Stony Brook University

The Identification of a Novel Binding Partner for Phospholipase Cβ1, Translin-Associated Factor X: A Link to RNA Interference

A Dissertation Presented

by

Omozuanvbo R. Aisiku

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Abstract of the Dissertation

Identification of a Novel Binding Partner for Phospholipase Cβ1, Translin-Associated Factor X: A Link to RNA Interference A Dissertation Presented by

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Mammalian inositol-specific phospholipase Cβ1 (PLCβ1) is activated by $G\alpha_q$ subunits on the plasma membrane where it catalyzes hydrolysis of the minor membrane phosphatidylinositol 4,5 bisphosphate. While PLCβ1 localizes primarily to the plasma membrane it also exists in other compartments of the cell. It is not currently known what function PLCβ1 has in the cytosol and nucleus nor is it known what factors localize PLCβ1 to these compartments. In this study, we have identified a novel binding partner, translin-associated factor X (TRAX). Our experiments show that both proteins interact in solution and in cells. While TRAX exerts little effect on the membrane binding of PLCβ1, it does reduce the affinity of PLCβ1 for $G\alpha_q$ and inhibits the stimulation of PLCβ1 activity by $G\alpha_q$. TRAX, a cytosolic protein that can localize to the nucleus, is involved in the down-regulation of protein expression by RNA interference. Our study shows that

cytosolic PLCβ1 can drive TRAX out of the nucleus and reduce siRNA activity. Furthermore, activation of $G\alpha_q$ results in apparent displacement of cytosolic PLCβ1 from TRAX and concurrently allows TRAX to relocalize to the nucleus. Importantly we have found that increased cytosolic expression of PLCβ1 appears to reverse the siRNA-induced down-regulation of GAPDH. Our data suggests a potential link between $G\alpha_q$ activation, PLCβ1 localization and siRNA activity.

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Chapter 1: Introduction

Phospholipase Cβ is the main effector of the Gαq family of G proteins that are coupled to a diverse array of agents such as angiotensin, dopamine, serotonin, bradykinin, etc. PLCβ catalyses the hydrolysis of the minor membrane signaling lipid phosphatidylinositol 4,5 bisphosphate (PI(4,5)P2) to generate the second messengers inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG) which stimulate the release of Ca^{2+} from intracellular stores and activate protein kinase C, respectively. There are 4 known forms of PLCβ, and this work focuses on PLCβ1 and its localization in the cytosol. The enzyme mainly localizes at the plasma membrane but exists in other cellular compartments. For example, PLCβ1 along with other PLC isozymes have been found in the nucleus but the function of these enzymes in the nucleus and other compartments remains a mystery. The goal of this project was to find alternative binding partners for PLCβ1 with the ultimate aim to elucidate its function in other compartments of the cell.

PLCβ1 is a prominent in neuronal cells and is strongly activated by Gαq (Dowal, L. et al., 2006). PLCβ interacts with G proteins and $PI(4,5)P_2$ on the plasma membrane (Dowal, L., et al., 2006) and, not surprisingly, a large population of the enzyme is localized on the plasma membrane where it associates with Gαq even in the basal state. However, depending on the cellular conditions, PLCβ1 can also localize to the nucleus and cytosol where Gαq is absent and the role of the enzyme in these compartments is presently unclear.

One potential role of the cytosolic PLCβ population is to maintain a constant level of $PI(4,5)P_2$ in internal vesicles. It is also possible that the cytosolic pool serves as a reservoir for plasma membrane activators. Supporting data for this idea comes

from the Henis laboratory who find a much higher plasma membrane population of PLCβ2 when HEK293 cells were transfected with constitutively active Rac2 as compared to wild type Rac2 suggesting that the cytosolic population serves as a PLCβ reservoir (Illenberger, et al., 2003)). However, we found this is not the case for eYFP-PLCβ1 where the cytosolic population remained constant upon $G\alpha_q$ activation suggesting that PLCβ1 does not have this function (Dowal, L., et al., 2006). While there are pools of PIP_2 stored in internal compartments of the cell, the basal level of PLCβ1 activity is considered too low to affect these populations of PIP_2. The absence of an activator of PLCβ1, such as $G\alpha_{q,}$ makes it highly unlikely that PLCβ1 could alter PIP_2 levels in the cytosol and the nucleus where PLCβ1 has been found.

It is possible that the cytosolic PLCβ1 population serves as a reservoir for the nuclear population rather than the plasma membrane one. The function of PLCβ1 in the nucleus remains an intriguing question. There is substantial evidence of that the nucleus contains lipid signaling networks independent of the plasma membrane (Irvine, 2003). Also, there is increasing evidence that phospholipase Cs (PLC) and their phosphoinositide (PI) products are involved in nuclear processes, specifically, gene expression and chromatin remodeling, mRNA processing and transport, and cell cycle progression (Irvine, 2003). The fact that PLCs may be involved in nuclear processes marks a departure from the classical lipid signaling pathways at the plasma membrane.

The necessary question to ask is: what is the physicochemical structure of nuclear lipids? Nuclei have been isolated using buffers that contained detergents, which presumably would indicate that any remaining lipids were not part of a lipid bilayer. In experiments that allowed for quantifiable titration of nuclei with Triton X-100, it was discovered that approximately 50% of the PtdIns (4,5)P_2 (PIP_2) content measured by mass assay remained in these preparations, along with more than 80% of

the PI lipid enzymes, i.e. DAG kinase, PtdIns 4-kinase and PtdIns4P 5-kinase activities. Furthermore, experiments using the pleckstrin homology domain (PH) of $PLC\delta_1$, which binds strongly and specifically to the head group of PIP_2 and IP_3 (see Rebecchi, and Pentyala, 2000) have shown the presence of intranuclear PIP_2. These results lead to the following question: Are there nuclear components that bind inositol lipids? Some candidates include the laminar layer, which are structures associated with splicesomes, and invaginations of the laminar layer into the nucleus which are are similar to those that have been proposed for the nuclear envelope. Efforts to characterize the subnuclear localization of lipids at this current time have been elusive (Irvine, 2003).

