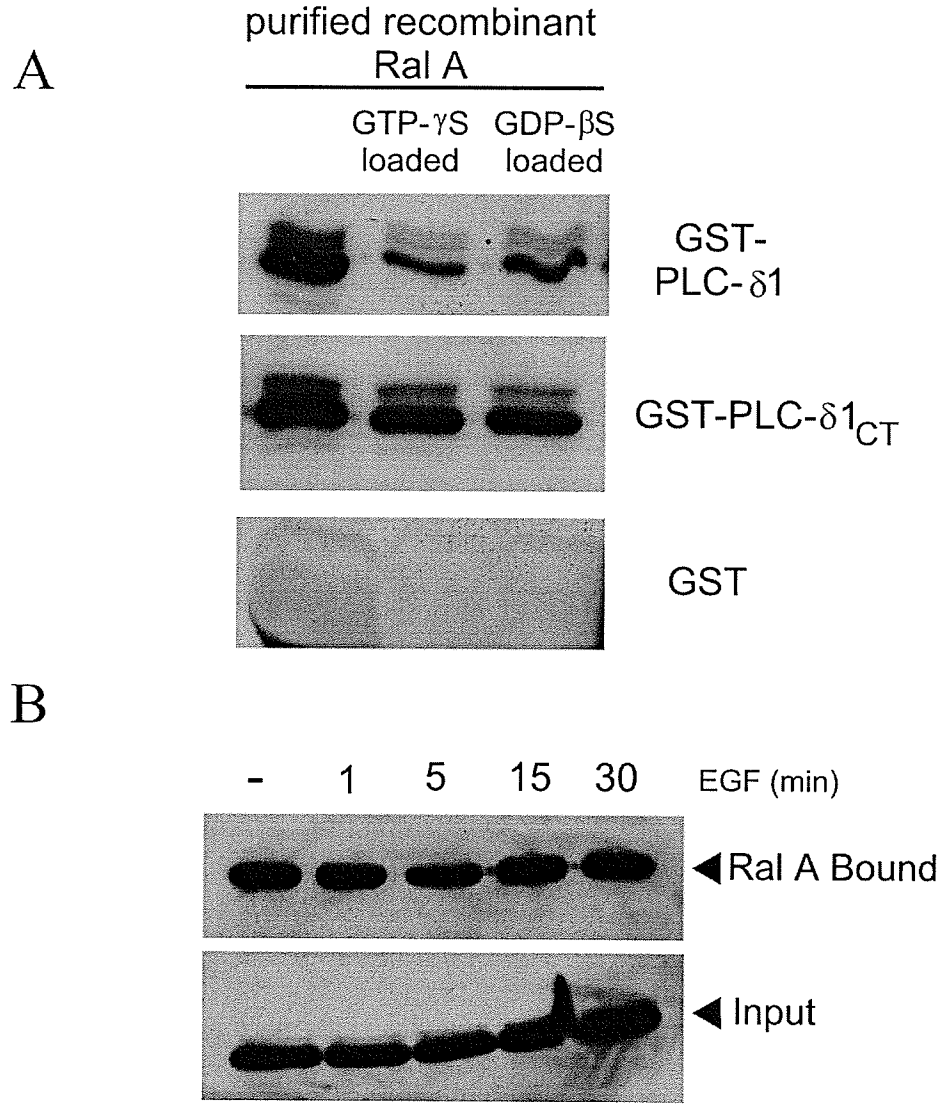


Figure 31: Ral binds PLC-δ1 irrespective of the guanine nucleotide status. (A) Purified recombinant Ral pre-loaded with 10μM GDP-βS or 10μM GTP-γS was incubated with full-length GST-PLC-δ1, GST-PLC-$\delta 1_{CT}$ or GST as described in methods. (B) Quiescent HeLa cells treated with 2ng/ml of EGF for various times (min) were lysed in buffer (50mM Tris-HCl, pH 7.5, 200mM NaCl, 2mM $MgCl_2$, 0.1% NP-40, 10% glycerol, plus protease inhibitor cocktail) and incubated with GST-PLC-δ1 for 2 hr at 4°C. Western blotting was used to detect RalA. Control experiments to confirm Ral-activation were performed using GST-Ral Binding domain of RIP1 (not shown).

