Characterization of Ral and Ras p21 interaction sites in Phospholipase C-delta1

By

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

Master of Science

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Dedication and Acknowledgements

I would like to dedicate this thesis to my dear parents, Biljana and Zdravko Grujic. Their belief in my ability to succeed, and their continuous support and encouragement are greatly appreciated.

I would like to thank my brother, Dalibor Grujic, for always being there for me.

Alexandra Dudley, thank you for bringing joy and happiness to my life.

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Finally, I would like to thank Canadian Institutes of Health Research and the University of Manitoba for the financial assistance.

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Summary

Small GTP-binding proteins have been shown to bind and regulate phosphoinositide-specific phospholipase C (PLC), and thus play an important role in intracellular calcium signaling. Previously, it has been shown in our laboratory that RalA, a calmodulin (CaM)-binding protein, binds to the C2 region of PLC-δ1 (PLC-δ1 CT), and increases PLC-δ1 enzymatic activity. Since PLC-δ1 contains a CaM-like region in its N-terminus, we have investigated if RalA can also bind to the N-terminus of PLC-δ1. Therefore, we generated a GST-PLC-δ1 construct consisting of the first 294 amino acids of PLC-δ1 (GST-PLC-$\delta1_{1-294}$). *In vitro* binding experiments indicated that PLC-$\delta1_{1-294}$ was capable of binding directly to RalA and PLC-δ1. W-7 coupled to polyacrylamide beads bound pure PLC-δ1, demonstrating that PLC-δ1 contains a CaM-like region. Competition assays with W-7, peptides representing RalA and the newly identified RalB CaM-binding regions, or the IQ peptide from PLC-δ1 were able to inhibit RalA binding to PLC-$\delta1_{1-294}$.

A previous study from our laboratory has shown that in addition to RalA, overexpression of constitutively active forms of RalB and H-Ras GTPases in HeLa cells caused activation of PLC-δ1 *in vitro* suggesting that H-Ras may interact with PLC- δ1. GST-PLC-δ1 and GST-PLC-δ1 CT, but not GST, bound recombinant H-Ras and endogenous H-Ras from HeLa cell lysates in a calcium dependent manner. In the reverse experiment, GST-H-Ras, but not GST, was capable of binding PLC-δ1 from HeLa cells. The co-immunoprecipitation assay indicated that the H-Ras / PLC-δ1 complex exists in HeLa cells. The competition assay showed that H-Ras and RalA compete for binding to

PLC-δ1 CT. Even though H-Ras bound PLC-δ1 independent of the guanine nucleotide status, it was found that a dominant negative form of H-Ras bound PLC-δ1 less efficiently than the constitutively active or wild type forms of H-Ras.

The results demonstrate that there are two binding sites for RalA in PLC-δ1, and that the C2 domain of PLC-δ1 also contains a binding site for H-Ras. Altogether, the study provides further insight into the regulation of PLC-δ1 function by small GTPases.

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List of Abbreviations

apo-CaM : calcium free calmodulin

Ca^{2+}: calcium

Ca^{2+}-CaM: calcium bound calmodulin

CaM: calmodulin

CNBr: Cyanogen bromide

DAG: diacylglycerol

DMEM: Dulbecco's modified Eagle's medium

EGFR: Epidermal growth factor receptor

EGTA: ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid

FBS: fetal bovine serum

G proteins or GTPases: Guanine nucleotide binding proteins

GAPs: GTPase activating proteins

GDP: guanosine-5'-diphosphate

GEFs: Guanine-nucleotide exchange factors

GSH: glutathione

GST: glutathione-S-transferase

GTP: guanosine-5'-triphosphate

IP_3: inositol 1,4,5-trisphosphate

IPTG: isopropyl 1-thio-β-D-galactopyranoside

kDa: kiloDalton

PBS: Phosphate buffered saline

PH: pleckstrin homology (PH)

PI(4)P: phosphatidylinositol 4-phosphate

PI: phosphatidylinositol

PIP_2: phosphatidylinositol 4,5-bisphosphate

PLC: phosphoinositide-specific phospholipase C

PLD: phospholipase D

PVDF: polyvinylidene fluoride

RalBP1: Ral binding protein 1

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TIM: triose phosphate isomerase

wt: wild type version of protein

1. Introduction

In order to respond to a continually changing external environment, a cell needs to detect and respond to extracellular stimuli (Gonos and Spandidos, 1993). The net effect of this is alterations in cell metabolism and/or other properties of the cell, such as differentiation and proliferation (Gonos and Spandidos, 1993; Costello and Franklin, 2006). In order to accomplish these important biological functions, the external information needs to be relayed (transduced) to appropriate intracellular target proteins (Giamarellos-Bourboulis *et al.*, 2006). This signal transduction process is achieved through the activation or inhibition of appropriate proteins in signaling pathways (Wheeler-Jones, 2005). In response to signals, some proteins are translocated within the cell (Sidhu *et al.*, 2003). For example, a protein that is inactive when attached to the plasma membrane may move to the cytosol, or vice versa, and become active. In addition, there are two additional mechanisms for the cellular proteins to be turned ON or OFF during signal transduction. One way is by phosphorylation of target proteins, and another is by exchanging a guanine nucleotide bound to a protein (Wheeler-Jones, 2005). In this thesis, the latter process will be the focus of attention.

1.1 Guanine nucleotide binding proteins

Guanine nucleotide binding proteins (G-proteins or GTPases) play an important role in the signal transduction pathways of the cell. All GTPases bind guanosine-5'-triphosphate (GTP) or guanosine-5'-diphosphate (GDP), essential molecules for the signaling process in the cell (Dhanasekaran and Gutkind, 2001; Neves *et al.*, 2002). The G-proteins participating in signal transduction are divided into two groups:

1. Heterotrimeric G-proteins
2. Small-GTP binding proteins

Heterotrimeric G-proteins are associated with the cell membrane and are composed of three subunits: α, β and γ (Neer, 1995). In the resting cell, the three subunits form a complex. Upon ligand binding to the G-protein coupled receptor, GDP on the α subunit is exchanged for GTP, allowing the subunit to dissociate from the β and γ subunits. Both the GTP-bound α subunit and the β/γ complex activate/inhibit a number of effector proteins such as adenylyl cyclase and phospholipase C-β, leading to the generation of second messengers and alterations in cell function (Neer, 1995; Harden and Sondek, 2006).

The second group of G-proteins is comprised of small GTP-binding proteins (small GTPases). These GTPases are monomeric proteins ranging in size from 20-40 kDa (Takai *et al.*, 2001). They have received a lot of attention as they have been shown to be crucial for a number of cellular functions that ensure the survival of the cell. Some of these functions include: cell proliferation, differentiation, cellular transport and trafficking, regulation of gene expression, and cytoskeletal reorganization (Takai *et al.*,

2001). The small GTPases are closely related to the Ras p21 protein, and are thus grouped under the Ras superfamily of GTP-binding proteins (Wennerberg *et al.*, 2005). This superfamily consists of ~150 members, and is divided into five sub-families: Ras, Rho, Rab, Ran and Arf (Wennerberg *et al.*, 2005). Members of the Ras superfamily proteins are 30-55% homologous to Ras p21 with respect to their amino acid sequence. The Ras sub-family shares the highest homology (~55 %) with Ras p21 (Paduch *et al.*, 2001).

In their active state all small GTPases are bound to GTP (**Figure 1**). Upon hydrolysis of GTP to GDP, small GTPases are converted from the active state to their inactive state. This intrinsic GTPase activity is accelerated by GTPase activating proteins (GAPs) (Takai *et al.*, 2001). Guanine-nucleotide exchange factors (GEFs) activate small GTPases by enhancing the exchange of GDP for GTP. GEFs accomplish this by stabilizing the nucleotide free state of small GTPases (Cherfils and Chardin, 1999).

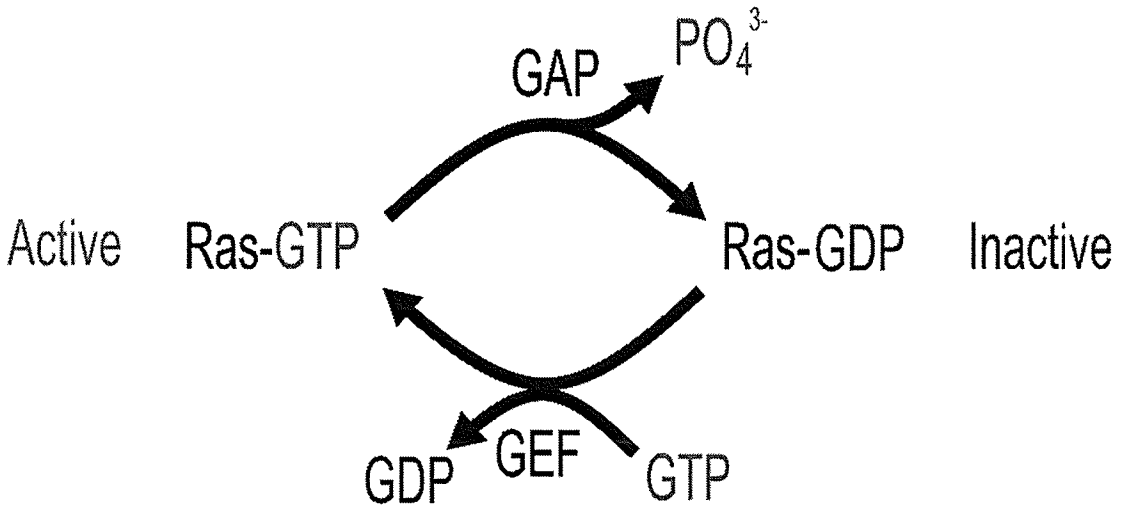


Figure 1. GTPase cycle. The Ras GTPase is active when bound to GTP. GTPase activating protein (GAP) stimulates the intrinsic GTPase activity of Ras, resulting in the hydrolysis of GTP to GDP, and loss of a phosphate group. GDP-bound Ras is inactive until GDP is exchanged for GTP with the help of guanine nucleotide exchange factor (GEF).

1.2 Ras sub-family

The Ras sub-family consists of Ras p21 (H-Ras, K-Ras, N-Ras), R-Ras, Ral, Rap, Rheb, Rin and Rit (Reuther and Der, 2000). The Ras p21 protein is often used as a prototype member to describe the structure of the proteins belonging to the Ras superfamily.

Ras p21 or Ras is encoded by 189 amino acids and has molecular weight of 21 kDa. The structure of Ras bound to GTP and GDP has been solved (**Figure 2A**). Structurally, it consists of five α helices and six β sheets that are connected by eleven loops (Pai *et al.*, 1990). The catalytic domain consists of amino acid residues 1 to 166 (**Figure 2A and 2B**) (Pai *et al.*, 1989). Within this domain there are several important regions. The regions encompassing amino acids 10 to 17 and 57 to 60 interact with the β- and γ- phosphates of GTP, respectively. The regions encompassing amino acid residues 116 to 119 and 146 to 148 make contact with the guanine base. The effector domain, encoded by amino acids 32 to 40, interacts with target proteins (Pai *et al.*, 1990).

Based on the structural studies, it has been suggested that upon binding of GTP to Ras there is a conformational change in the switch region I (amino acids 30 to 38), and the switch region II (amino acids 60 to 76) of the protein, exposing the effector domain, thus enabling the protein to interact with its downstream targets (eg. Raf) via the effector domain (**Figure 2B**) (Milburn *et al.*, 1990; Lucas *et al.*, 2002).

As oncogenes, Ras proteins have been shown to be responsible for approximately 30% of all human cancers, notably pancreatic and colorectal cancers (Downward, 1996; Kubuschek *et al.*, 2006; Benhattar *et al.*, 1993; Miranda *et al.*, 2006). How these cancers may arise can be explained by considering the following Ras mutants. Ras proteins

having mutations in amino acids 12, 13, 59, or 61, 116, and 119 have either lost their GTPase activity, are unable to interact with GAPs, or have an increased affinity for GTP (Trahey and McCormick, 1987). For example the loop that binds the β-phosphate of the guanine nucleotide is enlarged (Tong *et al.*, 1989). As a result these Ras mutants cannot hydrolyze GTP to GDP and are constantly in the active state.

A

Adopted and modified from: Ito *et al.*, 1997; Courtesy of the Protein Data Bank (www.pdb.org). ID number 1AA9.

B

Figure 2. Structure of Ras. A) GTP-bound Ras, B) Ribbon diagram of Ras showing the switch regions and the catalytic domain (1-166 amino acids).

The Ras sub-family GTPases are post-translationally modified through prenylation of their conserved C-terminal C*AA*X box (C stands for cysteine, *A* for aliphatic, and X for any amino acid) (Hancock *et al.*, 1989, 1990; Sidhu *et al.*, 2005a). This modification targets the Ras sub-family proteins to the plasma membrane (Hancock *et al.*, 1989, 1990). Following prenylation, the last three amino acids of the C*AA*X box are cleaved, and a methyl group is added at the terminal cysteine residue (Schillo *et al.*, 2004). The protein is farnesylated when the last amino acid is alanine, cysteine, glutamine, methionine, or serine, and geranylgeranylated when the last amino acid is leucine or phenylalanine (Schillo *et al.*, 2004). The last amino acid in Ral is leucine, and therefore Ral is modified through geranylgeranylation (Jilkina and Bhullar, 1996; Schillo *et al.*, 2004). On the other hand, Ras is farnesylated at its C-terminus. In addition to Ras farnesylation, Ras membrane localization requires either a polybasic region found in the hypervariable domain immediately upstream of the C*AA*X box, or palmitoylation at the hypervariable domain (Hancock *et al.*, 1990). The Ras proteins are divergent in their C-terminal plasma membrane targeting sequences, and this characteristic is one reason for the functional specificity of each member. Since all Ras proteins are polyisoprenylated, but only some are palmitoylated, it appears that isoprenylation is crucial for the membrane localization step (Hancock *et al.*, 1989). The importance of post-translational modifications is highlighted by the fact that mutation of the Ras C-terminal Cys186 prevents the membrane localization of Ras and blocks transformation (Hancock *et al.*, 1989).