Classical Lipid Signaling

Signaling through PI lipids involves the binding of an agonist, such as a hormone, to a heterotrimeric G protein coupled receptor at the plasma membrane (Rebecchi and Pentyala, 2000; Rhee, 2001; Suh, 2008). Agonist-bound receptor activates heterotrimeric G proteins by catalyzing the exchange of GTP for GDP on the $G\alpha$ subunit. Activated $G\alpha_q$ and $G\beta\gamma$ subunits can in turn activate $PLC\beta$, which, as mentioned above, hydrolyze to generate diacylglycerol (DAG) and inositol triphosphate ($InsP_3$) to mobilize calcium (Ca^{++}) stores within the cell (Rhee, 2001).

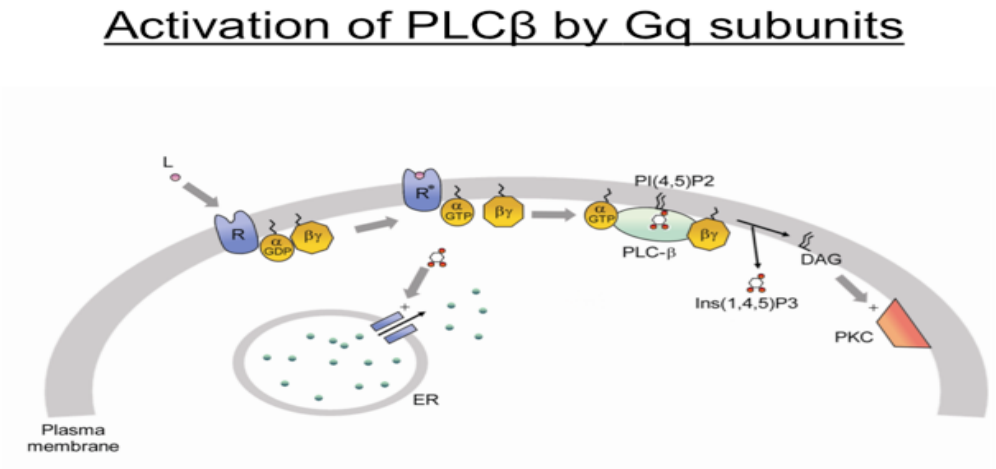

Figure -1 - Classic Lipid Signaling by PLCβ

Structural Organization of PLC Isozymes

The PLC family is a complex and diverse group of enzymes comprised of 13 mammalian isozymes. They can be divided into six types: four PLC-β, two PLC-γ, three PLC-δ, PLC-ε (2), PLC-η (3) and the recently discovered PLCζ isozyme (Lai et al., 2002). The molecular size of the β- γ-type enzymes are in the range of 120-155 kDa, whereas PLC-ε is 230-260 kDa. Only the δ-type isozymes are found in lower eukaryotes, which suggests that the β-, γ-, ε-type isoforms found in higher eukaryotes evolved from PLC-δ .

PLC isozymes are homologous in the X and Y domains that form the catalytic core. The β-, γ-, and δ-type isozymes all contain an NH_2-terminal PH domain necessary for membrane binding and prevalent in many signaling proteins; however,

the newly discovered ζ-type lacks a PH domain (McLaughlin, 2007). Another feature the β- and γ-type isozymes have in common with the δ-type isozyme is the inclusion of an EF-hand domain located between the PH and X domains.

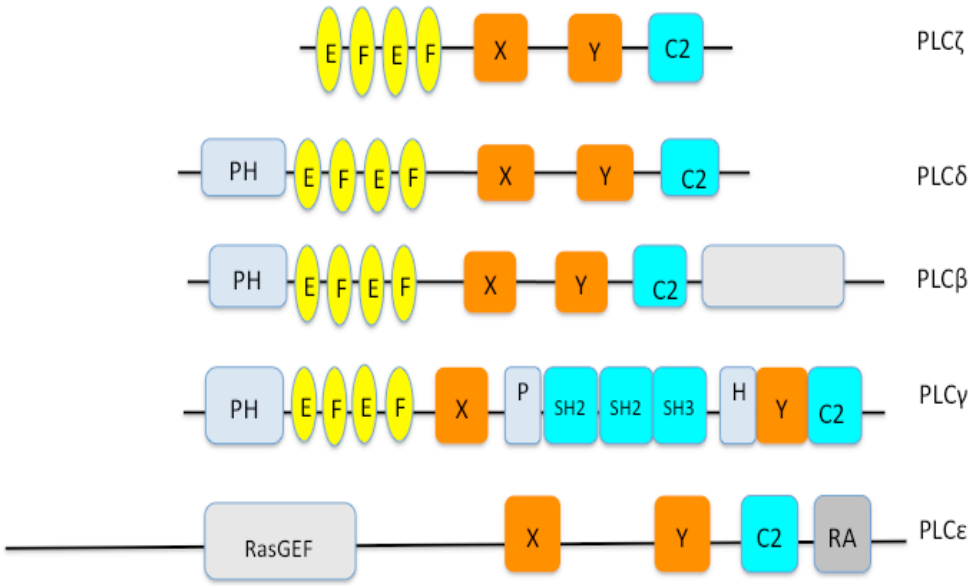

Figure 1 - Domain Architechture of PLC isozymes - Only PLC-ζ, -δ, -β are found in the nucleus (Crijen, V. et al., 2004; Suh, P. et al., 2008).