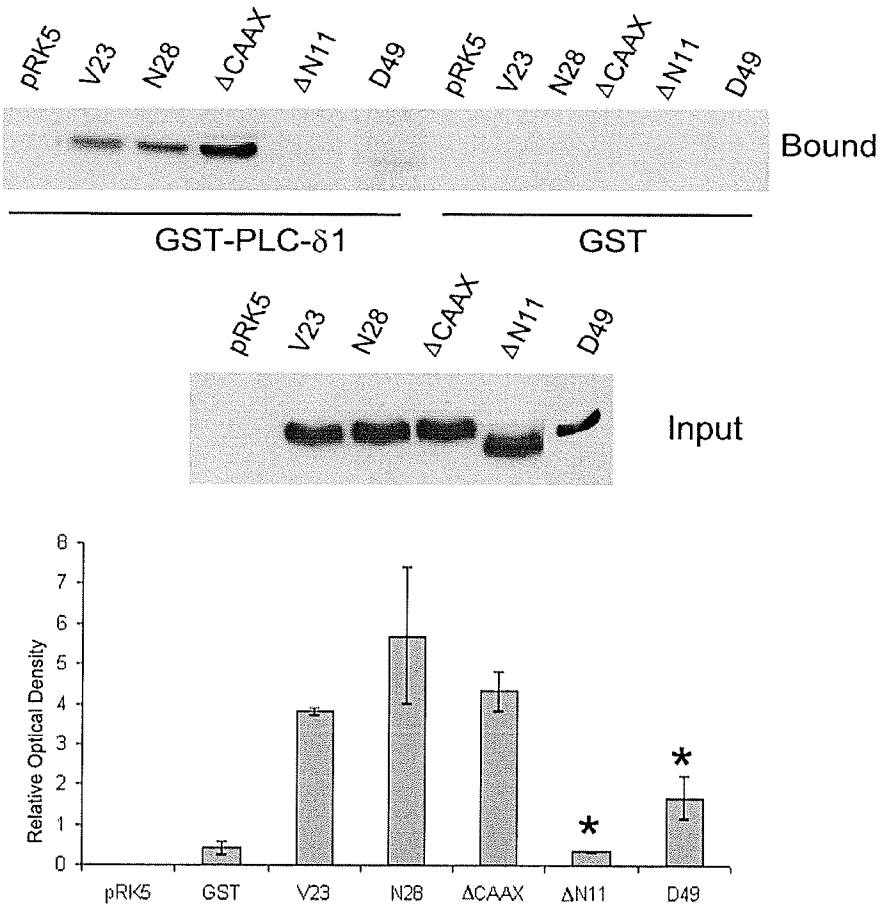


Figure 32: Ral mutants bind to PLC-δ1 selectively *in vitro* and require the N-terminal portion of Ral. (A) GST-PLC-$\delta 1_{CT}$ was incubated with HeLa cell lysates (500μg protein) expressing the Ral mutants RalB(V23), RalB(N28), RalB(ΔN11), RalB (Δ*CAAX*) or RalB (V23,D49). RalB bound to GST-PLC-δ1 was determined by Western blotting. All experiments were repeated a minimum of three times and quantified using Quantity One (Bio-Rad). *P values <0.05 were considered significantly different from RalB (V23), (N28) or (Δ*CAAX*) (n ≥3).

Activation of the Ras pathway is known to result in binding of the Ral effector molecule RalGDS to Ras-GTP thereby activating Ral signaling (Hofer *et al.*, 1994).

Hence, we wished to determine whether constitutively active Ral or activation of the Ras/Ral pathway, using a constitutively active Ras(V12) mutant, has an effect on PLC-δ1 activity *in vivo*. When RalB(V23) or Ras(V12) was overexpressed and PLC-δ1 immunoprecipitated from thrombin stimulated HeLa cells, a significantly greater PIP_2 hydrolysis was seen compared to control empty vector transfected cells (**Figure 33**). Further, PLC-δ1 immunoprecipitated from cells overexpressing the Ral mutants RalB (N28), RalB (V23, D49) and RalB (ΔC*AA*X), but not RalB (ΔN11), had significantly increased PIP_2 hydrolysis (**Figure 33**). Since PLC-δ1 does not associate with RalB (ΔN11) this confirms that physical interaction between Ral and PLC-δ1 is necessary for enzyme activation.

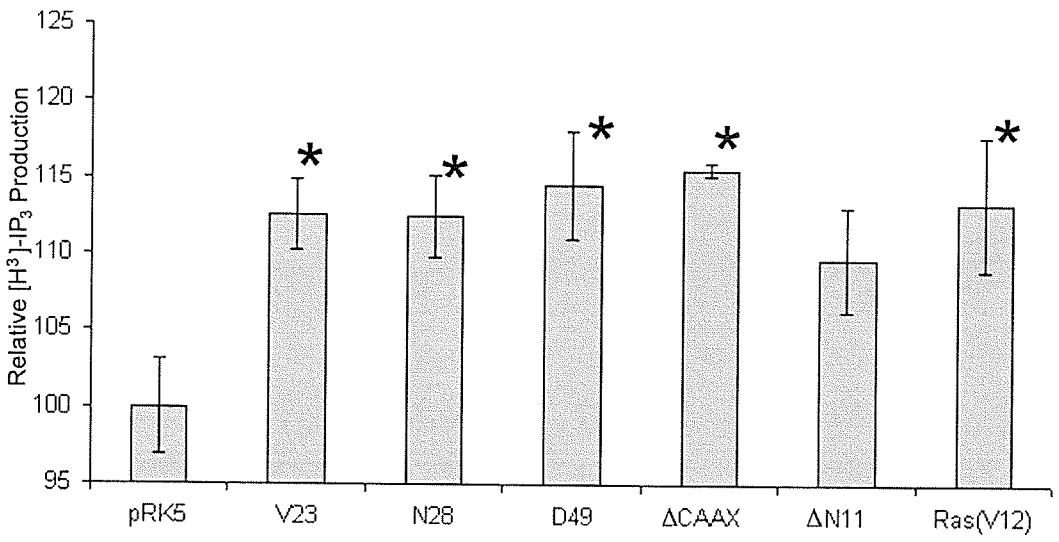


Figure 33: Ral mutants selectively activate of PLC-δ1 *in vivo* and require N-terminal portion of Ral. Cells transfected with the various RalB mutants and the Ras(V12) mutant for 24 hr were stimulated with 0.2ng/ml thrombin for 3 min and then lysed and PLC-δ1 immunoprecipitated as described in Materials and Methods section. PLC-δ1 activity in immunoprecipitates was measured as explained in Materials and Methods section. Values were quantified as percent hydrolysis measured against empty vector (pRK5) treated samples and were considered significantly different when *P values <0.05 compared to pRK5 treated samples ($n \geq 3$). Abbreviations used: V23 = RalB(V23); N28 = RalB(N28); D49 = RalB(V23, D49); ΔN11 = RalB(V23, ΔN11); ΔCAAX = RalB(V23, Δ*CAA*X).