Since RalGEF is a downstream target of Ras, as discussed later in Section 1.3, Ral proteins are also important in oncogenic transformation (Feig, 1996).

1.3 Ral

Ral, a member of the Ras family, consists of the members RalA and RalB. The two proteins are 85% identical, and most of the differences are clustered in the C-terminal region (Chardin and Tavitian, 1989). However, the C-terminal region of both RalA and RalB have common characteristics, one being their overall positive charge (**Figure 3**).

RalA 178 RKMEDSKEKNGKKKRKSLAKRIRERCCIL 206

RalB 179 KKMSENKDKNGKKSSKNKKSFKERCCLL 206

Figure 3. RalA and RalB C-terminal amino acid sequence. Positive amino acids are highlighted.

Ral proteins are associated mainly with the plasma membrane, but are also shown to be associated with transport, synaptic, and secretory vesicles (Mark *et al.*, 1996; Urano *et al.*, 1996). Just like other small GTPases, Ral is also regulated by the GTPase cycle (Feig, 2003). The existence of a Ral GTPase activating protein (GAP) has been reported but its identity has not been established (Emkey *et al.*, 1991; Bhullar and Seneviratne, 1996). Compared to Ras, Ral has an extra eleven amino acids at its N-terminal. However, the overall 3D structure of the two proteins is similar (Geyer and Wittinghofer, 1997). The function of the two proteins, Ras and Ral, is what sets them apart from one another, a feature of many proteins belonging to the same sub-family.

RalA is necessary for anchorage-independent proliferation of tumor cells, while RalB is needed by transformed cells for evasion from programmed cell death (Chien and

White, 2003). RalA, Ral-GDS, as well as Ras activate early embryonic gene expression and thus have been associated with myocardial hypertrophy (Kawai *et al.*, 2003).

Ral has been shown to interact with phospholipase D (PLD) and is therefore implicated in exocytosis (van Dam and Robinson, 2006). Ral-GTP is important in filopodia formation and cell secretion as it has been shown to bind to Sec5 of the exocyst complex (Sugihara *et al.*, 2001; van Dam and Robinson, 2006). Ral-binding protein 1 (RalBP1) interacts with Reps1 and Pob1, proteins that are linked with endocytosis (Bos, 1998). Since RalBP1 is a downstream Ral effector, Ral is also believed to play a role in endocytosis (Bos, 1998; van Dam and Robinson, 2006). As discussed in the remainder of this thesis, Ral has also been demonstrated to interact with CaM as well as PLC-δ1.

1.4 Calmodulin

The intracellular calcium concentration ranges from approximately 10 nM to 1000 nM (Ahlemeyer *et al.*, 1992). A change in calcium concentration affects function of other proteins directly and/or through the calcium sensory protein, calmodulin (CaM). A variety of proteins are regulated by calcium/CaM including: calcium/CaM-dependent kinases, small-GTPases, and the *Bordetella pertussis* and anthrax adenylyl cyclase toxins (Franklin *et al.*, 2006; Wang *et al.*, 1997; Guo *et al.*, 2005).

CaM is an ubiquitously expressed, acidic, highly conserved and calcium sensitive, cell signaling protein. It consists of 148 amino acid residues (Klee and Vanaman, 1982). In order to understand how calcium can regulate various proteins through CaM it is necessary to understand the structure of CaM (**Figure 4**).

CaM contains four calcium binding EF-hand motifs. Each EF-hand motif can bind one calcium ion (Ca^{2+}). It is important to note that CaM can exist in the calcium free state (apo-CaM), and the calcium bound state (Ca^{2+}-CaM) (**Figure 4A**) (Klee and Vanaman, 1982; Means *et al.*, 1982; Nakayama and Kretsinger, 1994). It has two globular domains which are linked by a solvent accessible α-helix. Each of the two globular domains (lobes) consists of three α-helices and two loops, giving the entire lobe a helix-loop-helix motif that repeats (Klee and Vanaman, 1982; Means *et al.*, 1982; Nakayama and Kretsinger, 1994).

In the absence of calcium, the central linking α-helix of CaM is partially unwound. Binding of calcium to CaM leads to reorganization of the secondary structure in CaM, and the CaM helices become reoriented. The central helix connecting the two domains becomes complete and flexible. This flexibility allows CaM to interact with a

variety of proteins, as CaM wraps around the target peptide (**Figure 4B**) (Klee and Vanaman, 1982; Means *et al.*, 1982; Nakayama and Kretsinger, 1994).

CaM binding sequences typically range from 16 to 30 amino acids in length, and have a net positive charge (O'Neil and DeGrado, 1990). They form amphipathic α-helices that have a basic and a hydrophobic face (Yamniuk, 2004), and can bind both Ca^{2+}-CaM and apo-CaM (Bahler and Rhoads, 2002).

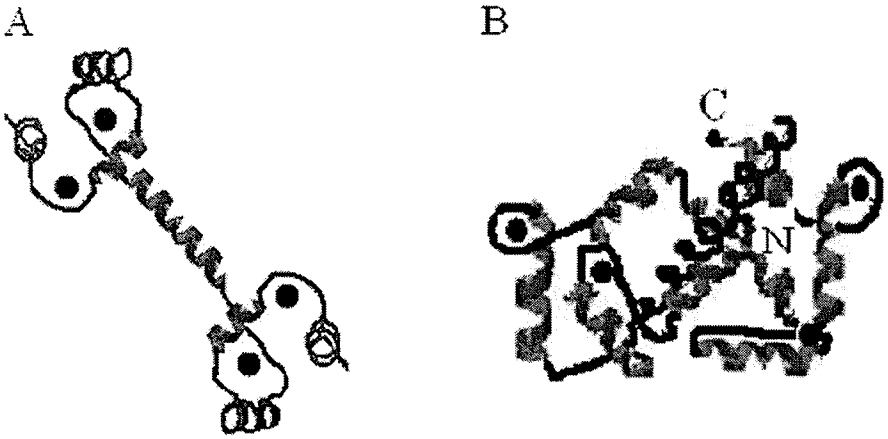


Figure 4. Structure of CaM. A) Calcium-bound CaM (calcium is represented by blue circles). B) Calcium-bound CaM interacting with a target peptide (shown in blue).

Adopted and modified from: A) chemistry.umeche.maine.edu/CHY431/Proteins7.html; B) Clapperton *et al.*, 2002.

1.5 Regulation of Ras and Ral by CaM

CaM has been shown to bind and regulate both Ras and Ral. CaM binds K-RasB in a calcium dependent and GTP-independent manner in platelets and MCF-7 cells. H-Ras and N-Ras, on the other hand, do not bind CaM (Villalonga *et al.*, 2001; Sidhu *et al.*, 2003). It has been shown that CaM inhibits protein kinase C (PKC) mediated activation of K-Ras in 3T3 fibroblast cells (Villalonga *et al.*, 2002). CaM also causes K-Ras dissociation from membranes in platelets and MCF-7 cells (Sidhu *et al.*, 2003).

Ras activates Ral by its effector protein RalGDS (Hofer *et al.*, 1994). However, Ral can also be activated in response to high intracellular calcium concentrations suggesting that Ral regulation can be independent of Ras (Feig, 2003). Also, Ral is activated by the calcium ionophore ionomycin, and activation by lysophosphatidic acid or epidermal growth factor can be blocked by a phosholipase C inhibitor (Hofer *et al.*, 1998). A basic/hydrophobic amino acid rich region that forms an amphipathic α-helix is found in the C-terminal region of RalA, and has been shown to bind CaM (Wang *et al.*, 1997). Calcium stimulates GTP binding to RalA while it reduces the binding of GDP to RalA (Park, 2001). The binding of GTP to RalA increases 3-fold in the presence of calcium/CaM (Wang and Roufogalis, 1999). In addition, calcium/CaM stimulate the GTPase activity of Ral (Park, 2001). It has been shown that CaM is required for the thrombin-induced activation of RalA and RalB in human platelets (Clough *et al.*, 2002). As mentioned above, differential functions of RalA and RalB could be due to the difference in the C-terminal region of these two GTPases. It has been shown *in vitro* that both RalA and RalB have a calcium dependent CaM binding site in their C-terminal

region, and a calcium independent binding site in their N-terminal region (Clough *et al.*, 2002). The C-terminus of RalB may act as an inhibitory region, preventing the interaction between RalB and CaM (Clough *et al.*, 2002). A similar scenario has been reported for H-Ras (Villalonga *et al.*, 2001). Using various Ral mutants it was shown that the C-terminal isoprenylated region of Ral was required for CaM interaction (Sidhu *et al.*, 2005a).

The above information clearly indicates the importance of Ras, Ral, and CaM in cell signaling. Ras, Ral, and CaM have also been shown to regulate phosphoinositide-specific phospholipase C (PLC).

1.6 PLC

Phosphoinositide-specific phospholipase C (PLC) is a cellular protein that is activated by G_q coupled receptors (eg. muscarinic) and hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid found in the cell membrane, into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Williams, 1999). DAG activates protein kinase C, while IP_3 causes release of calcium from the endoplasmic reticulum (Williams, 1999). There are six different isoforms of PLC (PLC-β1-4, PLC-γ1, -2, PLC-δ1, -3, -4, PLC-ε, PLC-ζ, and PLC-η1, -2) (Rebecchi and Pentyala, 2000; Rhee, 2001; Song *et al.*, 2001; Saunders *et al.*, 2002; Stewart *et al.*, 2005; Hwang *et al.*, 2005; Harden and Sondek, 2006). The following table summarizes each of the six isoforms (**Table 1**).

Table 1. PLC isoforms: Molecular weight, and tissue expression in mammals

	PLC-β	**PLC-γ**	**PLC-δ**	**PLC-ε**	**PLC-ζ**	**PLC-η**
MW (kDa)	150	150	85	250	70	115
Mammalian Tissue Expression	brain, hematopoeitic cells, liver, retina	ubiquitous	widely expressed (highest in skeletal muscle, spleen, testis, and lung)	brain, lung, kidney, testis, colon, heart	sperm	brain, lung

What makes PLC-δ interesting is the fact that PLC-δ is one of the most sensitive isoforms to calcium (Rebecchi and Pentyala, 2000). It is activated by an increase in

intracellular calcium following the activation of PLC-β, PLC-γ and PLC-ε, and is responsible for the amplification of initial calcium spike (Rebecchi and Pentyala, 2000).

1.7 Structural characteristics of PLCs

PLC-δ can be used as a model to describe common structural characteristics of all six PLCs. PLC-δ1 is composed of several conserved domains as described below (**Figure 5**).

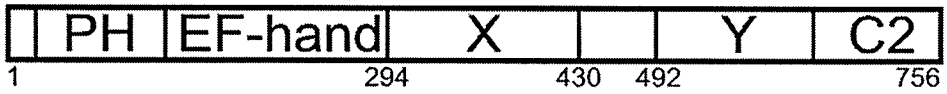


Figure 5. Structure of PLC-δ. PLC- δ1 consists of the PH, EF-hand, X/Y catalytic, and C2 domains.

1.8 Pleckstrin homology (PH) domain

The pleckstrin homology (PH) domain, composed of amino acid residues 29 – 130, was named after the protein pleckstrin (Tyers *et al.*, 1988; Haslam *et al.,* 1993). The PH domains by themselves do not have any catalytic activity (Ferguson *et al.*, 1995), and are not conserved between different PLCs (Rebecchi and Pentyala, 2000). However, they contribute to the high specificity and efficiency of the enzymatic reaction. The PH domains are found mainly within proteins that are associated with the plasma membrane, and thus through interaction with phosphoinositides the domain is required for membrane localization of the protein (Ferguson *et al.*, 1995; Rebecchi and Scarlata, 1998; Katan and Allen, 1999). The domain has a barrel like structure. Two anti-parallel β-strands form one side, while three other anti-parallel β-strands form the second half of the barrel. A C-terminal α -helix is found at the bottom of the barrel (Rebecchi and Scarlata, 1998).

1.9 EF-hand domain

The EF-hand domain, amino acid residues 133-279, is composed of four EF-hand motifs (**Figure 6A**) (Ellis *et al.*, 1993; Nakashima *et al.*, 1995; Essen *et al.*, 1996). Each EF-hand motif is composed of a helix-loop-helix arrangement (Essen *et al.*, 1996). On its own, the EF-hand domain has a weak affinity for calcium (Kobayashi *et al.*, 2005). However, in the presence of arachidonic acid and the PH domain, the calcium affinity increases (Kobayashi *et al.*, 2005). Structurally, the EF-hand domain of PLC-δ1 is similar to the EF-hand domain found in CaM (**Figure 6B**) (Essen *et al.*, 1996). The EF-hand domain is required for catalytic activity of PLC-δ1 (Ellis *et al.*, 1993). In addition, PLCs, such as PLC-ζ, are regulated through their calcium sensitive EF-hand domain (Kouchi *et al.*, 2005).