In addition to these features, the β-type isozyme contains a long COOH-terminal sequence downstream of the Y-domain. Of particular interest to this project, the β1- isozyme bears a lysine-rich NLS signal important for transport into the nucleus. The β1-type isozyme has two splice variants, β1a and β1b that are identical in sequence except the 1b variant has a 43-residue deletion in the C-terminus. Both have identical activity and G protein activation, and both have a nuclear localization signal (NLS) between residues 1055-1072 (Rhee, 2001;

Rebecchi, 2000). No nuclear export signal (NES) has been found in PLCβ1. PLCβ1a appears to be found preferentially in the cytosol, whereas PLCβ1b is found predominantly in the nucleus, although this depends on the particular cell line (Rhee, 2001).

PLCβ1 can be phosphorylated by extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) 1 or 2. Luccio Cocco's laboratory has immunoprecipitated PLCβ1 from the nuclear fraction of IGF-1 stimulated cells and found that PLCβ1 is phosphorylated on serine (Ser) 982 (Xu, A., et al., 2001). While there is no change in enzyme activity in vitro as a result of this phosphorylation, one laboratory used two-dimensional phosphopeptide mapping and site-directed mutagenesis to demonstrate that PKC promoted phosphorylation of PLCβ1 at serine 887 in the nucleus of IGF-1 treated cells (Gilmour, 2001). In cells that expressed a mutant form of PLCβ1 in which the Ser887 is replaced with alanine, a sustained activation of nuclear PLC following IGF-1 stimulation was observed suggesting that phosphorylation of this serine residue regulates nuclear activity by inactivating the enzyme (Gilmour, 2001).

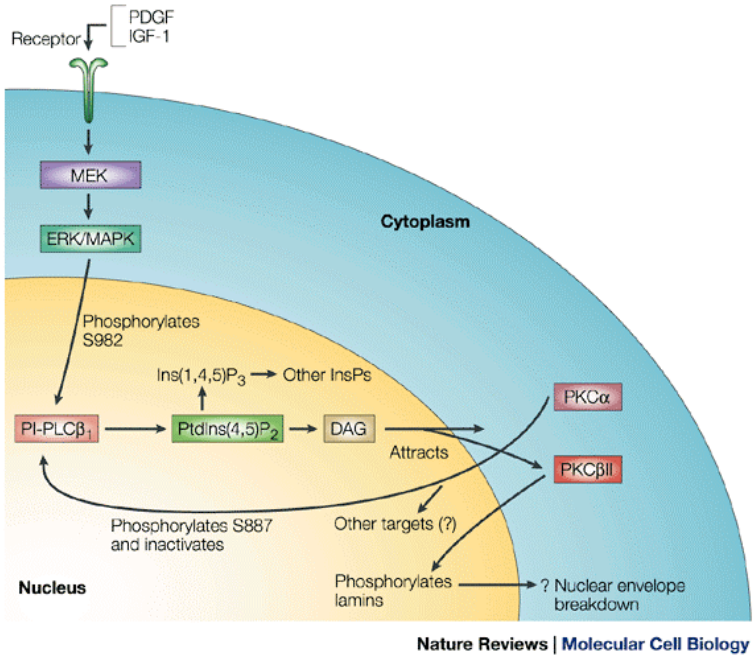

Figure 1-3 - <u>Targeting of PLCβ1 to the Nucleus By Growth Factors.</u> Reports have indicated that PLCβ1 is targeted to the nucleus where it hydrolyzes PIP_2 to generate the classical second messengers resulting in the attraction of downstream effectors like PKCα and PKCβII (Irvine R., 2003).

Activation of PLCβ by $G\alpha_q$ Subunits at the Plasma Membrane

While little information is available about how PLCβ1 is activated in the nucleus, it is regulated on the plasma membrane by heterotrimeric G proteins. Heterotrimeric G proteins consist of Gα and Gβγ stably associated in the inactive GDP-bound state. When a G protein coupled receptor (GPCR) is occupied by an agonist, GDP is exchanged for GTP and the Gα and Gβγ subunits dissociate. The

extent that the heterotrimer dissociates upon activation is under debate (Dowal et al, 2006). Gα has an intrinsic GTPase activity that mediates the rate-limiting step of GTP hydrolysis and subsequent reassociation of Gα with Gβγ.

The Gα subunit is divided into four subfamilies based on their amino acid sequence and effector interaction (Dowal et al., 2006). $G\alpha_q$ activates all four of the PLCβ isozymes with the greatest impact on PLCβ1. The activated $G\alpha_q$ subunit interacts with the C-terminus of PLCβ1 downstream of the Y domain, which consists of residues 803-1216 (Runnels, 1998). This stretch of residues is unique to PLCβ1. The activated $G\alpha_q$ subunit also interacts with the C2 domains of PLCβ isozymes (Runnels, 1998). PLCβ1 and PLCβ2 bind GTP-γ-S-activated $G\alpha_q$ with high affinity but bind the GDP form with much lower affinity (Runnels, 1998).

Activation of PLCβ by Gβγ Subunits

All of the PLCβ isozymes are activated by Gβγ with the exception of PLCβ4. PLCβ1 is the least sensitive to Gβγ (Rhee, 2001) and for that reason the interaction between Gβγ and PLCβ1 will not be explored in greater detail.