Chapter 8

DISCUSSION

We have found a novel protein interaction between the small GTPase Ral and Phospholipase C-δ1 that occurs at low levels of calcium and increases proportionally with increased calcium concentrations. Activation of PLCs by the small GTPase Ras has been demonstrated previously for PLC-ϵ (Kelley *et al.*, 2001; Lopez *et al.*, 2001; Song *et al.*, 2001) and the homologous nematode PLC210 enzymes (Shibatohge *et al.*, 1998). Consistent with v-Src-induced phospholipase D (PLD) regulation by Ral, the N-terminal 11 amino acids of Ral are essential for PLC-δ1 binding. However, the interaction of Ral to PLC-δ1 is hampered when the Ral (V23, D49) double mutant containing an effector domain mutation at residue 49 is used in binding experiments with PLC-δ1. This is in sharp contrast to what is observed in the case of PLD where the binding of Ral(V23,D49) is enhanced (Jiang *et al.*, 1995; Luo *et al.*, 1998). Another important difference between Ral/PLC-δ1 and Ral/PLD binding is that Ral alone is sufficient to activate PLC *in vitro* and *in vivo* but PLD likely requires another factor, such as the GTPase ARF6 for activation (Xu *et al.*, 2003). Unlike typical Ras effectors, the Ral interaction failed to demonstrate guanine nucleotide dependence for PLC-δ1 binding. Thus, Ral behaves as an atypical effector in that it stimulates PLC-δ1 enzymatic activity independent of its guanine nucleotide status.

Expression of all the Ral mutants, except the N-terminal truncated clone, enhanced PLC-δ1 activity *in vivo*. Even though the effector domain mutant of RalB(V23,D49) bound very weakly to PLC-δ1, the enzyme activity was still significantly increased. Whether the weak binding alone is sufficient to activate PLC-δ1 or RalB(V23,D49) activates another effector capable of enhancing enzyme activity is unclear.

PLC enzymes contain two domains, PH and C2, which are involved in membrane attachment (Katan and Allen, 1999). The PLC-β, -γ and -ϵ isoforms also have specific regions of interaction with upstream regulatory elements that provide additional membrane tethering points (Essen *et al.*, 1996). PLC-δ1 is known to cycle between a membrane bound and cytosol form through interactions with PIP_2 or soluble IP_3, respectively. Binding of Ral at the C-terminal region of PLC-δ1 could provide an additional mechanism for membrane targeting of PLC-δ1. Ral is accepted as a downstream target of Ras-p21 through activation of RalGDS (Wolthius and Bos, 1999) while the role of PLC-δ1 is believed to be that of amplifying or sustaining IP_3 production initiated by upstream PLC-β, γ and ϵ isoforms (Kim *et al.*, 1999; Nakamura *et al.*, 2003). The enhanced membrane targeting of PLC-δ1 by Ral could provide an additional mechanism for regulating PLC-δ1 activity following various physiological stimuli.

Our results indicate that exogenous CaM inhibits PLC-δ1 activity, likely through binding at the X/Y linker region in the catalytic α/β barrel. CaM-binding to PLC-δ1 could deny the catalytic core access to the substrate PIP_2. Studies on PLC-β have previously shown that CaM binds to an N-terminal region within the EF-hand domain (McCullar *et al.*, 2003). We show that a peptide mimicking the CaM-binding IQ motif also disrupts the ability of PLC-δ1 to catalyze PIP_2 hydrolysis. Since the IQ motif region is solvent accessible it remains to be seen whether this region can bind to the EF-hand domain in PLC-δ1. In addition, IQ motifs often bind internally to proteins at distal regions to regulate CaM binding (Yamniuk and Vogel, 2004). Further work is required to determine whether such a mechanism also exists for PLC-δ1. An alternative explanation for this observation is that IQ binding at a distal region, such as the EF-hand domain, maintains the enzyme in an active conformation that is disrupted by the addition of exogenous IQ peptide or CaM. A similar "two-faced" CaM binding pattern is seen for other proteins such as cAMP-phosphodiesterase (James *et al.*, 1995).

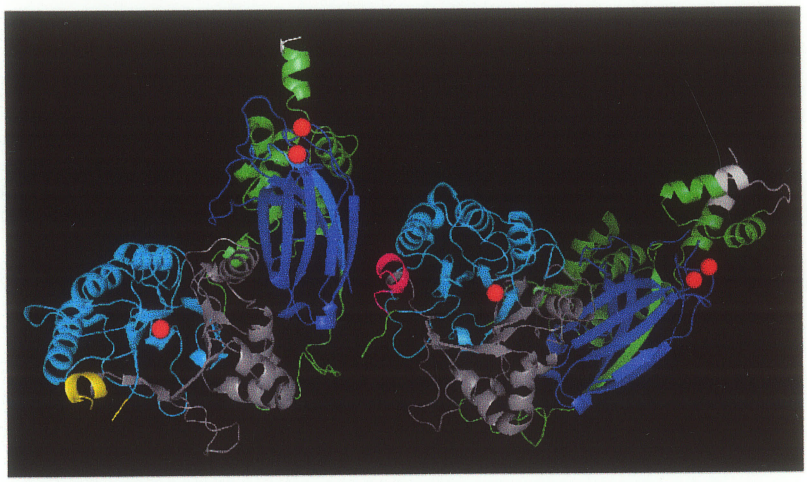
In competition studies using the PLC-IQ peptide, CaM binding to PLC-δ1 is disrupted only in the absence of Ca^{2+}. IQ-motifs typically bind CaM in the absence of Ca^{2+} (Apo-CaM), consistent with the results seen with PLC-δ1 in the absence of calcium. However, an additional Ca^{2+}-independent CaM binding site in PLC-δ1 may exist that is different from the IQ motif but perhaps analogous to the CaM binding

region in the EF-hand domain of PLC-β (McCullar *et al.*, 2003). Thus, PLC-IQ likely inhibits the binding of CaM directly but is unable to compete for CaM at an alternate site in PLC-δ1, which is accessible at higher Ca^{2+} concentrations.