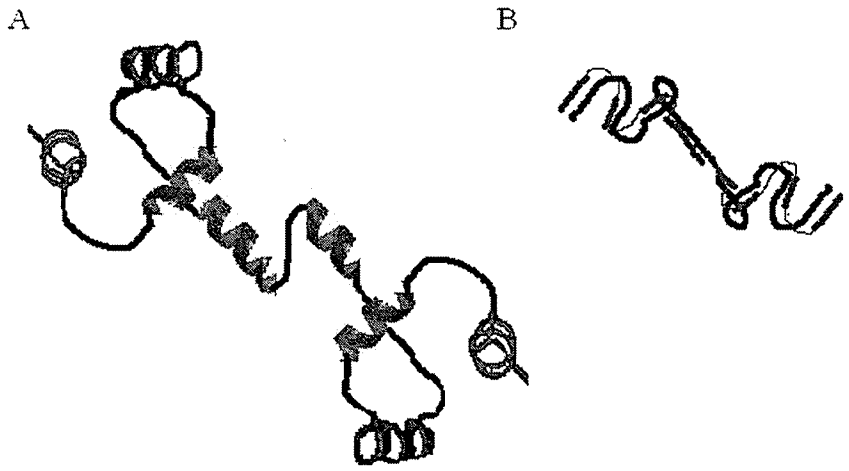


Figure 6. Structure of A) the EF-hand domain of PLC-δ1 and B) the EF-hand domains of PLC-δ1 (red) and CaM (black) superimposed. Adopted and modified from Essen *et al.*, 1996.

1.10 Catalytic domains

The X (296-430 amino acids) and Y (492-609 amino acids) domains form the catalytic domains that hydrolyze the O-P bond connecting phosphoinositol to DAG. The two domains are separated by a flexible linker region mostly encoded by charged amino acids (Williams, 1999; Essen, *et al.*, 1996). The catalytic domain resembles a triose phosphate isomerase (TIM) α/β- barrel, as it is composed of α-helices and β-strands (Essen *et al.*, 1996). The enzyme prefers $PI(4,5)P_2$ over phosphatidylinositol 4-phosphate [PI(4)P], and even more over PI (Rebecchi and Pentyala, 2000). However, the affinity of the PH domain for PIP2 is higher than that of the catalytic domain. The enzyme is unable to hydrolyze the 3-phosphorylated phosphoinositides (Rebecchi and Pentyala, 2000). A calcium ion binds to the active site, and is indispensable for the catalytic activity of all PLCs (Essen *et al.* 1997a).

1.11 C2 domain

The C2 domain is found at the C-terminus encompassing amino acid residues 647 to 756 (Essen *et al.*, 1996). It is composed of eight anti-parallel β-strands (Essen *et al.*, 1996; Singer *et al.*, 1997). In the absence of membranes, the domain has a weak affinity for up to three calcium ions: 1 Ca^{2+}(643–653 a.a.), 2 Ca^{2+}(675– 680 a.a.), and 3 Ca^{2+}(706 –714 a.a.) (Essen *et al.*, 1997a). The domain, in the presence of calcium, has a specific binding affinity for the membrane lipid, phosphatidylserine. It has been suggested that whereas the PH domain attaches the enzyme to the plasma membrane, the binding of the C2 domain to phosphatidylserine places the catalytic domain in the proper orientation with respect to the plasma membrane (Lomasney *et al.*, 1999).

1.12 PLC and Disease

It has been suggested that PLC isozymes are responsible for onset of Alzheimer's disease (Shimohama *et al.*, 1995a). Specifically, inactivation of PLC-δ is linked to the pathophysiology of Alzheimer's disease (Shimohama *et al.*, 1995b). PLC-δ1 has also been shown to be required for skin homeostasis and hair growth (Nakamura *et al.*, 2003) and is implicated in cardiac hypertrophy. It has been shown that PLC-β, PLC-γ1 and PLC-δ1 gene expression is increased in hypertrophied hearts (Dent *et al.*, 2006). It is interesting that Ras, Ral, and PLC are all associated with cardiac hypertrophy. However, this common physiological function is not surprising since, as described in the next section, Ras and Ral, as well as CaM regulate various PLCs.

1.13 Regulation of PLCs by small GTP-binding proteins and CaM

Small GTP-binding proteins belonging to the Ras superfamily (Ral, Ras, Rho), have been shown to bind and regulate various isoforms of PLC (Song *et al*, 2001; Wing *et al.*, 2003; Sidhu *et al.*, 2005; Harden and Sondek, 2006). PLC-β isoforms, through a long C-terminal extension, interact with heterotrimeric G-proteins (Ellis *et al.*, 1993; Park *et al.*, 1993). The N-terminus of PLC-β binds CaM (McCullar *et al.*, 2003). PLC-ε has separate Ras and Rho binding domains (Song *et al.*, 2001; Wing *et al.*, 2003). Recently, it has been shown that RalA binds PLC-δ1 at the C2 domain, and causes PLC-δ1 activation (**Figure 7**) (Sidhu *et al.*, 2005). Previously, it has been shown that CaM-binding peptides and CaM inhibit PLC activity (Richard *et al.*, 1997). W-7 is a potent small molecule inhibitor of CaM, and has been shown to inactivate PLC-δ1 *in vitro*

(Osawa *et al.*, 1998; Sidhu *et al.*, 2005). IQ motifs have an amphipathic character and commonly bind CaM in a calcium independent fashion (Bahler and Rhoads, 2002). An IQ type domain in the region linking the X- and Y- domains of PLC-δ1 has been identified (Sidhu *et al.*, 2005). In addition it was demonstrated that the IQ-peptide binds CaM and that the IQ-peptide inactivates PLC-δ1 *in vitro* (**Figure 7**) (Sidhu *et al.*, 2005).

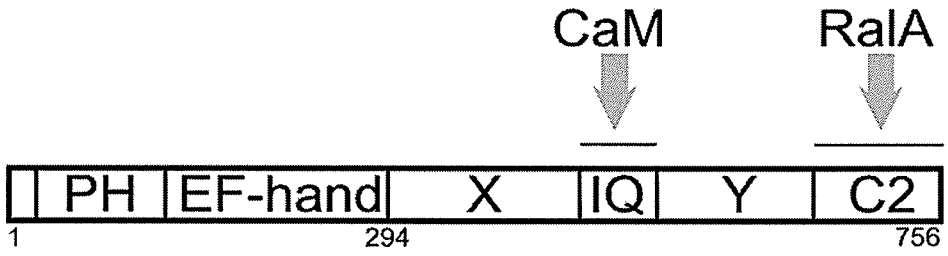


Figure 7. Representation of PLC-δ1 RalA and CaM binding sites.

In our laboratory, we have been able to bind RalA, but not H-Ras from HeLa cell lysates using GST-PLC-δ1 CT, a construct containing only the C2 domain of PLC-δ1. However constitutively active H-RasV12 caused activation of PLC-δ1 in a fashion similar to RalA or constitutively active RalBV23 (Sidhu *et al.*, 2005). The activation of PLC-δ1 by RasV12 was attributed to the fact that activation of Ras can lead to activation of the Ral pathway by the Ras effector molecule RalGDS (Hofer *et al.*, 1994; Sidhu *et al.*, 2005). It has been shown, however, that the binding of RalA to PLC-δ1 was independent of the guanine nucleotide status of RalA (Sidhu *et al.*, 2005), suggesting that activation of RalA is not a prerequisite for interaction between RalA and PLC-δ1. Figure 8 represents our current model for Ral mediated activation of PLC-δ1.

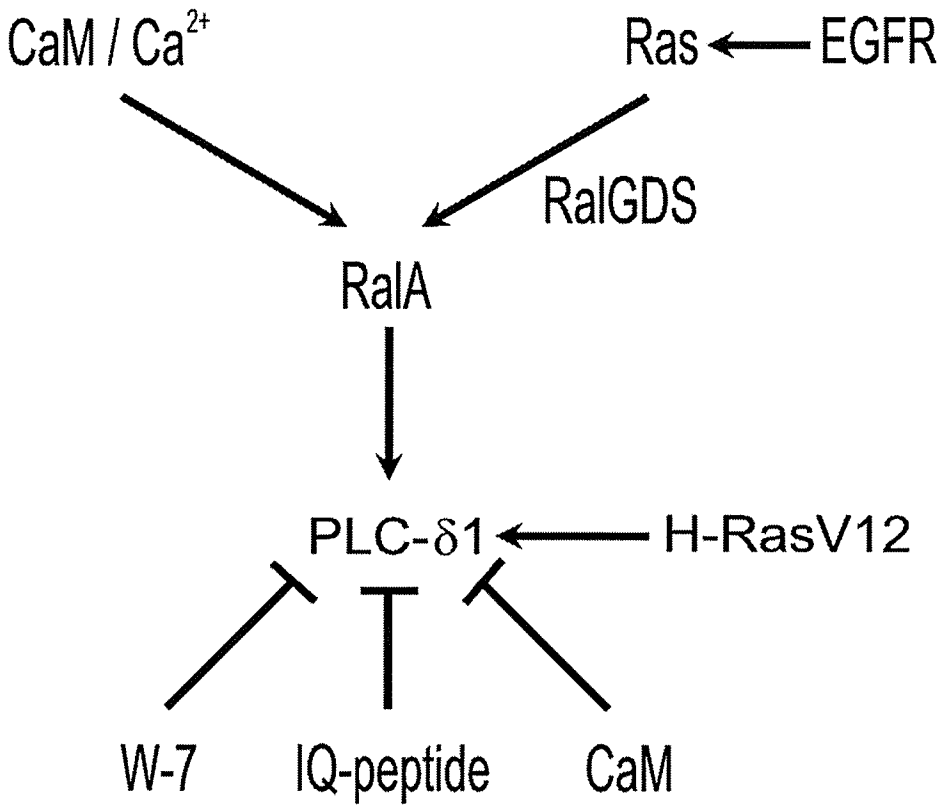


Figure 8. Summary of Ral and PLC-δ1 activation. Ral is activated by Ras-GTP through RalGDS, or through CaM/Ca^{2+} independently of Ras. PLC-δ1 binds and is activated by RalA. H-RasV12 activates PLC-δ1. PLC-δ1 binds to CaM through the IQ-peptide, and is inhibited by CaM. The IQ-peptide and W-7, which potentially bind to the EF-hand region, also inhibit PLC-δ1 activity. Ras is activated upon the binding of mitogens to EGFR.

2. Hypothesis

In the present report we have investigated the existence of a second RalA binding site in PLC-δ1. We propose that since the EF-hand domains of CaM and PLC-δ1 are structurally similar, and since RalA is able to bind to CaM, that in turn RalA will also be able to bind the EF-hand domain of PLC-δ1.

PLC-δ1 is activated upon by overexpression of the constitutive active form of Ras (H-RasV12) in HeLa cells. Therefore, we also hypothesize that H-Ras and PLC-δ1 are capable of direct interaction.

3. Materials and Methods

3.1 Materials

Rat PLC-δ1 in pGEX-2T (pGEX-2T-PLC-δ1) was a generous gift of Dr. M. Katan (The Institute of Cancer Research, London, UK). Restriction enzymes, DNA Polymerase I Large Fragment (Klenow), and the T4 Quick Ligation Kit were obtained from NEB, while Wizard® DNA Clean-Up System was from Promega. RalA (NH_2-SKEKNGKKKRKSLAKRIR-COOH) and RalB (NH_2-KSSKNKKSFKERC-COOH) C-terminal peptides (Jilkina and Bhullar, 1996), and the IQ (NH_2-VRSQVQHKPKEDKLKLVPELS-COOH) peptide (Sidhu *et al.*, 2005) were commercially synthesized. Bovine brain calmodulin, W-7 covalently coupled to polyacrylamide beads, W-7•HCl and W-5•HCl were purchased from Calbiochem. DH5α *E. coli* chemically competent cells, isopropyl 1-thio-β-D-galactopyranoside (IPTG), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and Lipofectamine 2000 were supplied by Invitrogen. Glutathione (GSH)-agarose beads, lysozyme and thrombin were from Sigma. Culture plates were purchased from Corning. CNBr-activated Sepharose™ 4 Fast Flow beads, PVDF membrane, and the ECL plus Western Blotting Detection System were from Amersham Biosciences. Mouse anti-human-RalA, mouse anti-human-PLC-δ1, and mouse anti-H-Ras were purchased from BD Biosciences. Mouse-anti-human-calmodulin was purchased from Upstate Biotechnology, and goat anti-mouse IgG horseradish peroxidase conjugate was obtained from BioRad. Protein A/G Plus-Agarose beads were from Santa Cruz Biotechnology. Mouse IgG was from NeoMarkers. The Ponceau S

solution used to stain proteins was from Boehringer. The Calcium calibration buffer was from Molecular Probes. All other chemicals were reagent grade.

3.2 Plasmid constructs

pGEX-2T-PLC-δ1 was digested with the restriction enzymes *Acc*I and *Sma*I. The resulting DNA fragments were separated on a 1% agarose gel. Two bands were visualized (~5781 bp and ~1386 bp). The larger band encoding for pGEX-2T and PLC-δ1 consisting of amino acids 1 to 294 was cut out of the gel. This DNA fragment was isolated using steel wool, and cleaned using Wizard® DNA Clean-Up System. The purified DNA fragment was Klenow-blunted, ligated, and transformed into DH5α *E. coli* chemically competent cells. The transformed cells were plated on LB plates supplemented with 100 μg/mL ampicillin, and incubated overnight at 37°C. The resulting truncated GST-PLC-δ1 construct was named GST-PLC-$\delta1_{1\text{-}294}$.