Identification of a Novel Binding Partner of PLCβ1, TRAX

It is known that PLCβ isozymes, and in particular PLCβ1 are activated by $G\alpha_q$ in the classical model. To date, $G\alpha_q$ has not been found in the nucleus. However, there is evidence that PLCβ1 is in the nucleus. How is PLCβ1 activated in the nucleus and what is its nuclear function? PLCβ1 has been visualized in nuclear

speckles, hinting at a possible role in RNA splicing and/or in mRNA processing (Cocco, 2006). These nuclear speckles or interchromatin granule clusters are storage deposits for splicing factors and other transcription machinery. Upon phosphorylation these factors translocate out of speckles and migrate to transcription binding sites (Spector and Lamond, 2003). A yeast two-hybrid assay was performed and identified a protein-binding partner of PLCβ1, TRAX. Since TRAX along with its putative partner translin is anti-proliferative the scope of my thesis only concentrates on the relationship between TRAX and PLCβ1.

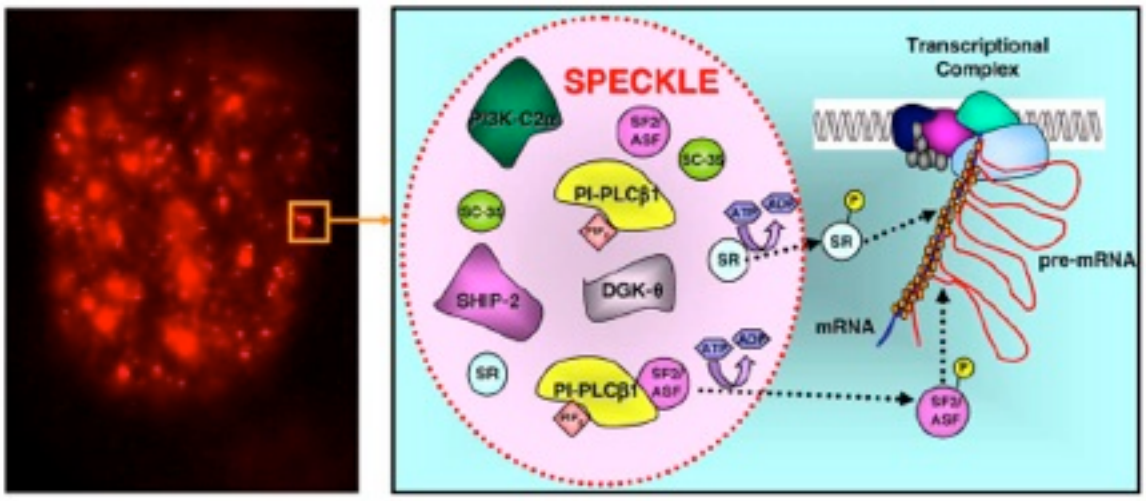

Figure 1-4 - PLCb1 has been found in nuclear speckles. Nuclear speckles are nuclear organelles that contain RNA, splicing factors and other transcriptional and post-transcriptional machinery. PIP2 has also been found in the nucleus.

TRAX or translin-associated factor X is post-transcriptionally regulated by its partner translin (or its mouse orthologue testis-brain RNA-binding protein) and in one study, both have been found to be essential for normal cell proliferation (Chock et al., 2004; Hecht et al., 2004; Jaendling, 2010). TRAX forms a DNA and RNA binding complex with translin (Chock et al., 2004; Hecht et al., 2004). Translin is a

single-stranded DNA and RNA-binding protein with proposed functions in chromosomal translocations in lymphoid cells and mRNA transport and storage in brain and testis (Chock et al., 2004; Hecht et al., 2004). In *in vitro* assays, translin has been found to bind to chromosomal DNA breakpoint junctions as an octameric ring and recognizes breaks at human meiotic recombination hotspots (Chock et al., 2004; Hecht et al., 2004). Experimental data has also shown that mice deficient in translin also show a coordinate loss of TRAX (Chock et al., 2004; Hecht et al., 2004). In addition, truncated forms of translin showed that its leucine zipper, which is critical for nucleotide binding, is necessary for binding TRAX as well. In cells, the two proteins also colocalize and the levels of TRAX protein closely parallel the levels of translin throughout the cell cycle as shown in double-thymidine releasing experiments (Chock et al., 2004; Hecht et al., 2004). RNAi experiments have shown that both TRAX and translin are essential for normal cell proliferation (Chock et al., 2004; Hecht et al., 2004).

The ability of translin, which forms a complex with TRAX, to bind RNA has been shown to be GTP-dependent (Rao and Sengupta, 2002). This finding, coupled with the fact that the C-terminus of PLCβ1 has GTPase activity, is very intriguing and gives rise to the hypothesis that PLCβ1 may have a role in accelerating the hydrolysis of GTP to GDP bound to translin. GTP binding has been implicated as a regulator of the RNA/DNA binding function of mouse translin according to published data (Rao and Sengupta, 2002). Additionally, GTP binding as confirmed by CD spectral analysis induces conformational changes in translin (Rao and Sengupta, 2002). Structural

studies and sequence analyses of GTPases show conserved switch-like motifs that are critical in GTP/GDP exchange, GTP-induced conformational change and GTP hydrolysis. Translin shares homology with switch regions conserved in most GTPases (Rao, 2006).