The three-dimensional structure data available for catalytically active PLC-δ1 indicate the C2 domain is in close proximity to the EF-hand domains and both regions are essential for PLC-δ1 function (Essen *et al.*, 1996; Ellis *et al.*, 1993). At Ca^{2+} concentrations equivalent to those found in the active cell (Allen *et al.*, 1997; Kim *et al.*, 1999) Ral potentiates PLC-δ1 activity possibly by relieving CaM inhibition. In the present study we have shown that the Ral-CaM-PLC complex can be co-precipitated from cell lysates using the IQ peptide of PLC-δ1 even in the absence of Ca^{2+} but not in its presence. At low Ca^{2+} concentrations Apo-CaM binding likely occurs to the IQ motif in the linker region of PLC-δ1 thus keeping the PLC-δ1 in an inactive state. As Ca^{2+} concentrations increase CaM interaction is removed. However, the Ca^{2+} dependent increase in Ral binding to PLC-δ1 was not observed when PLC-IQ was used as the bait peptide. Since the C2 domain (Ral binding region) is in close proximity of the EF-hand domain in Ca^{2+}-bound PLC-δ1 (**Figure 27**) it is plausible that PLC-IQ peptide binding to PLC-δ1 may hinder Ral binding to the C2 region. Whether the EF-hand domain is the site of internal binding with the IQ motif present in the linker region remains to be determined. This could explain the absence of Ral and thus CaM, through Ral binding, at higher Ca^{2+} concentrations. Thus,

exogenous PLC-IQ peptide binding to PLC-δ1 may prevent proper refolding of the Ca^{2+}-bound catalytically active PLC-δ1 form, which can be relieved in the presence of Ral. Another potential role for the IQ motif can be in dimerization or multimerizatoin. In support of this, crystals of PLC-δ1 form intermolecular contacts between asymmetric dimers (**Figure 34**) (Essen *et al.*, 1996). The unstructured nature of the linker region opens the possibility for its role in dimerization of PLC-δ1 that may occur irrespective of Ca^{2+} concentrations. Although previous work has shown that the X/Y linker region may be dispensable for catalytic activity (Ellis *et al.*, 1993), in the present study we have shown that this region plays a critical role in CaM-dependent inhibition of PLC-δ1.

A



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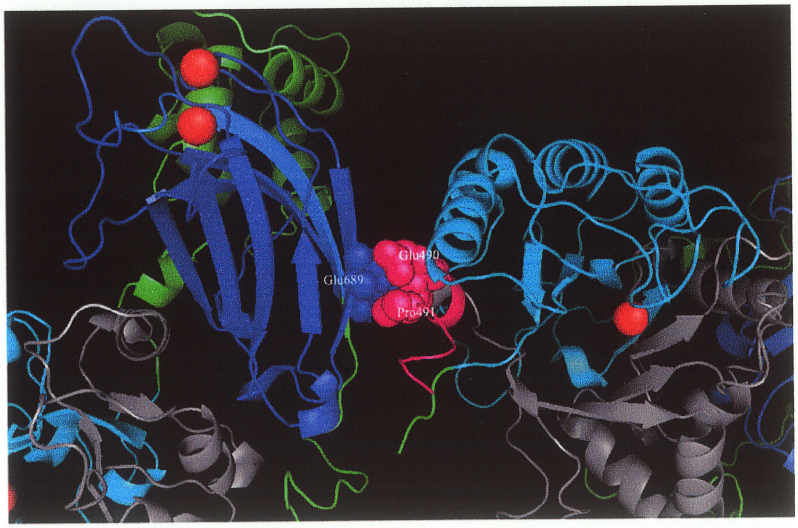


Figure 34: A structural model showing the formation of asymmetric dimers of PLC-δ1. (A) The IQ motif (pink) of one PLC monomer is in close proximity to the C2 domain (blue) of an adjacent monomer. Calcium ions (red) and the catalytic X (cyan) and Y (grey) boxes are also shown. The free IQ motif (yellow) in the second monomer is also shown. (B) A close up view of potential intermolecular contact sites between the two monomers. The residues of the IQ motif (Pro490 and Glu491, pink) are shown in spacefill making contact with Glu689 (blue) of an adjacent PLC monomer. The structures were created from PDB coordinates 1DJI using PyMOL v. 0.97 (DeLano Software).

PLC-β, -γ and -ϵ isoforms contain specific regions that mediate interactions with regulatory proteins. In the present studies we have demonstrated that CaM binding to the linker X/Y region and Ral binding to the C-terminal portion of PLC-δ1 regulate its activity. The work in the present study has expanded the model proposed by Essen *et al.*, (1996) to include regulation of PLC-δ1 by Ral and CaM. The new scheme is shown in **Figure 35**. The significance of PLC-δ1 activation by the Ral pathway and inhibition by CaM provides a key link between Ca^{2+} regulation of PLC-δ1 through ion channels and/or upstream pathways of PLC-β, -γ and -ϵ isoforms. Thus, malfunctioning of these proteins can lead to dysregulation of Ca^{2+}-homeostasis resulting in conditions such as cardiovascular disease, stroke or cancer.