3.3 Protein Expression, Isolation and Purification

Ral, H-Ras, Raf and PLC-δ1 GST fusion proteins were expressed in DH5α *E. coli* cells in the presence of 0.5 mM IPTG as previously described (Jilkina and Bhullar, 1996). Briefly, one large isolated colony was picked from an LB plate, inoculated into 5 mL LB media supplemented with 50 μg/mL ampicillin, and incubated overnight at 37°C with shaking. The following day, the culture was transferred into a large flask containing 500 mL LB media, 50 μg/mL ampicillin, and incubated overnight at room temperature with shaking. The protein expression was stimulated with IPTG (0.5 mM final concentration) for 2-4 hours at room temperature. The cell culture was centrifuged at 6,000 x g for 20

minutes at 4°C. Cell pellets were resuspended in 40 mL NETT buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100), followed by addition of 40 mg of lysozyme and 80 μM PMSF. After incubation for 10 minutes at room temperature the suspension was sonicated. The lysate was centrifuged at 27,000 x g at 4°C, and the supernatant containing the GST-fusion protein of interest was frozen at –20°C in the presence of 20% glycerol (v/v). To isolate the recombinant protein, the supernatant was incubated with NT buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl) washed glutathione (GSH)-agarose beads for 30 minutes at 4°C with rocking. The GSH-agarose bound GST-fusion proteins were washed 3 times in NETT buffer followed by 3 times in NT buffer. A small aliquot was used to test for protein expression and purity using 12% SDS-PAGE and Coomassie staining, while the rest was used in binding experiments.

3.4 Cleavage of GST-fusion proteins by thrombin

To remove the GST tag, purified GST-fusion proteins were resuspended in phosphate buffered saline (PBS) (20 mM NaH_2PO_4, pH 7.4, 300 mM NaCl) in the presence of 0.5 units of thrombin and incubated at room temperature overnight. The protein mixture was centrifuged at 325 x g for 10 minutes. The supernatant containing the recombinant (minus GST) protein was aliquoted and stored at –20°C.

3.5 Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (v/v), 1.5 g/L $NaHCO_3$, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C and 5% CO_2 on 6 well or 100 mm plates.

3.6 Cell lysis

HeLa cells were lysed in ice cold lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol) containing protease inhibitors (Sigma).

3.7 Peptide coupling to CNBr-activated Sepharose beads

RalB C-terminal peptide (2.5 mg/ml) was coupled to CNBr-activated Sepharose beads as per the manufacturer's protocol.

3.8 W-7 polyacrylamide beads

W-7 covalently coupled to polyacrylamide beads or control polyacrylamide beads were hydrated as per the manufacturer's instructions.

3.9 Binding assays - endogenous protein

Cell lysates were centrifuged at 13,000 x g at 4°C for 10 minutes. The supernatant containing soluble proteins was kept for further experiments. Approximately 500 μg of HeLa cell lysate protein was incubated with 50 μg of an appropriate GST-fusion protein coupled to GSH-agarose beads or the RalB C-terminal peptide coupled to Sepharose beads, in the presence or absence of EGTA plus varying concentrations of $CaCl_2$ for 2 hours at 4°C with gentle rocking. The binding reaction mixture was centrifuged at 1,300 x g for 1 minute, washed 3 times in the lysis buffer containing appropriate concentration of EGTA and $CaCl_2$, separated by 12% SDS-PAGE and analyzed by Western blotting using appropriate antibodies.

3.10 Binding assays-pure protein

An aliquot of purified fusion protein (cleaved from GST) or pure calmodulin was incubated with the appropriate GST-fusion protein, the RalB C-terminal peptide coupled to Sepharose beads, W-7 covalently coupled to polyacrylamide beads, or appropriate control beads in the presence or absence of EGTA plus $CaCl_2$ for 2 hours at 4°C with gentle rocking. The binding reaction mixture was centrifuged at 1,300 x g for 1 minute, washed 3 times in the lysis buffer containing the appropriate concentration of EGTA plus $CaCl_2$, separated by 12% SDS-PAGE and analyzed by Western blotting using appropriate antibodies.

3.11 Competition assays

RalA, RalB or IQ peptides, W-7•HCl or W-5•HCl were incubated with 50 μg of GST-PLC-$\delta 1_{1\text{-}294}$ in the presence of 5 mM EGTA plus 1 mM $CaCl_2$ for 15 minutes at 4°C with gentle rocking, after which thrombin cleaved RalA was added and the mixture incubated for an additional 2 hours at 4°C with gentle rocking. The binding reaction mixture was centrifuged at 1,300 x g for 1 minute, washed 3 times in the lysis buffer containing appropriate concentrations of EGTA plus $CaCl_2$, separated by 12% SDS-PAGE and analyzed by Western blotting using appropriate antibodies.

3.12 H-Ras and RalA Competition assays

HeLa cell lysate alone (500 μg protein), or HeLa cell lysate (500 μg protein) plus RalA (500 ng), cleaved from GST-RalA by thrombin, were incubated in the presence of

cell lysis buffer alone, or cell lysis buffer plus 5 mM EGTA / 1 mM $CaCl_2$ or 5 mM EGTA / 5 mM $CaCl_2$. Additionally, RalA (250 ng, 500 ng or 1500 ng) and H-Ras (500 ng), cleaved from GST-RalA and GST-H-Ras using thrombin, were incubated in 68 nM calcium calibration buffer in the presence of GSH-agarose beads bound to GST-PLC-δ1 CT for 2 hours at 4°C. Bound proteins were separated using 12% SDS-PAGE and Western blotting was performed with anti-H-Ras and anti-RalA antibodies.

3.13 Guanine nucleotide loading of GST-fusion proteins

GSH agarose beads bound GST proteins were resuspended in 1 volume of exchange buffer (25 mM MES-NaOH, pH 7.5, 50 mM NaCl, 2.5 mM EDTA, 0.05% Triton X-100 and 0.5 mM GTP or GDP), incubated at room temperature with occasional shaking for 5 minutes, and washed once with 1 mL of ice cold buffer (10 mM sodium phosphate, pH 6.8, 20 mM $MgCl_2$, and 0.5 mM GTP or GDP) to lock in nucleotides. Proteins were either cleaved from the beads with thrombin, or used directly in subsequent binding assays.

3.14 Transfection of Ras mutants

HeLa cells were cultured in 6 well plates in 2 mL of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (v/v), 1.5 g/L $NaHCO_3$ until 70% confluent. The cells were then transfected with 4 μg of DNA corresponding to various Ras mutants (wt, RasV12 or RasS17 obtained from cDNA) using Lipofectamine 2000 as per the manufacturer's protocol. After 24 hours of incubation at 37°C and 5% CO_2 the cells were lysed in the lysis buffer.

3.15 Co-immunoprecipitation

HeLa cells, one 35 mm^2 plate per sample, were washed in PBS, lysed in 0.5 mL of the lysis buffer (Section 3.5) containing protease inhibitors and centrifuged at 13,000 x g for 10 minutes at 4°C. The supernatant was precleared with 30 µl Protein A/G Plus-Agarose beads for 1 hour, after which the solution was centrifuged at 13,000 x g for 25 sec at 4°C. Anti-H-Ras antibody (4 µg) (BD Transduction) or mouse IgG (NeoMarkers) was added to the supernatant and incubated for 1.5 hours at 4°C. 20 µl of Protein A/G Plus-Agarose beads were added to the reaction mixture and incubated for an additional 1.5 hours with shaking at 4°C. The reaction mixture was centrifuged at 13,000 x g for 25 seconds at 4°C, and the beads were washed 3 times with the lysis buffer. Bound proteins were separated by 12% SDS-PAGE, and Western blot analysis was performed using anti-PLC-δ1 antibody.

3.16 SDS-PAGE and Western blot analysis

The beads were resuspended in 30 µl of Laemmli's sample buffer (Laemmli, 1970), heated at 100°C for 3 minutes, and the resulting supernatant was separated by 12% SDS-PAGE. The proteins were transferred onto a PVDF membrane overnight at 4°C. The blot was blocked in 5% skim milk powder in 0.4% NT-Tween 20, probed using appropriate antibodies at the following dilutions: RalA (1:5000), PLC-δ1 (1:250), H-Ras (1:500) and CaM (0.2 µg/mL). The blot was visualized by enhanced chemiluminescence.

3.17 Quantification of Western blots

Experiments were repeated three times and the resulting Western blots were scanned and bands were quantified using Quantity One software obtained from Bio-Rad (Sidhu *et al.*, 2005). Calculations were performed by taking the ratio of the detected binding to protein expression level of each H-Ras mutant or H-Ras wild type, in order to correct for any differential protein expression.

3.18 Calculation of free calcium concentrations

Free calcium concentrations in solutions were determined by using the Maxchelator software obtained from www.stanford.edu/~cpatton/maxc.html (Sidhu *et al.*, 2005).

3.19 Protein Determination

The amount of protein in samples was determined using Bio-Rad protein dye reagent.

4. Results

4.1 Expression of GST-PLC-δ1$_{1-294}$

In order to determine whether there is a RalA binding site in the N-terminal domain of PLC-δ1, it was necessary to express a GST-PLC-δ1 construct that encoded for the N-terminal of PLC-δ1. We chose to express a PLC-δ1 form that consisted of amino acids 1 to 294, and therefore, consisting of the PH and EF-hand domains. The truncation of PLC-δ1 into GST-PLC-δ1$_{1-294}$ was successful (**Figure 9**). The expressed protein had a molecular weight of approximately 70 kDa as expected. The expression level of the fusion protein was high, and there was no significant proteolytic degradation observed.

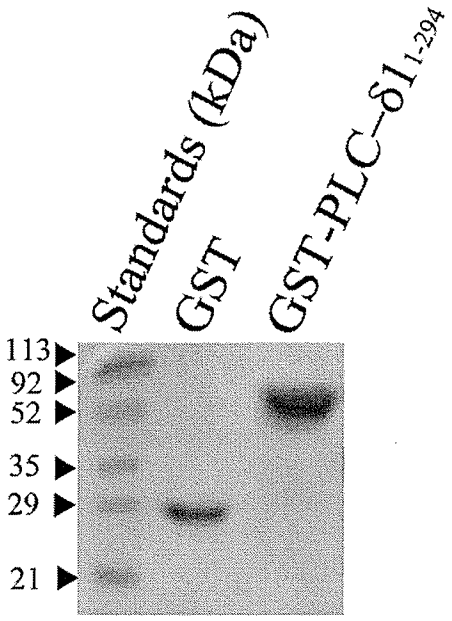


Figure 9. Expression of the GST-PLC-δ1$_{1-294}$ construct. pGEX-2T plasmid encoding for GST-PLC-δ1$_{1-294}$ was transfected into *E. coli* DH5α cells, and the protein was expressed, isolated and purified as described in the Materials and Methods section. Proteins were separated using 12% SDS-PAGE and stained with Coomassie blue stain.

4.2 RalA and PLC-δ1, but not CaM, bind to PLC-δ1$_{1-294}$

We hypothesized that since RalA is capable of binding CaM, then RalA could be capable of binding to the CaM-like domain in the N-terminal region of PLC-δ1. In order to test this hypothesis we created a GST-PLC-δ1 construct (GST-PLC-δ1$_{1-294}$) consisting of the first 294 amino acids, thus encompassing the PH domain and the entire EF-hand domain. We used this construct in "pull down" experiments using HeLa cell lysates (**Figure 10**). The binding of RalA to the agarose beads containing GST-PLC-δ1$_{1-294}$ increased with increasing calcium concentration, signaling that the binding was calcium dependent. In addition to RalA, PLC-δ1 was also detected and this binding was also calcium dependent (**Figure 10**). Since Ral, CaM and PLC-δ1 can exist in a complex (Sidhu *et al.*, 2005), we investigated if CaM was also present in the pull down sample. However, we did not detect any CaM (**Figure 10**). This suggests that a CaM binding region is not present in PLC-δ1$_{1-294}$.

To assess if RalA and PLC-δ1 can interact directly with PLC-δ1$_{1-294}$, pure recombinant RalA and PLC-δ1 were incubated with GST-PLC-δ1$_{1-294}$. GST-PLC-δ1$_{1-294}$ pull down of cleaved RalA and PLC-δ1 showed the interactions were direct, as well as calcium dependent (**Figures 11A and 11B**). GST-PLC-δ1$_{1-294}$ did not cross-react with the antibodies used to detect RalA and PLC-δ1 (**Figures 10 and 11A**). To examine if the observed interactions were due to the presence of the GST tag, we repeated the pull down experiments with GST alone. GST did not bind RalA (**Figure 11A**) or PLC-δ1 (**Figure 11B**).

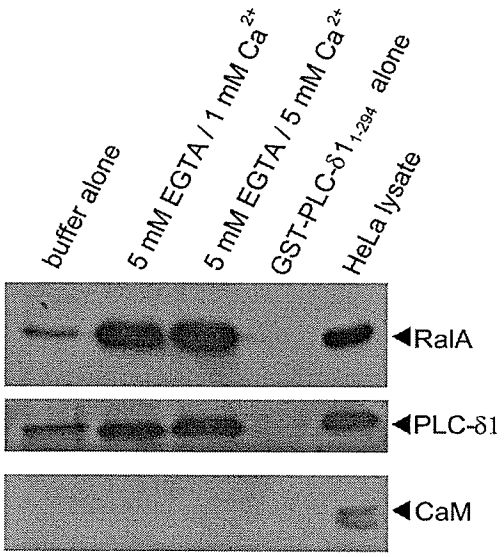
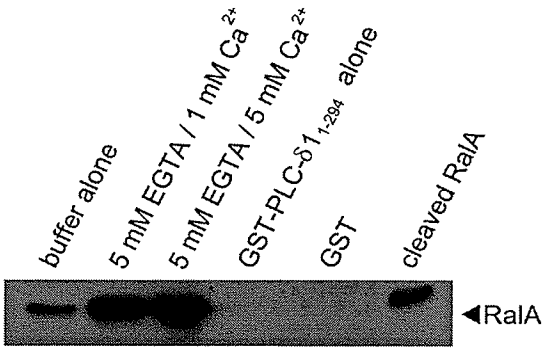


Figure 10. GST-PLC-δ1$_{1-294}$ binds RalA and PLC-δ1, but not calmodulin from HeLa cell lysates in a calcium dependent manner. HeLa cell lysate was incubated with GSH-agarose beads bound to GST-PLC-δ1$_{1-294}$ for 2 hours at 4°C in the presence of buffer alone or exogenous Ca^{2+} plus EGTA, separated by 12% SDS-PAGE and analyzed by Western blot using anti-RalA, anti-PLC-δ1, and anti-CaM antibodies. Free calcium concentrations: buffer alone contains no additional Ca^{2+} and EGTA in the binding reaction; 5 mM EGTA / 1 mM Ca^{2+} (12.3 nM free Ca^{2+}); and 5 mM EGTA / 5 mM Ca^{2+} (17.0 μM free Ca^{2+}).