TRAX and its Role in RNA Interference

RNA interference is a conserved biological response to double-stranded RNA that was first discovered in *Caenorhabditis elegans* (Fire, 1998). It results in the suppression or silencing of gene expression. This response can be caused by delivery of dsRNA using into the C. *elegans*, which can lead to systemic silencing and silencing in its first generation progeny (Hannon, 2002). It has now been confirmed that RNAi exists in many eukaryotic organisms (Hannon, 2002).

RNAi results in the loss of target mRNAs without an effect on the rate of transcription (Hannon, 2002). It was proposed and proven that RNAi induces the degradation of target mRNA. Current understanding of RNAi mechanism comes from genetic studies in C. *elegans* and plants as well as biochemical studies of *Drosophila* extracts. In the case of *Drosophila*, injection of dsRNA led to post-transcriptional silencing (Hannon, 2002). The dsRNA is cleaved by the enzyme Dicer generating small interfering RNAs that recognizes its substrate, target mRNA, through Watson-Crick base-pairing (Hannon, 2002).

After the siRNAs have been generated they then associate with members of the Argonaute family of proteins. Argonaute forms a core of a complex of nucleases

now called RISC (RNA-induced silencing complex) that is responsible for the degradation of the targeted mRNA sequence in an ATP-dependent manner (Hannon, 2002). Argonaute binds the guide strand, ejects the passenger strand of the siRNA or miRNA-miRNA* duplex (* indicating the passenger strand) during loading, and subsequently recognizes the targeted region (Jinek, 2009). Structural information for full length Argonaute reveals two structural domains required for binding nucleotides, coined MID and PIWI, joined by way of an extensive conserved interface that is centered on the buried C terminus of the protein (Jinek, 2009). The PIWI domain adopts an RNasH fold suggesting that Argonaute proteins are responsible for the 'slicer' activity of the RISC. Additionally, the PIWI domain serves as a module for anchoring the 3' end of the guide strand (Jinek, 2009). The full-length crystal structure also reveals that the PAZ and MID domains (Janek, 2009**).**

The most interesting aspect of RNAi is that it can be amplified and spread. The process has been coined 'transitive RNAi' and refers to the movement of the silencing signal along a particular gene (Mallory, 2001**)**. Meaning that along with gene suppression of that mRNA there is production of siRNAs homologous to the targeted mRNA (Mallory, 2001). There is also an increase of target transcripts that are silenced if they are complimentary to the siRNAs produced.

Other components of the RNAi machinery have been unknown for quite some time. Of particular interest were the components necessary for unwinding siRNAs, which leads to the activation of RISC. A recent study has also indicated that TRAX plays a vital role in activating the RISC (Liu et al. 2009). Using a seven step

chromatographic process the Liu group was able to identify TRAX and Translin as the third component (C3PO) necessary for RISC activation. It was determined that TRAX along with translin were necessary for RNAi in vitro and in vivo. Specifically, TRAX promotes siRNA unwinding and RISC activation (**Fig. 1-5**). By doing so it facilitates the transition from the RISC loading complex to RISC activation. Moreover TRAX appears to have endonuclease activity, allowing it to rapidly degrade 9 nt and 12 nt fragments generated by Ago2 cleavage of the siRNA passenger strand (Liu et al., 2009). Mutational analysis revealed three critical residues (Glu^{123}, Glu^{126}, and Asp^{204}) necessary for catalysis.