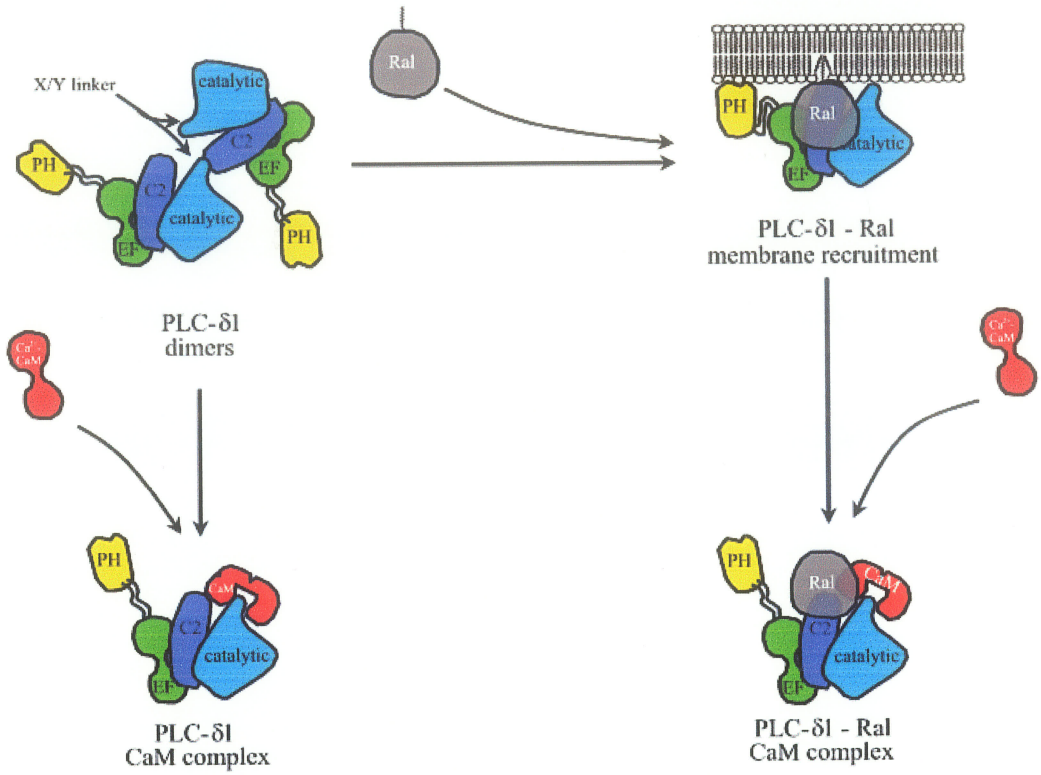


Figure 35: Proposed model for the regulation of PLC-δ1 by Ral and Calmodulin. PLC-δ1 dimers (top left) bind through interactions of the X/Y linker region in PLC-δ1 to the C2 region in the adjacent monomer. The IQ motif likely makes internal contact with distant regions of PLC-δ1 in the active enzyme. Binding of either Ca^{2+}-CaM or Apo-CaM at the IQ motif in the X/Y linker region causes the dissociation of the dimers and inhibition of catalytic activity (bottom left). In the presence of Ral, but absence of CaM (top right), Ral provides an additional membrane tethering point for PLC-δ1 facilitating access to substrate and enhancing enzyme activity. In the presence of both Ral and CaM the complex can be precipitated formed by either binding of CaM directly to PLC-δ1 or through Ral.

Chapter 9

SUMMARY AND CONCLUSION

In the present work Phospholipase C-δ1 was discovered to be a novel Ral interacting protein. The dependence on calcium of Ral binding to PLC-δ1 indicated a positive correlation. The finding that binding is calcium dependent led to a second possibility that the calcium sensor protein, calmodulin, may be involved in the regulation of PLC-δ1, either directly or through Ral. Using a database search for potential CaM binding sequences we postulated, and further confirmed experimentally, that calmodulin binds to PLC-δ1 directly. Binding of Ral to PLC-δ1 potentiates enzymatic activity while CaM binding inhibits it. Further, using a synthetic peptide with the sequence of a CaM binding target region in PLC-δ1 and CaM-specific inhibitors we found inhibition of PLC-δ1 activity. This data suggests that the putative CaM binding IQ-motif sequence in PLC-δ1 may itself regulate enzymatic activity.

Ral effector proteins generally bind Ral preferentially in the GTP-bound state leading to downstream target activation or signaling. However, we found that PLC-δ1 binds Ral irrespective of the guanine nucleotide status. These results were iterated in cell stimulation experiments, with the known Ral activator EGF, and in binding experiments using various mutant Ral proteins overexpressed in mammalian cells.

The binding of Ral to PLC-δ1 requires the N-terminal 11 amino acids of Ral. In addition, a Ral effector domain mutation significantly reduced binding of Ral to PLC-δ1 indicating interactions of PLC-δ1 may occur with the Ral effector domain. Lastly, measurements of PLC-δ1 catalytic activity of immunoprecipitated PLC-δ1 enzyme from cells overexpressing various Ral mutants demonstrated that all Ral mutants, except the N-terminal truncated form, as well as a constitutively active Ras mutant, increased PIP_2 hydrolysis. In conclusion, these studies have established a complex regulatory mechanism of PLC-δ1 by Ral and the calcium sensor protein calmodulin.

Chapter 10

FUTURE DIRECTION

The research presented here has established novel regulatory mechanisms for PLC-δ1 catalytic activity. Further research to advance this work can now be performed to characterize detailed structural and functional aspects of this regulation. We have discovered that Ral interaction occurs with the C-terminal portion of PLC-δ1, which contains the C2 domain region. X-ray crystallographic structures of PLC-δ1 dimers show that the X/Y linker region of PLC-δ1 makes contacts with the C2 region of the adjacent dimer (**Figure 34**). Enzyme assay results using Ral and the IQ peptide demonstrated that Ral mediated activation of PLC-δ1 is partially reversed by the IQ peptide (**Figure 30**). This suggests that the IQ peptide could be a competitive inhibitor of Ral. However, further enzyme kinetic studies need to be performed to validate whether the IQ peptide is a competitive antagonist of Ral. In addition, further elucidation of the residues involved in Ral contact with the PLC-δ1 C-terminal region is necessary to understand the function. Although, we have no evidence that the IQ peptide binds to other PLC isoforms, enzyme kinetic studies of other PLC isoforms in the presence of the IQ peptide should be performed in comparison with PLC-δ1 to determine the selectivity of the peptide as a PLC-δ1 antagonist.