A



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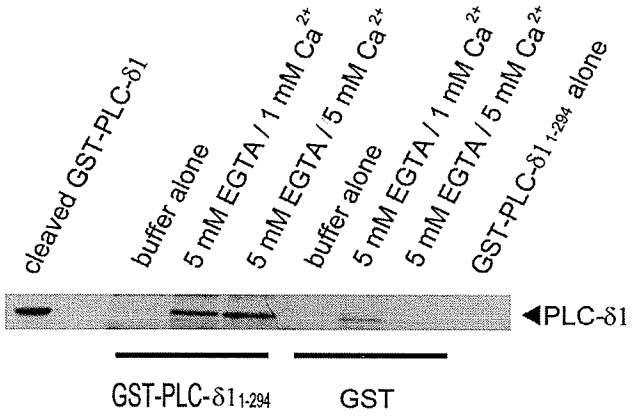


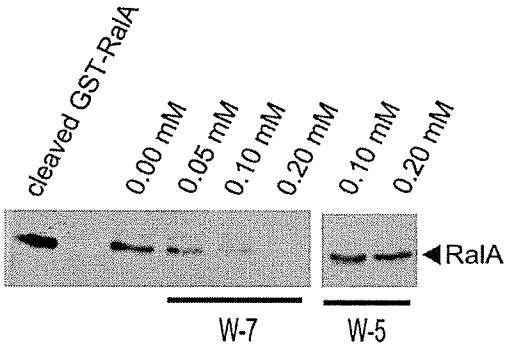
Figure 11. Recombinant RalA and full length PLC-δ1 bind GST-PLC-δ1_{1-294} in a calcium dependent manner. A) Recombinant RalA, cleaved from GST-RalA with thrombin, was incubated for 2 hours at 4°C with GSH-agarose beads bound GST-PLC-δ1_{1-294} in the presence of buffer alone or the indicated concentrations of EGTA plus Ca^{2+}, or GST in the presence of 5 mM EGTA / 5 mM Ca^{2+}, separated by 12% SDS-PAGE and analyzed by Western blot using the anti-RalA antibody. Any products in the GST-PLC-δ1_{1-294} did not cross-react with the anti-RalA antibody. B) Recombinant PLC-δ1, cleaved from GST-PLC-δ1 with thrombin, was incubated with GSH-agarose bound GST-PLC-δ1_{1-294} or GST in the presence of buffer alone or the indicated concentrations of EGTA plus Ca^{2+} for 2 hours at 4°C, separated by 12% SDS-PAGE and analyzed by Western blot using anti-PLC-δ1 antibody raised against the C-terminal of PLC-δ1. GST-PLC-δ1_{1-294} did not cross-react with anti-PLC-δ1 antibody.

4.3 W-7 inhibits RalA binding to PLC-δ1$_{1-294}$, and binds to PLC-δ1

W-7 is a potent inhibitor of CaM and can occupy the site within the CaM EF-hand domains and thus prevent CaM from interacting with its target proteins (Osawa *et al.*, 1998). Since the EF-hand domains of CaM and PLC-δ1 are structurally similar, we next investigated if W-7 can inhibit interaction between RalA and GST-PLC-δ1$_{1-294}$. *In vitro* competition assays showed that in the presence of W-7, at a chosen calcium concentration of 12.3 nM, the binding of RalA to GST-PLC-δ1$_{1-294}$ was inhibited (**Figure 12A**). On the other hand, W-5, a less potent inhibitor of CaM, had no effect on RalA binding to GST-PLC-δ1$_{1-294}$, when used at a concentration similar to W-7 (**Figure 12A**). These results suggest that W-7 can specifically bind to PLC-δ1$_{1-294}$, and that the noted inhibition was not simply due to the presence of a small molecule.

If W-7 could indeed bind to the CaM-like domain of PLC-δ1, then it should be possible to pull down PLC-δ1 out of solution using W-7. We showed that this was indeed possible using W-7 covalently coupled to polyacrylamide beads (**Figure 12B**). No interaction was observed when control polyacrylamide beads alone were incubated with PLC-δ1 (**Figure 12B**). This suggests that W-7 can possibly interact with the CaM-like region in PLC-δ1$_{1-294}$.

A



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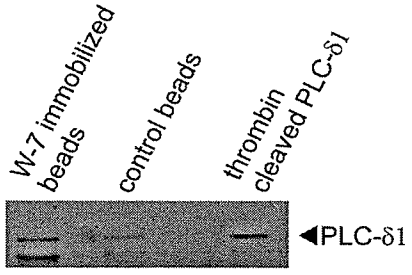


Figure 12. W-7 inhibits RalA binding to GST-PLC-δ1$_{1-294}$, and binds to full length PLC-δ1. A) GST-PLC-δ1$_{1-294}$ was incubated with the indicated concentrations of W-7•HCl or W-5•HCl for 15 minutes in the presence of 5 mM EGTA / 1 mM Ca^{2+} prior to addition of equal amounts of recombinant RalA that was obtained by cleaving GST-RalA with thrombin. The mixture was incubated for 2 hours at 4°C, separated by 12% SDS-PAGE and analyzed by Western blot using anti-RalA antibody. B) PLC-δ1, obtained by cleaving GST-PLC-δ1 with thrombin, was incubated with W-7 covalently coupled to polyacrylamide beads or control beads in lysis buffer for 2 hours at 4°C with shaking, separated by 12% SDS-PAGE, and analyzed by Western blot using anti-PLC-δ1 antibody.

4.4 The IQ peptide inhibits RalA binding to PLC-δ1$_{1-294}$

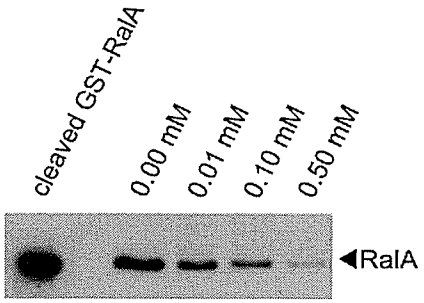
PLC-δ1 contains an IQ-type motif (473-493 amino acid residues) capable of binding CaM, but not RalA, and a peptide from this motif inhibits PLC-δ1 activity *in vitro* (Sidhu *et al.*, 2005). Based on this information we wanted to test if the IQ peptide could also bind PLC-δ1$_{1-294}$. We conducted a competition assay by incubating the IQ peptide and RalA with GST- PLC-δ1$_{1-294}$. The GST-PLC-δ1$_{1-294}$ bound RalA was detected by Western blotting. The IQ peptide partially inhibited RalA binding to PLC-δ1$_{1-294}$ at a concentration of 10 μM (**Figure 13A**). Full inhibition of binding was observed at 500 μM IQ peptide. These results suggest that there was direct interaction between PLC-δ1$_{1-294}$ and the IQ peptide of PLC-δ1.

4.5 RalA and RalB C-terminal peptides inhibit RalA binding to PLC-δ1$_{1-294}$

As mentioned, RalA and RalB have an N-terminal independent and a C-terminal calcium dependent CaM binding site (Clough *et al.*, 2002). The C-terminal calcium-dependent binding site of RalA has been identified (Wang *et al.*, 1997). To establish if the RalA C-terminal CaM binding peptide can inhibit Ral binding to PLC-δ1$_{1-294}$ we did an *in vitro* competition assay. The results demonstrated that the RalA CaM binding peptide (189 - 206 amino acid residues) strongly inhibited binding of full length RalA to GST-PLC-δ1$_{1-294}$ at concentrations as low as 10 μM (**Figure 13B**). Since the IQ and RalA peptides have different effective concentrations, the observed inhibition was specific and was not due to the presence of the peptides alone. Since RalB also has a potential C-terminal CaM binding region we repeated the competition assay using a RalB C-terminal peptide. The RalB peptide completely inhibited the binding at a concentration

of 1 mM (**Figure 13C**). This finding suggests that RalB potentially also binds to the N-terminal of PLC-δ1.

A



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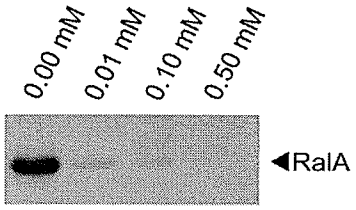

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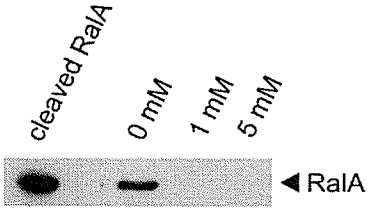
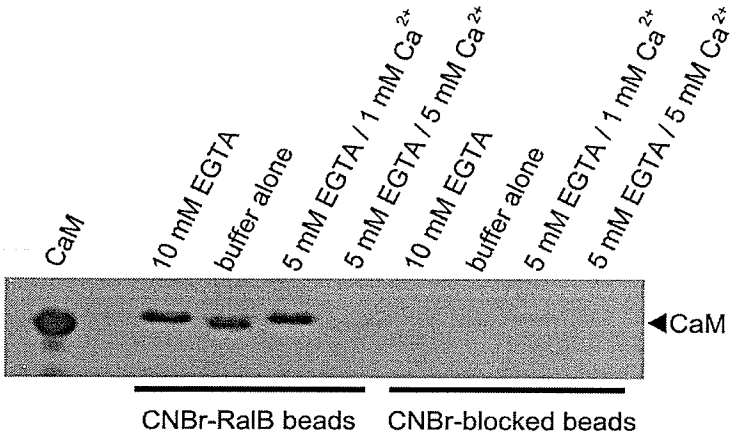


Figure 13. The IQ peptide from the X-Y linker region of PLC-δ1, and Ral C-terminal peptides inhibit binding of RalA to GST-PLC-δ1_{1-294}. GST-PLC-δ1_{1-294} was incubated with the indicated concentrations of A) IQ peptide, B) C-terminal RalA peptide, C) C-terminal RalB peptide for 15 minutes in the presence of 5 mM EGTA / 1 mM Ca^{2+} prior to addition of equal amounts of thrombin cleaved GST-RalA. The mixture was incubated for 2 hours at 4°C, separated by 12% SDS-PAGE and analyzed by Western blot using anti-RalA antibody.

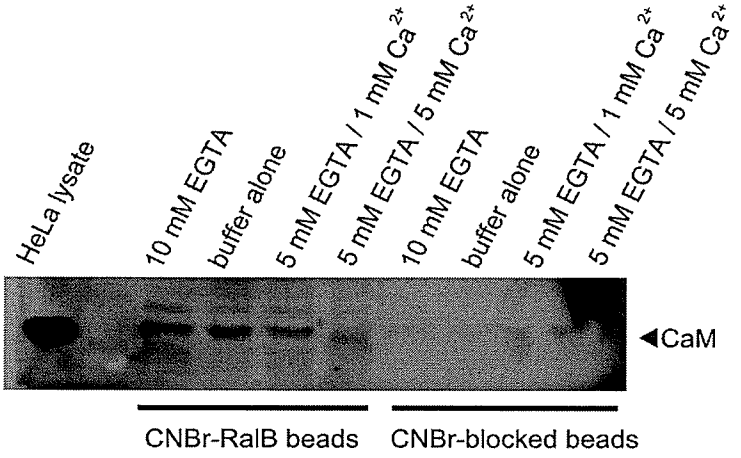
4.6 The RalB C-terminal peptide binds CaM

In order to understand the mechanism by which the RalB C-terminal peptide inhibited RalA binding to PLC-$\delta 1_{1\text{-}294}$ we decided to test if this peptide can bind to CaM. Here we show that a peptide consisting of the 13 C-terminal amino acids of RalB (194 - 206 amino acid residues) coupled to CNBr-beads was sufficient to bind CaM. The binding of the CNBr-RalB peptide to pure CaM appeared to be constant in the presence of EGTA and at free calcium concentrations of 0.22 nM and 12.3 nM (**Figure 14A**). At very high calcium concentrations (17.0 μM), the binding was not observed (**Figure 14A**). The same trend was observed for binding of CaM from HeLa cell lysates (**Figure 14B**). In addition to CaM, PLC-δ1 was also pulled down from HeLa cell lysate by this peptide, potentially through interaction with the CaM-like region of PLC-δ1, in a calcium dependent manner similar to that for CaM (**Figure 14C**). Blocked CNBr-Sepharose beads did not bind CaM or PLC-δ1 (**Figures 14A, 14B, and 14C**).