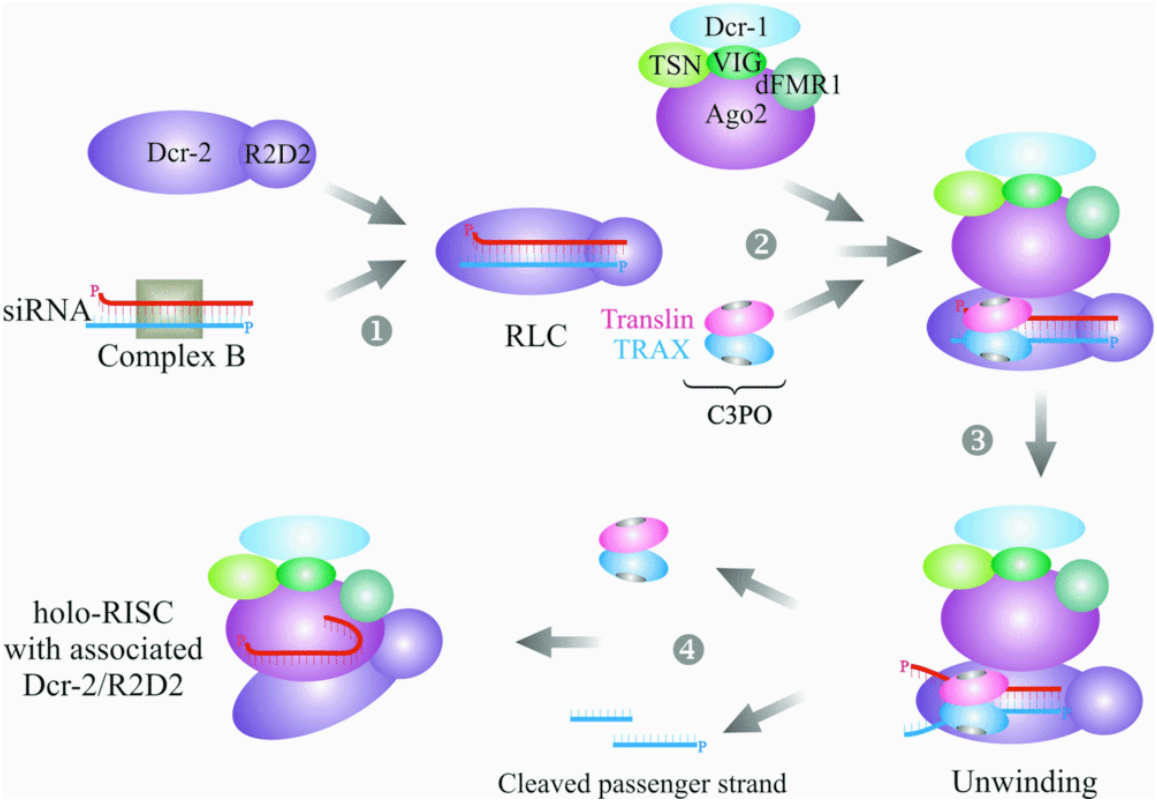

Figure 15 - Schematic of the proposed role of translin and TRAX (C3PO) in Drosophila RNAi. **Biochemical Journal 2010 Volume 429, 225-234**

Binding of TRAX and its Functional Implications

However, many questions remain. The fundamental question being, does TRAX help localize PLCβ1 to nuclear speckles where PLCβ1 regulates their activity or vice versa? Since the main function of TRAX is to transport translin into the nucleus we first tested the hypothesis that TRAX transports PLCβ1 to the nucleus. This hypothesis raised a few questions. First, do TRAX and PLCβ1 interact and how strong is this interaction? To this end we purified PLCβ1 and TRAX to determine whether they interact with each other using a combination of biochemical and biophysical techniques. Second, can these proteins interact in cells? To address this question we used a combination of immunohistochemistry and Fluorescence Resonance Energy Transfer (FRET). Finally, what is the biological endpoint and does the interaction between PLCβ1 and TRAX have any effect on the endpoint? The main techniques used to address these questions have been outlined below.

Determination of the Binding Between TRAX and PLCβ1

A series of binding experiments were carried out using a fluorescence-based approach. In these experiments, protein was labeled with the thiol-reactive probe called CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin). The unlabeled protein partner was titrated and the increase in CPM intensity was recorded. The subsequent data was plotted and fit to a bimolecular association.

From this curve the dissociation constant (K_d) for PLCβ1 and TRAX was calculated. Membrane binding experiments using POPC LUVs were carried out to see if TRAX could disrupt the membrane binding ability of CPM-labeled PLCβ1. Fluorescent intensity was measured as a function of unlabeled POPC LUVs in the presence and absence of TRAX.

Colocalization of PLCβ1 and Its Binding Partners Using Immunofluorescence

HEK293 cells were fixed and stained with an antibody to TRAX, while PLCβ1 will be detected using either an eYFP tag fused to it or an antibody directed to it. The cells were washed with PBS + Ca^{2+}, Mg^{2+}. Data was primarily analyzed using Image J (Rasband, 1997) except where noted. Additionally, RNAi knockdown of both binding partners were used to assess their effect on the localization of PLCβ1. Fluorescence Resonance Energy Transfer (FRET) experiments were carried out to measure the interaction between PLCβ1 and its proposed binding partners.

Fluorescence Resonance Energy Transfer

In this study, we monitored PLCβ1 association with TRAX. Fluorescence resonance energy transfer (FRET) is a powerful tool used to understand bimolecular interactions. FRET is commonly called fluorescence resonance energy transfer, but is not technically a fluorescence process, so it is often called Förster resonance energy transfer named after the German scientist who gave us the first description of the mechanism. In this mechanism, the transfer of energy between two

chromophores occurs (Lakowicz, 2006; Liu, 2001). Briefly, a fluorescent donor is excited at its specific fluorescence excitation wavelength. The excited energy is then transferred to a second molecule, the acceptor, which causes the donor to return to the electronic ground state. In the absence of an acceptor, this energy would be either emitted as a photon or lost in several non-radiative processes. Transfer of energy to acceptor is seen by a loss in donor emission coupled with a gain in acceptor emission. An increase in the acceptor emission follows the acceptance of fluorescent energy from the donor. The loss of donor emission coupled with an increase in acceptor emission can be measured and used to quantify the extent of protein-ligand interaction (Lakowicz, 2006; Liu, 2001).

The efficiency of FRET is governed by three major parameters: 1) the distance between the donor and the acceptor. 2) the spectral overlap of the donor emission spectrum and the acceptor absorption spectrum. 3) the relative orientation of the donor emission dipole moment and the acceptor absorption dipole moment (Lakowicz, 2006; Liu, 2001). The FRET efficiency E, which is defined as $E = 1 - \tau'_D/\tau_D$ where τ'_D and τ_D are the donor fluorescence lifetimes in the presence and absence of an acceptor, respectively or as $E = 1 - F'_D/F_D$ where F'_D and F_D are the donor fluorescence intensities with and without an acceptor, respectively. E depends on the donor-to-acceptor separation distance `r` with an inverse 6^{th} order law due to dipole-dipole coupling mechanism: $E = 1/(1+(r/R_0)^6)$ with R_0 being the Förster distance of this pair of donor and acceptor, at which the FRET efficiency is 50%. The Förster distance depends on the overlap integral of the donor emission spectrum

with the acceptor spectrum (Lakowicz, 2006; Liu, 2001).