Ral is bound to membranes through a C-terminal isoprenylated Cys residue and PLC-δ1 is bound to membranes through interaction of the PH and C2 domains with membrane lipids. Despite having two membrane attachment points, PLC-δ1 is not tightly bound to membranes and is removed following cell lysis. We have suggested a mechanism whereby Ral provides an additional membrane tethering point thereby enhancing access of PLC-δ1 to the substrate. To establish whether Ral improves membrane association of PLC-δ1 two alternate *in vitro* approaches can be used. We expect purified recombinant full-length PLC-δ1 enzyme incubated in the presence of lipid micelles of phosphatidylserine (PS)/phosphatidylcholine (PC) with and without post-translationally modified Ral protein should enhance its binding to membranes. Following incubation of Ral and PLC-δ1 in the mixed micelles, separation by centrifugation should increase the amount of PLC-δ1 in the precipitated fraction. Alternatively, PIP_2 bound Sepharose is commercially available (Molecular Probes) and can be used in binding assays for PLC-δ1 with and without purified recombinant Ral. We expect that the presence of Ral should lead to increased PLC-δ1 binding to PIP_2-Sepharose. An *in vivo* approach to determine whether Ral improves membrane targeting of PLC-δ1 can also be used. That is, co-expression of PLC-δ1 and Ral in mammalian cell cultures can be performed. To determine subcellular localization of PLC-δ1 and Ral, cells can be fractioned and Western blotting performed on the soluble and particulate fractions or immunocytochemistry techniques using fluorescent secondary antibodies can be performed on fixed cells.

We, and others, have previously determined that CaM regulates membrane targeting of the small GTPases K-RasB, Rab3A and RalA (Sidhu *et al.*, 2003; Park *et al.*, 1997; Park *et al.*, 2001). In the present experiments CaM inhibited PLC-δ1 activity. Whether inhibition of PLC-δ1 by CaM is correlated with dissociation of PLC-δ1 from membranes is not known. This potential regulatory mechanism of PLC-δ1 inhibition could be examined using *in vitro* or *in vivo* experiments. That is, isolated membranes containing membrane-bound PLC-δ1 incubated with purified CaM should cause release of PLC-δ1 from membranes. Following incubation, membranes can be separated from soluble fractions by centrifugation. PLC-δ1 protein released into the soluble fraction can be detected by Western blotting. An *in vivo* approach similar to that proposed for Ral and PLC-δ1 can be used. That is, CaM and PLC-δ1 can be co-expressed in cultured mammalian cells and the subcellular distribution assessed similarly.

In some proposed experiments we wished to overexpress full-length PLC-δ1 protein in mammalian cells. However, we were unable to obtain appreciable expression above endogenous protein levels for further functional studies. In order to increase the expression efficiency of full-length PLC-δ1 the cDNA could be inserted into cells using a stable expression system or into a more efficient transient expression vector. Overexpression of PLC-δ1 in mammalian cells will provide an *in vivo* model to study the effects on PLC-δ1 localization by Ral or CaM. We would expect that co-

expression of Ral with PLC-δ1 in cells should increase the localization of PLC-δ1 to membranes while co-expression of CaM with PLC-δ1 would increase levels of PLC-δ1 in cytosol. In addition, over-expression of PLC-δ1 in mammalian cells will provide an alternative method of performing PLC enzyme assays to the expensive immunoprecipitation protocol.

One mechanism by which membane bound Ral is activated is through the Ras effector RalGDS (Kishida *et al.*, 1997; Matsubara *et al.*, 1999). Whether PLC-δ1 is an atypical Ral effector and is downstream of the Ras signaling pathway is not known. We have presented some evidence that overexpression of constitutively active Ras results in increased PIP_2 hydrolysis of immunoprecipitated PLC-δ1. Knockdown experiments targeting various elements of the Ras/Ral signaling pathway and subsequent measurement of PLC-δ1 enzymatic activity would provide important information about the regulation PLC-δ1 signaling.

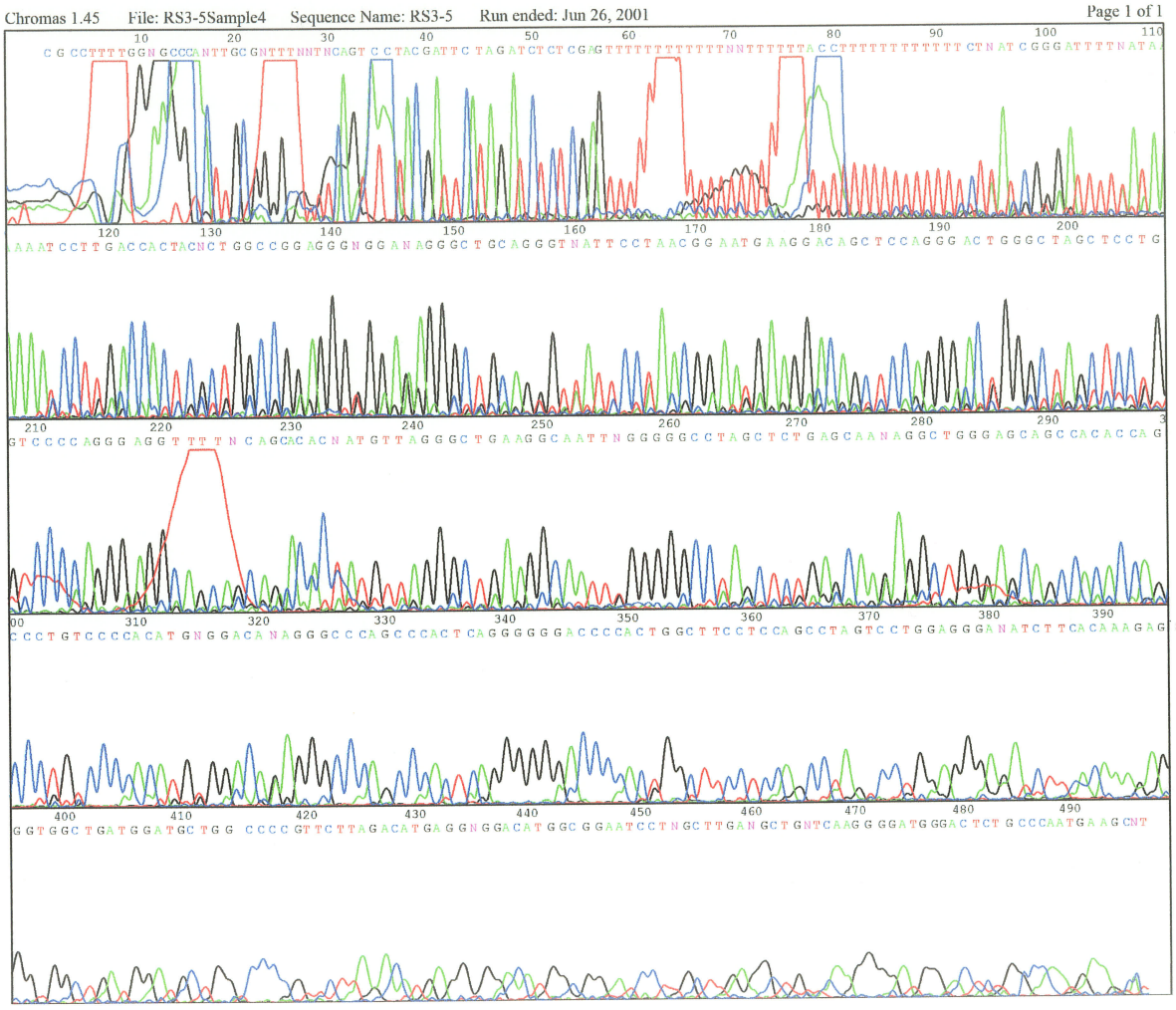
It has been shown previously that Ral binding to Filamin induces the formation of filopodia to regulate cell morphology (Ohta *et al.*, 1999). In addition, Ral binding to its effector RalBP1, which contains a GAP domain for Cdc42 and Rac GTPases, could be downstream of Ral/PLC-δ1 binding (Cantor *et al.*, 1995; Jullien-Flores *et al.*, 1995). Further support for a link of PLC-δ1 between Ral and Cdc42/Rac signaling is provided by the observation that PLC-δ1 binds to p122RhoGAP (Kawai *et al.*, 2004). p122RhoGAP activates PLC-δ1 (Homma *et al.*, 1995) and inhibits the