A



B



C

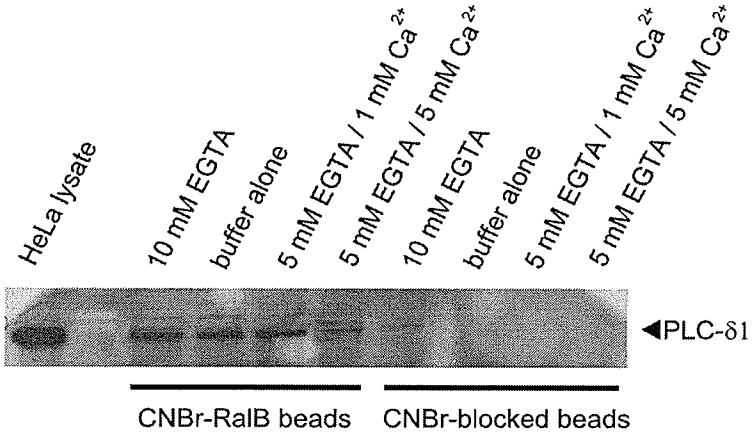


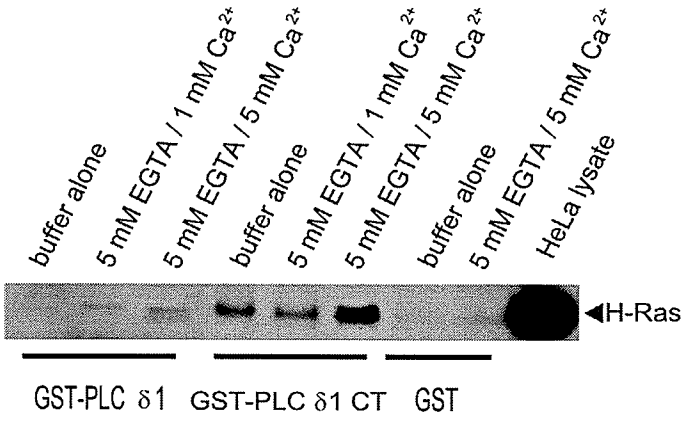
Figure 14. RalB C-terminal peptide binds pure calmodulin, as well as calmodulin and PLC-δ1 from HeLa cell lysate. A) Pure CaM, and B) and C) HeLa cell lysate was incubated in the presence of the C-terminal RalB peptide coupled to CNBr beads or CNBr blocked beads in the presence of the indicated concentrations of EGTA / Ca^{2+} for 2 hours at 4°C. Bound proteins were resolved by 12% SDS-PAGE and analyzed by Western blot using A) and B) anti-CaM antibody or C) anti-PLC-δ1 antibody.

4.7 Endogenous H-Ras binds to full length GST-PLC-δ1, GST-PLC-δ1 CT, but not GST

We have shown that constitutively active H-Ras(V12), when overexpressed in HeLa cells, activates PLC-δ1 *in vitro* (Sidhu *et al.*, 2005). Whether H-Ras(V12) activates PLC-δ1 by binding to it, or by some other mechanism has not been determined. In order to determine if H-Ras is capable of binding to PLC-δ1, HeLa cell lysate was incubated in the presence of GST-PLC-δ1 (**Figure 15A**). We detected a low degree of H-Ras binding by western blotting. The highest binding occurred in the presence of 5 mM EGTA / 5mM calcium, the highest calcium concentration used. The amount of bound H-Ras was the lowest in the presence of buffer alone. Since it has been shown that RalA binds the C2 domain of PLC-δ1 (Sidhu *et al.*, 2005), we wanted to test if H-Ras could also bind to the C2 domain of PLC-δ1. Therefore, HeLa cell lysate was incubated with GST-PLC-δ1 CT, a construct consisting of the PLC-δ1 C2 domain only (**Figure 15A**). The binding of H-Ras to PLC-δ1 CT was also calcium dependent in the same manner as the binding of H-Ras to full length PLC-δ1. GST alone did not bind H-Ras (**Figure 15A**). The amount of bound H-Ras appeared to be much higher when GST-PLC-δ1 CT was used in the assay. We have shown that the full length GST-PLC-δ1 is susceptible to degradation, and therefore we stained the blot using Ponceau S solution in order to visualize transferred proteins (**Figure 15B**). It was found that the amount of non-degraded GST-PLC-δ1 CT as well as GST present in the binding reactions was much higher than the amount of GST-PLC-δ1, even though we used the same starting amount of all three proteins. The fact that the amount of GST used was much higher than the amount of GST-PLC-δ1, and yet no H-Ras was bound in the presence of GST alone proves that the

binding was specific. This difference in the amount of GST-PLC-δ1 and GST-PLC-δ1 CT possibly explains why there was a difference in the amount of bound H-Ras to these fusion proteins.

A



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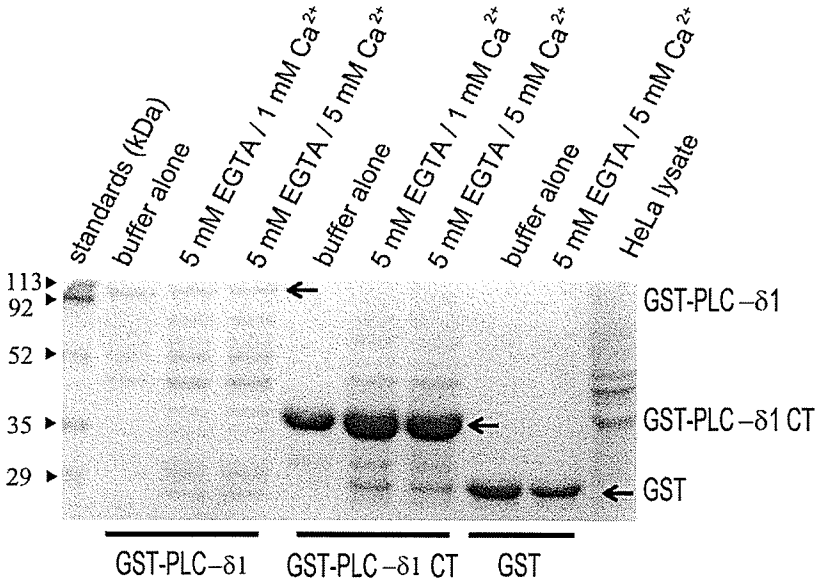


Figure 15. GST-PLC-δ1 and GST-PLC-δ1 CT bind H-Ras from HeLa cell lysate. HeLa cell lysate (500 μg protein) was incubated with GSH-agarose bound GST-PLC-δ1, GST-PLC-δ1 CT or GST in the presence of cell lysis buffer or cell lysis buffer plus 5 mM EGTA / 1 mM Ca^{2+}, or 5 mM EGTA / 5 mM Ca^{2+} for 2 hours at 4°C. Proteins were separated using 12% SDS-PAGE, and (A) western blotting was performed using anti-H-Ras antibody, and (B) the blot was stained with Ponceau S protein stain to visualize proteins.

4.8 Endogenous PLC-δ1 binds to GST-H-Ras

If GST-PLC-δ1 was capable of pulling down endogenous H-Ras, then GST-H-Ras should be able to pull down endogenous PLC-δ1. HeLa cell lysate was incubated in the presence of GST-H-Ras and the resulting Western blot was probed for PLC-δ1 (**Figure 16**). As before, the binding of PLC-δ1 to GST-H-Ras appeared to be calcium dependent. GST alone did not bind PLC-δ1, suggesting that the interaction was specific.

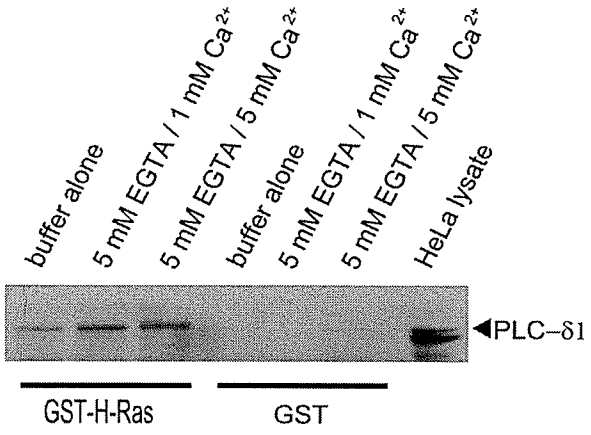


Figure 16. GST-H-Ras binds PLC-δ1 from HeLa cell lysate. HeLa cell lysate was incubated with GSH-agarose bound GST-H-Ras or GST in the presence of cell lysis buffer alone or cell lysis buffer plus 5 mM EGTA / 1 mM Ca^{2+}, or 5 mM EGTA / 5 mM Ca^{2+} for 2 hours at 4°C. Proteins were separated using 12% SDS-PAGE, and western blotting was performed using anti-PLC-δ1 antibody.

4.9 Pure H-Ras binds to GST-PLC-δ1, GST-PLC-δ1 CT, but not GST

Previously, it has been shown that CaM binds PLC-δ1 at a region outside the C2 domain. RalA, on the other hand, binds PLC-δ1 as well as CaM (Sidhu *et al.*, 2005). However, the possibility of H-Ras coming down in complexes with CaM was very low since H-Ras does not bind CaM. To confirm that H-Ras was able to bind PLC-δ1 directly, we incubated H-Ras, obtained by cleaving GST-H-Ras with thrombin, in the presence of GST-PLC-δ1, GST-PLC-δ1 CT or GST (**Figure 17**). The binding of pure H-Ras to GST-PLC-δ1 as well as GST- PLC-δ1 CT was calcium dependent, similarly to the binding of endogenous H-Ras. More specifically, the highest binding occurred in the presence of 5 mM EGTA / 5mM calcium, while the lowest binding took place in the presence of buffer alone. GST alone did not bind pure H-Ras (**Figure 17**).

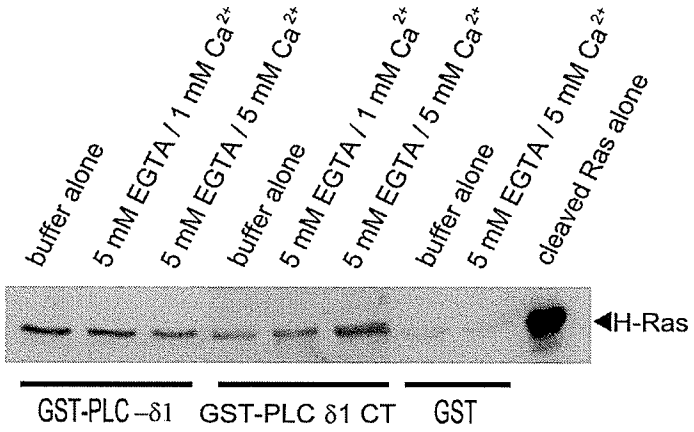


Figure 17. Recombinant H-Ras directly binds PLC-δ1 and PLC-δ1 CT. H-Ras cleaved from GST-H-Ras was incubated with GSH-agarose beads bound GST-PLC-δ1, GST-PLC-δ1 CT or GST in the presence of cell lysis buffer alone, or cell lysis buffer plus 5 mM EGTA / 1 mM Ca^{2+} or 5 mM EGTA / 5 mM Ca^{2+} for 2 hours at 4°C. Free calcium concentrations: buffer alone contains no additional Ca^{2+} and EGTA in the binding reaction; 5 mM EGTA / 1 mM Ca^{2+} (12.3 nM free Ca^{2+}); and 5 mM EGTA / 5 mM Ca^{2+} (17.0 μM free Ca^{2+}). Proteins were separated using 12% SDS-PAGE, and western blotting was performed using anti-H-Ras antibody.

4.10 PLC-δ1 co-immunoprecipitates with H-Ras from HeLa cell lysate

Next, it was necessary to determine whether the H-Ras / PLC-δ1 complex existed in HeLa cells. Co-immunoprecipitation experiments employing two different anti-human-H-Ras monoclonal antibodies showed that it was possible to co-precipitate PLC-δ1 out of HeLa cell lysate (**Figure 18**). PLC-δ1 co-precipitated with IgG_1 antibody, a negative control, in a lower amount, indicating that the assay was specific.

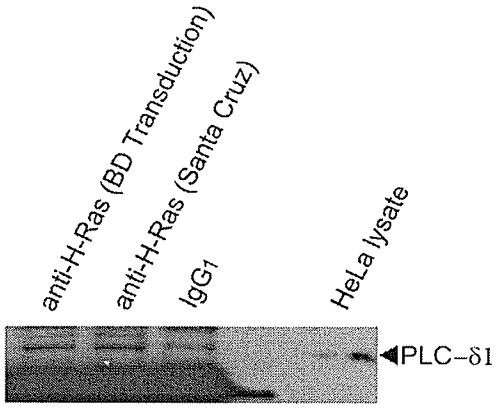


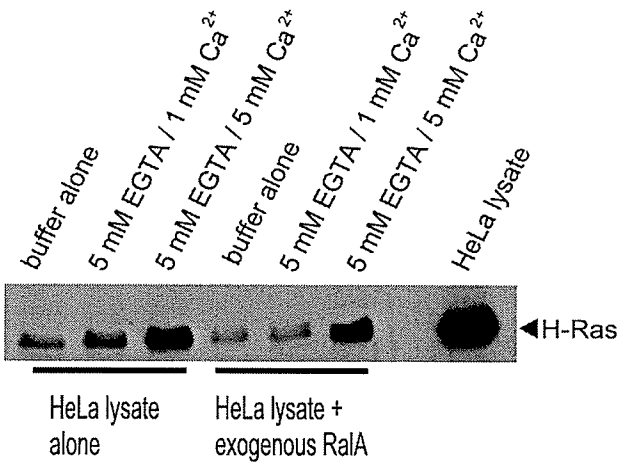
Figure 18. H-Ras and PLC-δ1 co-precipitate from HeLa cell lysate. HeLa cell lysate was incubated with mouse anti-human-H-Ras or mouse anti-human IgG_1. The immunoprecipitated proteins were separated by 12% SDS-PAGE, and western blotting was performed using anti-PLC-δ1 antibody.