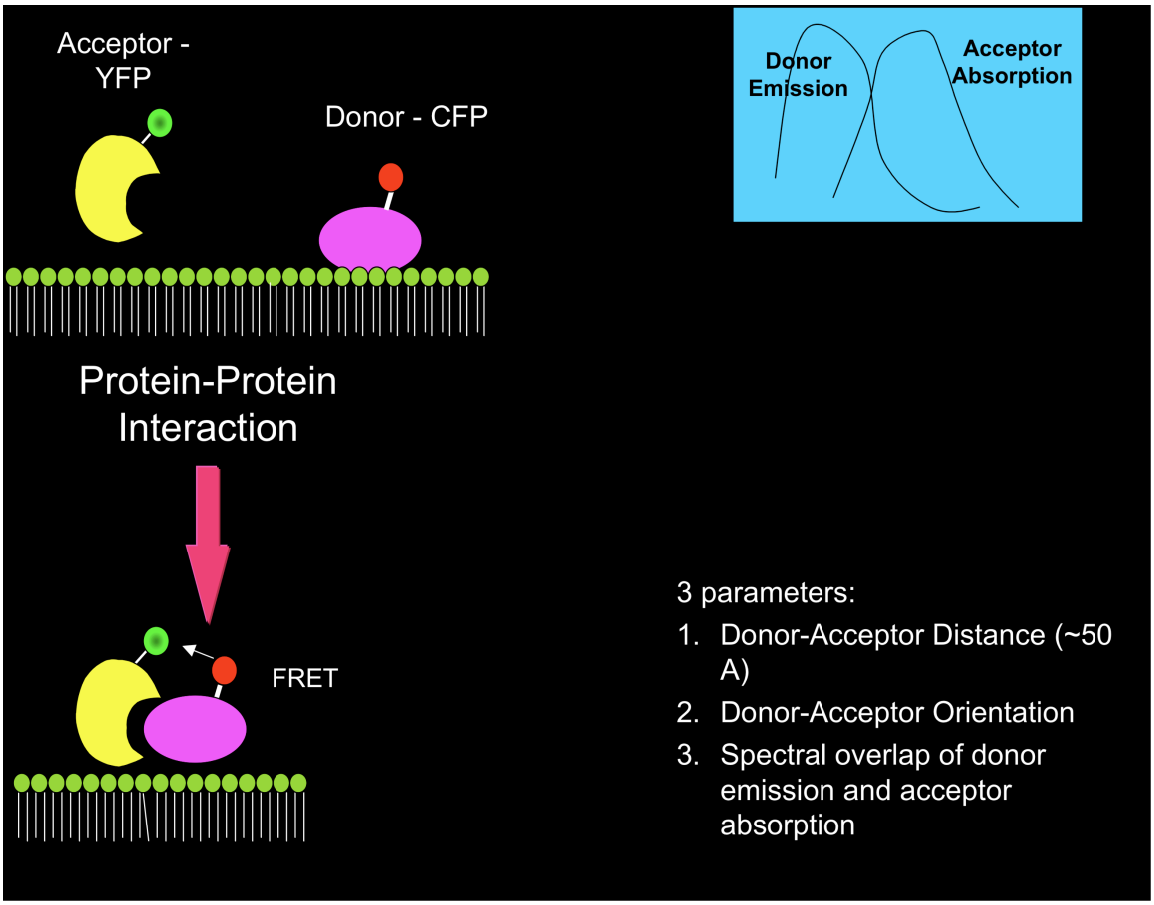

Figure 1-6 - Explanation of Fluorescence Resonance Energy Transfer

FRET experiments involving fluorescence are carried out by labeling both the donor and acceptor and these fluorophore-labeled molecules (PLCβ1 and TRAX) are mixed. If the two molecules are dissociated then both the donor and acceptor emissions are unchanged relative to when they are in solution by themselves. However, if the two are in close proximity (1-10 nm) due to the interaction of the two molecules, the acceptor emission is enhanced and the donor emission diminished because of the intermolecular FRET from the donor to the acceptor

(Lakowicz, 2006; Liu, 2001).

FRET is often quantified in cuvette-based experiments or in microscopy images on a pixel-by-pixel basis. Quantification is usually based on measuring changes in fluorescence intensity or fluorescence lifetime upon changing the experimental conditions.

The most popular FRET pair for live cell imaging a cyan fluorescent protein (CFP)-yellow fluorescent protein (YFP) pair, and these were used in FRET experiments designed for this project. Both fusion proteins are color variants of green fluorescent protein (GFP). A fully functional eYFP-tagged PLCβ1 construct has been generously provided to the lab by Loren Runnels (UMDNJ) and were used with TRAX constructs fused with eCFP for in vitro and in vivo experiments.

Scope of this project

The role of PLCβ1 in cytosolic and nuclear compartments is unclear. There is evidence that under certain circumstance PLCβ1 localizes to the nucleus and is part of a separate nuclear inositol signaling pathway. Additionally, PLCβ1 is found in the cytosol but its partner $G\alpha_q$ is absent so its role in the cytosol is still a mystery. We show that TRAX and PLCβ1 bind and interact. Our studies also suggest a novel a link between Ga_q activation, PLCb1 localization and siRNA activity through the control of TRAX localization.