formation of actin stress fibres and focal adhesions (Kawai *et al.*, 2004). Whether expression of constitutively active or dominant negative Ral mutants or conversely knockdown of Ral or elements of the Ral signaling pathway can affect cell morphology is not known. Also, correlation of PLC-δ1 activity with alteration of the Ral pathway could help to establish a link between these pathways. Measurement of local PIP_2 levels in cells and changes in PIP_2 distribution following manipulation of the Ral pathway could provide insight into changes in cell dynamics.

This research has established novel regulatory mechanisms for PLC-δ1 enzymatic activity involving Ral and calmoduiln. The work opens the possibilities for continued research into several diverse areas that involve Ral-CaM-PLC-δ1. PLC-δ1 could prove to be a key player in Ca^{2+}/CaM and Ral signaling pathways.

Chapter 11

APPENDIX I



Blast Output of isolated yeast clone (closest match).

>gi|30046471|gb|BC050382.1| Homo sapiens phospholipase C, delta 1, mRNA (cDNA clone MGC:51904 IMAGE:5769665), complete cds

Length = 2674

Score = 571 bits (288), Expect = e-160
Identities = 373/402 (92%), Gaps = 6/402 (1%)
Strand = Plus / Minus

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Sbjct: 2509 ggaggca--gcagcagacactatgttagggctgaaggcaatttgggggcctagctctgag 2452


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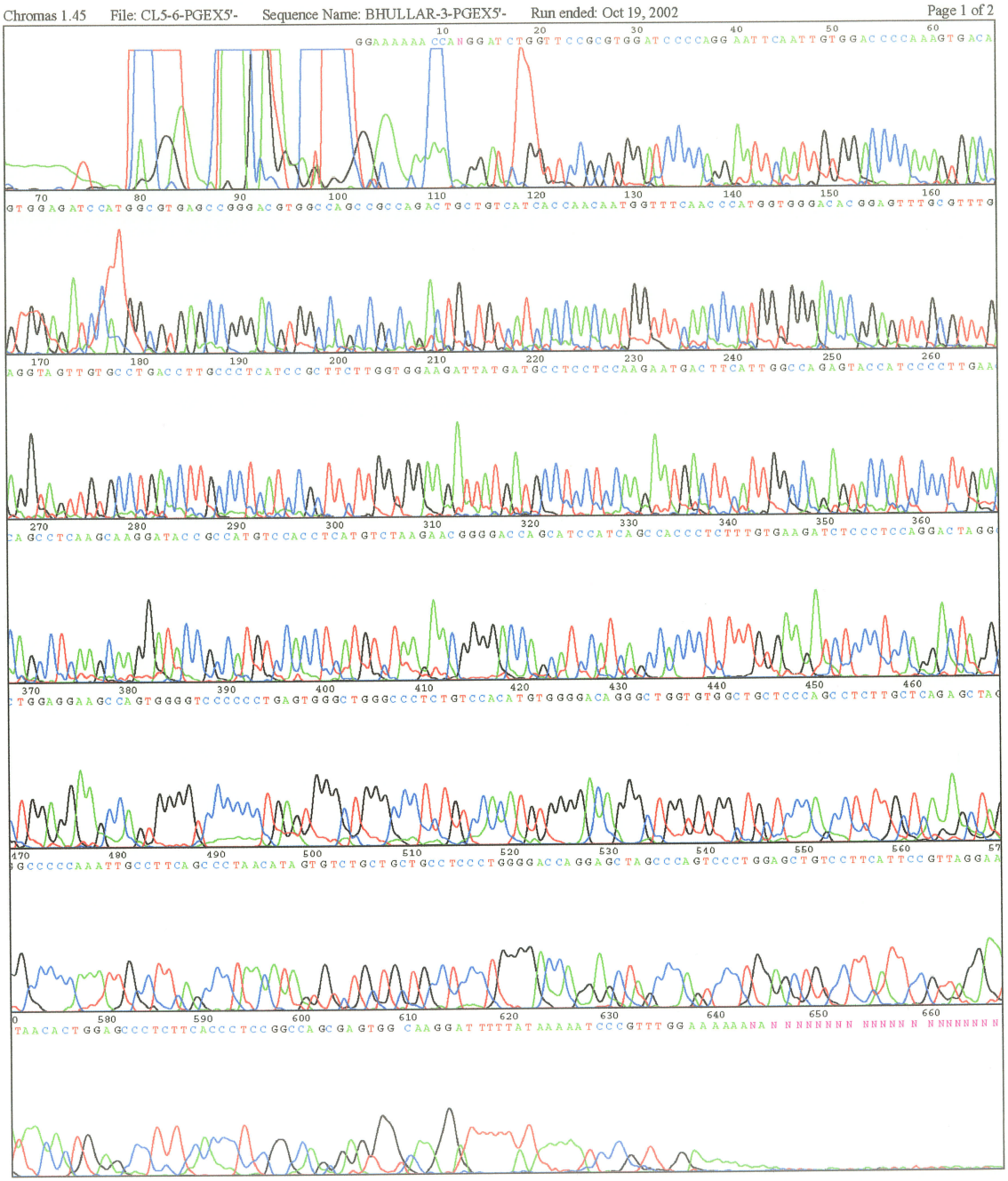