4.11 H-Ras and RalA compete for binding to GST-PLC-δ1 CT

Since RalA and H-Ras both bind to the C2 domain of PLC-δ1, we conducted a competition assay to determine if RalA and H-Ras bind to the same region in PLC-δ1 CT. First, we incubated GST-PLC-δ1 CT with HeLa cell lysate in the presence or absence of exogenous RalA (**Figure 19A**). By comparing H-Ras binding to GST-PLC-δ1 CT at the above conditions it was determined that H-Ras and RalA compete for binding to GST-PLC-δ1 CT.

In order to confirm this finding, we conducted a second assay using GST-PLC-δ1 CT, and thrombin cleaved GST-RalA or GST-H-Ras at 68 nM free calcium (**Figure 19B**). We chose this calcium concentration because at this calcium concentration both RalA and H-Ras strongly interacted with GST-PLC-δ1 CT. In addition, this calcium concentration is close to the physiological calcium concentration of 100 nM found in resting cells (Ahlemeyer *et al.*, 1992). The results indicated that RalA partially inhibited H-Ras binding to GST-PLC-δ1 CT. When the amount of H-Ras was kept constant and the amount of RalA increased, the binding of H-Ras to GST-PLC-δ1 CT decreased with increasing RalA amount (**Figure 19B**; left panel). The binding of RalA to GST-PLC-δ1 CT was higher in the absence of H-Ras (**Figure 19B**; left panel compared to right panel).

A



B

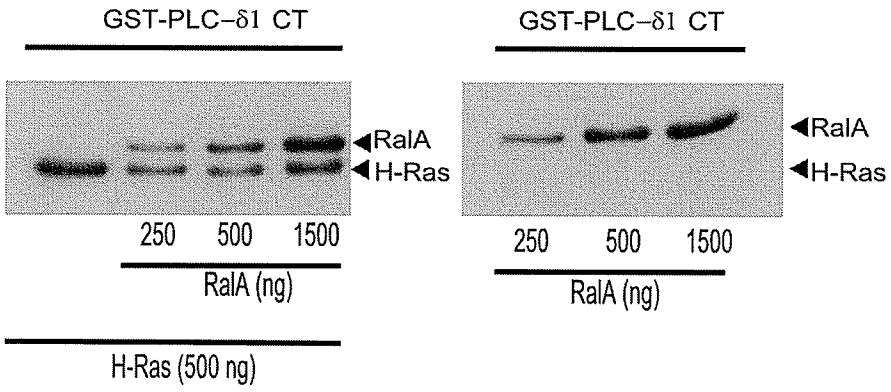
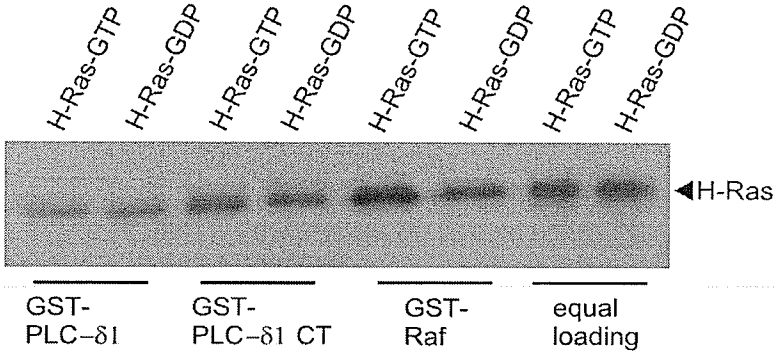


Figure 19. H-Ras and RalA compete for binding to GST-PLC-δ1 CT. A) HeLa cell lysate alone (500 μg protein), or HeLa cell lysate and RalA (500 ng), cleaved from GST-RalA by thrombin, in the presence of cell lysis buffer alone, or cell lysis buffer plus 5 mM EGTA / 1 mM Ca^{2+} or 5 mM EGTA / 5 mM Ca^{2+}. B) H-Ras (500 ng) alone or H-Ras (500 ng) plus RalA (250 ng, 500 ng, 1500 ng) (left panel), or RalA (250 ng, 500 ng, 1500 ng) alone (right panel), cleaved from GST-RalA and GST-H-Ras by thrombin, in 68 nM calcium calibration buffer, were incubated with GSH-agarose beads bound GST-PLC-δ1 CT for 2 hours at 4°C. Bound proteins were separated using 12% SDS-PAGE and Western blotting was performed with anti-H-Ras and anti-RalA.

4.12 PLC-δ1 binds H-Ras independently of the guanine nucleotide status of H-Ras

H-Ras can be in its active state (GTP bound), or inactive state (GDP bound). Therefore, we decided to test if PLC-δ1 had a preference for either one of the two H-Ras forms. In order to answer this question we employed a couple of approaches. First, pure H-Ras, obtained by thrombin cleavage of GST-H-Ras, which had been previously loaded with either GTP or GDP, was incubated separately with GST-PLC-δ1, GST-PLC-δ1 CT, or GST-Raf (**Figure 20A**). The protein Raf was used as a control to test for the GTP/GDP loading, as Raf binds GTP-bound H-Ras more efficiently (Williams *et al.*, 2000). In the second approach, GSH-agarose beads bound GST-H-Ras was loaded with GTP or GDP and incubated with HeLa cell lysate (**Figure 20B**). Our results, using both approaches, show that H-Ras binds PLC-δ1 in a GTP independent manner. The binding of H-Ras to GST-Raf was higher when H-Ras was GTP loaded, indicating that the loading procedure was successful.

A



B

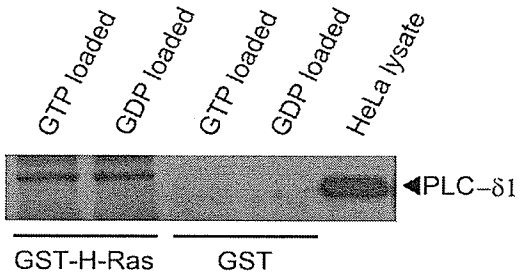


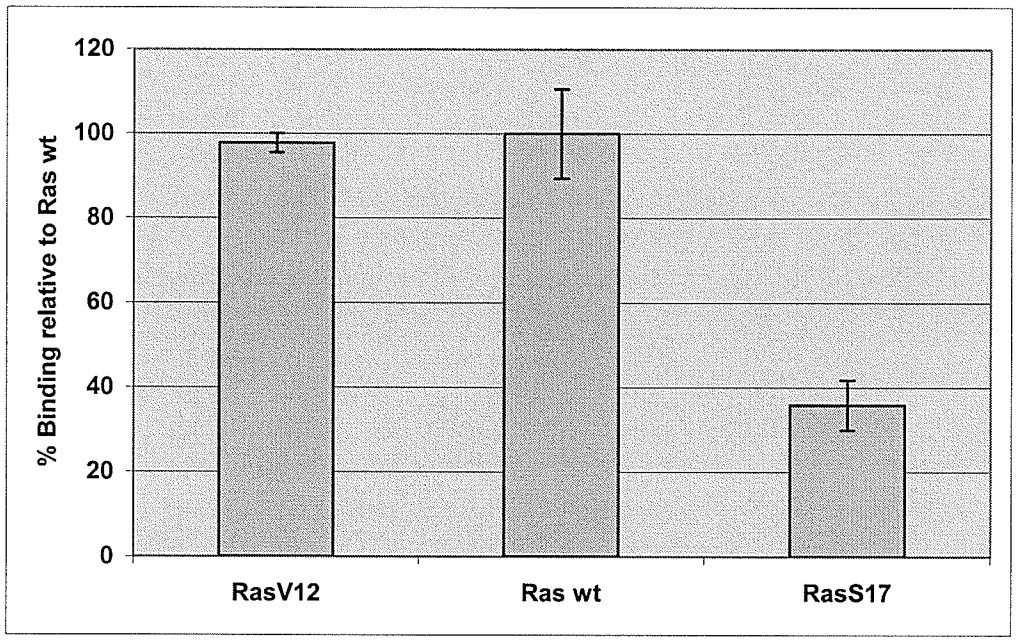
Figure 20. H-Ras binds PLC-δ1 independently of its guanine nucleotide status. A) Thrombin cleaved GTP or GDP loaded H-Ras was incubated with GST-PLC-δ1, GST-PLC-δ1 CT, or GST-Raf for 2 hours at 4°C. Bound proteins were separated using 12% SDS-PAGE and Western blotting was performed with anti-H-Ras, B) GST-H-Ras loaded with GTP or GDP was incubated with HeLa cell lysate for 2 hours at 4°C. Bound proteins were separated using 12% SDS-PAGE and Western blotting was performed with anti-PLC-δ1.

4.13 Wild type and mutant H-Ras show selectivity in interaction with GST-PLC-δ1 CT

Next, we wished to examine the binding preferences of wild type H-Ras, constitutively active H-Ras (RasV12), and dominant negative H-Ras (RasS17) for PLC-δ1. HeLa cells transfected with wild type or mutant H-Ras were lysed, and the lysates incubated with GST-PLC-δ1 CT. Relative to wild type H-Ras, RasV12 had equal binding, while RasS17 demonstrated lower binding to GST-PLC-δ1 CT (**Figure 21A**). It was found that the binding of Ras(S17) to GST-PLC-δ1 CT was 60% less than the binding of wild type or Ras(V12) to GST-PLC-δ1 CT. Figure 21B shows a western blot used to test for the expression of various Ras isoforms. The western blot indicates that all three forms of Ras were successfully expressed in HeLa cells. The fact that the three H-

Ras forms had differential expression levels was taken into account in binding experiment quantitation, as described in the Materials and Methods section.

A)



B)

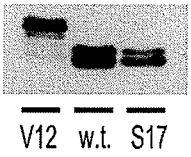


Figure 21. Wild type and mutant H-Ras selectively bind GST-PLC-δ1 CT. A) GST-PLC δ1 CT was incubated with lysates obtained from HeLa cells overexpressing wild type H-Ras or H-Ras mutants, H-Ras(V12) or H-Ras(S17), in the presence of cell lysis buffer for 2 hours at 4°C. Bound proteins were separated using 12% SDS-PAGE and Western blotting was performed with anti-H-Ras antibody. The experiment was repeated 3 times, the western blots were scanned and quantified using Quantity One software. The calculations were performed by taking the ratio of binding to protein expression level of each H-Ras mutant or H-Ras wild type. B) Expression of wild type and the H-Ras mutants.

5. Discussion and Conclusions

IP_3 and DAG are two products generated due to the action of PLC. IP_3 causes release of calcium from the endoplasmic reticulum, while DAG activates PKC (Williams, 1999). Calcium can regulate a number of cellular proteins directly, or through the calcium binding protein calmodulin (CaM) (Franklin *et al.*, 2006; Wang *et al.*, 1997; Guo *et al.*, 2005). PKC on the other hand regulates proteins through phosphorylation (Li and Gobe, 2006). This makes PLC an important molecule in the signal transduction processes of the cell. Along with PLC-η, PLC-δ1 is the most calcium sensitive of the PLC isoforms (Nakahara *et al.*, 2005). PLC-δ1 is activated in response to the initial calcium spike caused by the action of PLC-β, PLC-γ and PLC-ε (Rebecchi and Pentyala, 2000).

PLC isoforms bind, and are in turn regulated by several proteins including members of the Ras superfamily of proteins such as Ral and Ras, as well as CaM (Song *et al*, 2001; Wing *et al.*, 2003; Sidhu *et al.*, 2005; McCullar *et al.*, 2003). In addition, a transglutaminase, G_h/TGII, binds and regulates PLC-δ1 (Rebecchi and Pentyala, 2000). Transglutaminases are atypical GTPases since they have both GTP and ATP binding sites (Rebecchi and Pentyala, 2000). G_h/TGII links PLC-δ1 to α-adrenergic and oxytocin receptors (Rebecchi and Pentyala, 2000). However, recent focus has switched to defining the role of small GTPases in the regulation of various PLC isozymes (Harden and Sondek, 2006).

Ral is a small GTPase belonging to the Ras superfamily. Ral has been shown to interact with a variety of proteins including PLD, the exocyst complex, RalBP1, PLC-δ1,

and CaM, and thus is important in oncogenesis, exocytosis, endocytosis, and secretion (Chien and White, 2003; van Dam and Robinson, 2006).

Previously we have reported that RalA binds to the C2 domain region of PLC-δ1 in a calcium dependent manner (Sidhu *et al.*, 2005), and that the C-terminus of RalA binds CaM in a calcium dependent manner (Clough *et al.*, 2002). PLC-δ1 has a CaM-like structure in its N-terminal (Essen *et al.*, 1996). Based on this information we have investigated if RalA can interact with the N-terminus of PLC-δ1.

The results of the present study indicate that the N-terminus of PLC-δ1 (PLC-$\delta1_{1-294}$), comprised of the PH and EF-hand domains, contains a calcium dependent RalA binding domain (**Figure 22, pg. 57**). In fact, our competition assay results with the RalA C-terminal peptide provide evidence in support of the hypothesis that the CaM-binding C-terminus of RalA is responsible for binding of RalA to the N-terminus of PLC-δ1. This, therefore, represents a second RalA binding site within PLC-δ1 (**Figure 22**). The 3D-structure of PLC-δ1 places the EF-hand domain in close proximity to the C2 domain. In fact, these two domains make direct contact (Essen *et al.*, 1996). Thus each of the two domains could bind one molecule of RalA, or one site could be preferred over the other. The EF-hand domain appears to be more accessible for protein binding. At the present time we do not know how RalA interacts with the C2 domain. However, since the C2 domain is not a CaM-like structure, it is possible that a region in Ral, other than the C-terminal may interact with the C2 domain. If this were the case then one molecule of Ral may be able to interact with two molecules of PLC-δ1. As reported previously, RalA increases activity of PLC-δ1 *in vitro* (Sidhu *et al.*, 2005). However, the role of RalA in PLC-δ1 regulation *in vivo* is not known. One possibility is that Ral may help in

targeting PLC-δ1 to membrane. PLC-δ1 is thought to be activated by an increase in intracellular calcium concentration initiated by PLC-β, PLC-γ and PLC-ε (Rebecchi and Pentyala, 2000). Thus, it is possible that since the interactions of Ral with PLC-$\delta 1_{1\text{-}294}$ are stronger in the presence of calcium, that this may result in association of PLC-δ1 with the membrane. However, it has also been suggested that membrane attachment of PLC-δ1 could be a way to downregulate the enzyme (Peterson *et al.*, 1995).

W-7 and the PLC-δ1 IQ peptide have been shown to inhibit PLC-δ1 activity (Sidhu *et al.*, 2005), and both have been shown to bind CaM (Osawa *et al.*, 1998; Sidhu *et al.*, 2005). Previously, we have reported that the IQ peptide does not bind to RalA (Sidhu *et al.*, 2005). In the present study we show that W-7 can directly interact with full length PLC-δ1, and that both W-7 and the IQ peptide are capable of inhibiting RalA binding to the N-terminal of PLC-δ1. In addition, our results with RalA and the IQ peptide indicate that there was direct interaction between PLC-$\delta 1_{1\text{-}294}$ and the IQ peptide of PLC-δ1. The results also show that a C-terminal RalB peptide was able to inhibit binding of RalA to PLC-$\delta 1_{1\text{-}294}$, raising the possibility that RalB is also able to bind and regulate PLC-δ1. We further showed that the same RalB peptide was also capable of binding to CaM. These results thus offer additional evidence that the N-terminus of PLC-δ1 contains a CaM-like region. How these interactions affect PLC-δ1 activity is not known. RalA, W-7, and the IQ peptide could simply act by altering the 3D-structure of the enzyme, thus regulating its activity. It has been reported that the EF-hand domain of PLC-δ1 can act as an allosteric regulatory domain following its interaction with hydrophobic ligands (Kobayashi *et al.*, 2005). The RalA, RalB C-terminal and the IQ region are comprised of positively charged amino acids that confer an overall positive

charge on the peptides. Thus, these peptides could bind to stretches of negatively charged amino acids found in the N-terminal of PLC-δ1. The RalA and IQ peptides are similar in length; however, the RalA peptide has a greater overall positive charge (+9) than the IQ peptide (+3). This could explain why the RalA peptide is a better RalA binding inhibitor then the IQ peptide in our competition assays. A G_h/TGII C-terminal peptide comprised of amino acids 654-673 activates PLC-δ1 *in vitro* (Hwang *et al.*, 1995; Rebecchi and Pentyala, 2000). The G_h/TGII peptide has a net positive charge of +1, and it has been suggested that five charged amino acids found within this peptide are crucial for G_h/TGII binding to PLC-δ1 (Hwang *et al.*, 1995). It would be interesting to see if the Ral peptides used in our study could also activate PLC-δ1.

How the above interaction between the IQ peptide and PLC-δ1_{1-294} arises *in vivo* is not clear. One possibility is that the interaction occurs internally. However, the 3D-structure of PLC-δ1 places the EF-hand domain far from the IQ region. But the possibility exists that the EF-hand domain of one PLC-δ1 molecule may interact with the IQ region of an adjacent PLC-δ1 molecule. This is supported by the fact that we were able to pull down pure PLC-δ1 and PLC-δ1 from HeLa cell lysates using GST-PLC-δ1_{1-294}. It has been reported that PLC-δ1 could exist as a dimer under physiological salt concentrations (Ryu *et al.*, 1987; Ellis *et al.*, 1993). A peptide comprised of PLC-δ1 amino acids 139 to 475 (and therefore the EF-hand domain), interacts with a peptide comprised of amino acids 475 to 756 (encompasses IQ region) (Ellis *et al.*, 1993). These previous findings support our hypothesis that the EF-hand domain is likely responsible for the interaction with the IQ region and potential dimerization.

It has been shown that PLC-δ1 dimerization does not affect its activity *in vitro* (Ellis *et al.*, 1993). However, the effects of the dimerization *in vivo* are not known. If dimerization is necessary for the full activity of PLC-δ1, W-7 and the IQ peptide are certainly capable of disrupting it. On the other hand, dimerization may actually inactivate the enzyme, as the IQ peptide of one PLC-δ1 molecule binds to the EF-hand of an adjacent PLC-δ1 molecule. Following prolonged stimuli, the intracellular calcium concentration will increase, and stimulate PLC-δ1 dimerization, resulting in enzyme inactivation. However, since RalA also binds to the N-terminal of PLC-δ1 at high calcium levels it could disrupt the interaction between the EF-hand and the IQ region of PLC-δ1, thus maintaining the enzyme in its active state.

RalA is not the only protein capable of activating PLC-δ1. Previously, it has been shown that similar to the observtions for RalB(V23), overexpression of constitutively active H-Ras(V12) also activates PLC-δ1 in HeLa cells (Sidhu *et al.*, 2005). However, initial attempts to pull out H-Ras from HeLa cell lysate using GST-PLC-δ1 were not successful (Sidhu *et al.*, 2005). How is H-Ras(V12) causing activation of PLC-δ1? Although CaM binds and inhibits PLC-δ1, H-Ras unlike K-Ras does not bind CaM (Sidhu *et al.*, 2003; Villalonga, 2001). Therefore, the possibility that H-Ras was mediating its effects on PLC-δ1 by interacting with CaM was unlikely. It was possible, however, that the overexpressed H-Ras(V12) was activating the Ral pathway by the Ras effector RalGDS, leading to PLC-δ1 activation (Hofer *et al.*, 1994; Sidhu *et al.*, 2005). However, RalA binds and activates PLC-δ1 independently of its guanine nucleotide status (Sidhu *et al.*, 2005). H-Ras is known to regulate phospholipase D (PLD) via its

effector molecules Ral-GDS, PI3K, and Raf-1 (Lucas *et. al.*, 2002). It was possible that H-Ras(V12) was activating PLC-δ1 through similar proteins. It has been reported that PLC-ε interacts with Ras due to the presence of Ras-associated domain 1 and Ras-associated domain 2 in PLC-ε (Bunney *et al.*, 2006). Even though PLC-δ1 does not contain such a region, we decided to investigate further the possibility of Ras being able to interact directly with PLC-δ1.

The results of the present study suggest that H-Ras is capable of direct interaction with the C2 domain of PLC-δ1. Binding of recombinant H-Ras and endogenous H-Ras from HeLa cell lysates to GST-PLC-δ1 or GST-PLC-δ1 CT was found to be calcium dependent. The greatest binding occurred at the highest calcium concentration. In the reverse experiment, PLC-δ1 from HeLa cell lysates bound to GST-H-Ras in a similar calcium dependent manner. The calcium concentrations used in our assays were within physiological range, and thus similar calcium dependency could be operative in the cell (Ahlemeyer, 1992). Additionally, PLC-δ1 isoforms are activated by calcium concentrations in the range of 0.1 μM to 10 μM (Rebecchi and Pentyala, 2000). Co-immunoprecipitation results further confirmed that the H-Ras / PLC-δ1 complex exists in HeLa cells.

The results also indicate that H-Ras and RalA compete for binding to the C2 domain of PLC-δ1, and that the two GTPases bind to the same region in the C2 domain of PLC-δ1. Even though it appears that both RalA and H-Ras can independently activate PLC-δ1, one of the proteins could be a much better PLC-δ1 activator. Both proteins, RalA and H-Ras, bind to PLC-δ1 more efficiently at high calcium concentrations.

Therefore, which of the two small GTPases binds and activates PLC-δ1 could depend on the availability of each of the proteins at a particular calcium concentration.

In addition, as for RalA (Sidhu *et. al*, 2005), H-Ras binds PLC-δ1 independent of its guanine nucleotide status. Most effectors of Ras bind preferentially to Ras-GTP and not Ras-GDP (Clark *et al.*, 1996). One example is the Ras-binding-site of Raf-1 (Williams *et al.*, 2000). However, the Raf-1 cysteine-rich domain can bind H-Ras independently of H-Ras guanine nucleotide status (Williams *et al.*, 2000).

The calcium concentrations used in the present study were within the physiological range (Ahlemeyer, 1992; Sidhu *et al.*, 2005), stressing the importance of the current findings. As PIP_2 is converted to IP_3 by PLC-δ1, calcium is released from the endoplasmic reticulum, and the intracellular calcium concentration increases. Once the intracellular calcium concentration increases, H-Ras binds to PLC-δ1 more strongly, further activating PLC-δ1. Therefore, the calcium dependent binding of H-Ras to PLC-δ1 could be a mechanism for amplification of the initial PLC-δ1 activation. H-Ras could activate PLC-δ1 by changing the 3-dimensional conformation of PLC- δ1. As a membrane anchored protein, H-Ras could activate PLC-δ1 by recruiting it to the plasma membrane where PIP_2, the PLC-δ1 substrate, is found. It has been shown that H-Ras does not affect PLC-ε activity *in vitro*. However, constitutively active H-Ras(Q61L) stimulated PLC-ε activity in Cos-7 cells, suggesting that H-Ras was activating PLC-ε by recruiting it to the membrane (Kelley *et al.*, 2001; Bunney *et al.*, 2006). It has also been reported that normal and oncogenic forms of Ras activate PLD using different mechanisms (Lucas *et al.*, 2002). Our results indicate that equal amounts of the constitutively active H-Ras and wild type H-Ras bound to PLC-δ1 CT. However,

binding of wild type H-Ras and the constitutively active H-Ras was preferential to binding of the dominant negative H-Ras (H-RasS17) to PLC-δ1. The dominant negative H-Ras is capable of binding GTP as well as GDP, with GDP having only slight preference for the mutant (Farnsworth and Feig, 1991). However, H-RasS17 is unable to interact with its effector molecule even when GTP-bound (Farnsworth and Feig, 1991). Therefore, these results provide further support for the finding that the interaction between PLC-δ1 and H-Ras is GTP independent. In addition, the results suggest a way of how mutations in H-Ras could affect PLC-δ1 activity based on their ability to interact with PLC-δ1. A mutant such as H-RasS17 may not be able to bind PLC-δ1 as strongly, and therefore may not be able to activate PLC-δ1.

Finally, it is established that RalA is activated by calcium (Hofer *et al.*, 1998). Thus, the stronger the PLC-δ1 activation is, as a result of RalA / H-Ras binding to PLC-δ1, the stronger the activation of RalA is going to be due to an increased intracellular calcium concentration. Therefore, by activating PLC-δ1, RalA can be said to be responsible for self-activation, and thus may participate in additional signaling pathways.

In conclusion, the current study identifies a novel binding site for RalA in the N-terminal of PLC-δ1. Additionally, a novel interacting partner, H-Ras, for PLC-δ1 is identified. Even though these findings provide further insight into regulation of PLC-δ1, the regulation of PLC appears to be complex, and further studies are necessary in order to understand the exact mechanism controlling PLC-δ1 activity *in vivo*. A model depicting various regions of interaction for RalA, CaM and H-Ras with PLC-δ1 are shown (**Figure 22**).

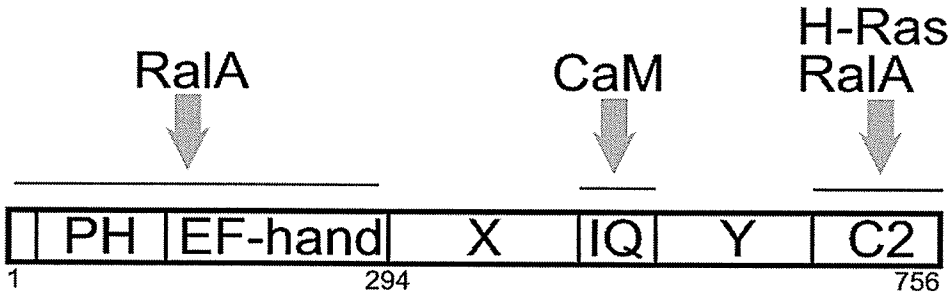


Figure 22. Representation of RalA, H-Ras and CaM binding sites PLC-δ1. The N – terminus and the C-terminus of PLC-δ1 contain the newly identified RalA and H-Ras binding sites, respectively.

6. Future directions

1. Using the yeast 2-hybrid system show that H-Ras binds to the C2 domain of PLC-δ1, and that RalA binds to the N-terminal of PLC-δ1.

2. Determine the exact site within the N-terminal region of PLC-δ1 responsible for binding to RalA. This can be achieved by creating PLC-δ1 constructs encoding either the PH domain or the EF-hand domain of PLC-δ1.

3. Determine the exact region within the C2 domain of PLC-δ1 responsible for binding H-Ras. This can be accomplished by truncating the C2 domain of PLC-δ1. Then, peptides can be synthesized to test for the region of interest. Finally, site-directed mutagenesis studies can be used to test the importance of specific amino acids in binding of H-Ras to full length PLC-δ1.

4. Determine the functional implication of RalA binding to PLC-δ1. Since the PH domain of PLC-δ1 binds to the plasma membrane lipids, it would be interesting to test whether RalA binding to the N-terminal of PLC-δ1 would affect membrane lipid binding ability of PLC-δ1. This can be accomplished *in vitro* by using artificial membranes or PIP_2 strips.

5. Determine the effect of various mutants of H-Ras (for example, oncogenic) on PLC-δ1 activity, both *in vitro* and *in vivo*.

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