Blast Output of isolated clone inserted into pGEX-4T3 vector

>gi|595725|gb|U13854.1|XXU13854 pGEX-4T2 cloning vector, complete sequence
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Identities = 32/32 (100%)
Strand = Plus / Plus

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Sbjct: 914  ggatctggttccgcgtggatccccaggaattc 945
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>gi|21748997|dbj|AK090774.1| Homo sapiens cDNA FLJ33455 fis, clone BRAMY2000354, highly similar to 1-PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE PHOSPHODIESTERASE DELTA 1 (EC 3.1.4.11)
Length = 2752

Score = 1142 bits (576), Expect = 0.0
Identities = 589/592 (99%), Gaps = 1/592 (0%)
Strand = Plus / Plus

```
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2150 gaattcaattgtggaccccaaagtgacagtggagatccatggcgtgagccgggacgtggc 2209

Query: 99   cagccgccagactgctgtcatcaccaacaatggtttcaacccatggtgggacacggagtt 158
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2210 cagccgccagactgctgtcatcaccaacaatggtttcaacccatggtgggacacggagtt 2269

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```

```
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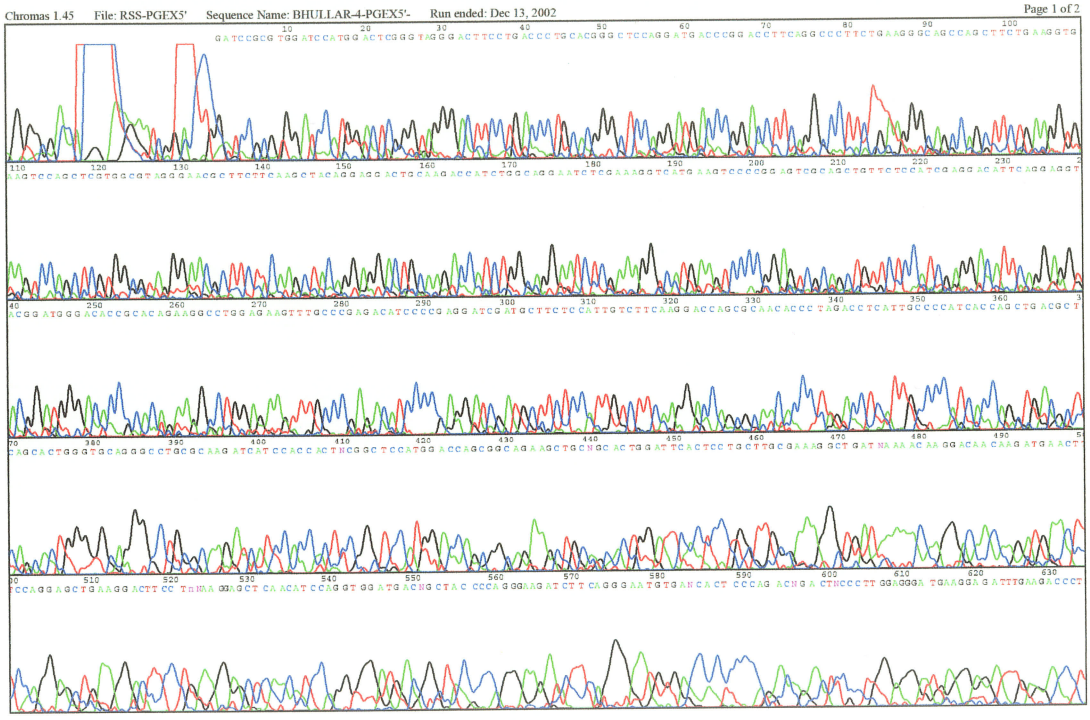

Blast Output of Rat PLC-δ1 clone in pGEX-2T vector

>gi|8393980|ref|NM_017035.1| Rattus norvegicus phospholipase C, delta 1 (Plcd1), mRNA
Length = 2791

Score = 1057 bits (533), Expect = 0.0
Identities = 573/586 (97%), Gaps = 2/586 (0%)
Strand = Plus / Plus

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Sbjct: 222 catggactcgggtagggacttcctgaccctgcacgggctccaggatgacccggaccttca 281


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Query: 495 gaacttccaggagctgaaggacttcctnnaaggagctcaacatccaggtggatgacngct 554
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Chapter 12

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