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Chapter 2: Identification of a Novel Binding Partner of Phospholipase Cβ1: Translin-Associated Factor X

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(*For this study, Loren Runnels carried out the yeast two-hybrid experiment. All binding experiments and CD data was generated by the first author.*)

ABSTRACT

Mammalian inositol-specific phospholipase Cβ1 (PLCβ1) is activated by $G\alpha_q$ subunits on the plasma membrane where it catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate. Under most circumstances, the major portion of PLCβ1 is localized on the plasma membrane, although a substantial fraction is also found in the cytosol and, under some conditions, in the nucleus. The factors that localize PLCβ1 in these other compartments are unknown but may involve an unknown binding partner. In searching for alternate PLCβ1 interacting proteins, we identified a novel binding partner, translin-associated factor X (TRAX). TRAX is a cytosolic protein that can transit into the nucleus through its nuclear localization signal. In purified form, PLCβ1 binds strongly to TRAX with an affinity that is only ten-fold weaker than its affinity for its functional partner, $G\alpha_q$. While TRAX has little effect on the membrane association or the catalytic activity of PLCβ1, TRAX directly competes with $G\alpha_q$ for PLCβ1 binding, and excess TRAX reverses $G\alpha_q$ activation of PLCβ1. In C6 glia cells, endogenous PLCβ1 colocalizes with

endogenous TRAX in the cytosol and the nucleus. In Neuro2A cells transfected with enhanced yellow fluorescent protein (eYFP)-PLCβ1 and enhanced cyano fluorescent protein (eCFP)- TRAX, Förster resonance energy transfer (FRET) is observed mostly in the cytosol and a small amount is seen in the nucleus, but FRET is absent at the plasma membrane. We propose that the association of TRAX to PLCβ1 serves to stabilize the cytosolic population of PLCβ1.

Introduction

Inositide-specific mammalian phospholipase Cβ (PLCβ) enzymes are the main effectors of the $G\alpha_q$ family of G proteins and are coupled to agents such as angiotensin, dopamine, serotonin, bradykinin, etc. (for review see (Suh, 2008; Rebecchi, 2000; Exton, 1996; Rhee, 2001)). PLCβ catalyzes the hydrolysis of the signaling lipid phosphatidylinositol 4,5 bisphosphate (PI(4,5)P2) to produce the second messengers inositol 1,4,5 trisphosphate and diacylglycerol that in turn stimulate the release of Ca^{2+} from intracellular stores and activate protein kinase C, respectively. In cultured cells, PLCβ1 resides mainly on the plasma membrane where it associates with its activator $G\alpha_q$ and can access its PI(4,5)P2 substrate. In addition to this plasma membrane population, a significant population of PLCβ1 resides in the cytosol, and under some circumstances, in the nucleus. The factors that localize PLCβ1 to these alternate compartments are unknown, especially since PLCβ1 is expected to have a high propensity to localize to the plasma membrane due to its strong, non-specific lipid binding behavior (Runnels, 1996). Additionally, the basal activity of PLCβ1 is very low and it is unclear how it can be

activated in these alternate compartments since $G\alpha_q$ appears to only reside at the plasma membrane (see (Dowal, 2006 and Hughes, 2001)).

There are several possible mechanisms that may underlie the cytosolic localization of PLCβ1. The first might be a saturation of binding sites on the plasma membrane. While we lack the knowledge to accurately quantify binding sites and the local cellular concentration of competing proteins, we note that the cellular concentration of $G\alpha_q$ appears to be higher than PLCβ1 allowing $G\alpha_q$ to interact with its other effectors, phosphatidylinositol 3-kinase and RhoGEF (see (Philip, 2007)). Another possibility is that one or more cytosolic proteins might promote the plasma membrane localization of PLCβ1. With this idea in mind, we searched for alternate protein partners of PLCβ1 using a yeast two-hybrid approach and identified the protein TRAX (translin- associated factor X). TRAX forms strong complexes with its only known partner, translin (Yang, 2004). Translin is a single-stranded DNA and RNA-binding protein with proposed functions in chromosomal translocations in lymphoid cells and mRNA transport and storage in brain and testis (Wang, 2004). Both translin and TRAX are part of the RNA-induced silencing complex (RISC) where they help guide double stranded RNA into the silencing machinery (Liu, 325). Additionally, TRAX has been implicated to function as a localization factor for translin. When the cellular level of translin exceeds TRAX, it remains in the cytosol (Cho, 2004). However, when the cellular level of translin is reduced, translin can partition into the nucleus through the nuclear localization signal (NLS) of TRAX.

Since TRAX appears to regulate the cellular localization of translin, it is possible

that TRAX may similarly modulate the localization of other cellular proteins. In this study, we show that TRAX and PLCβ1 interact strongly in solution and form complexes in living cells. We find that TRAX competes with $G\alpha_q$ for PLCβ1 binding and activation. Our studies link TRAX to G protein signaling and PLCβ1 regulation.

METHODS

Sample Preparation. Purified proteins were used in all in vitro experiments. His- tagged PLCβ1 and $G\alpha_q$ were expressed in Sf9 cells and purified based on previously described methods (see (Runnels, 1998 and Runnels, 1999)). Preparation of C-terminal truncated PLCβ1 has been described (Runnels, 1996). Large, unilamellar vesicles (LUVs), 100nm in diameter, were prepared by extrusion. All lipids (1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE), 1 -palmitoyl-2- oleoylphosphatidylserine (POPS), 1 -palmitoyl-2-oleoylphosphatidylcholine (POPC)) were purchased from Avanti Polar Lipids (Alabaster, AL) with the exception of the tritiated PI(4,5)P2 which was purchased from Perkin Elmer. TRAX cDNA purchased from Open Biosystems was cloned into pET32a expression vectors purchased from Novagen. His-tagged TRAX was expressed in Rosetta cells and purified on a Ni-NTA column. The integrity of the TRAX preparation was determined by western blot analysis and circular dichroism spectra (see results).

Protein Labeling and Reconstitution. In vitro binding studies were carried out by labeling either the PLCβ1 or $G\alpha_q$ constructs with the thiol reactive probe 7-diethylamino-3- (4'-maleimidylphenyl)-4-methylcoumarin (CPM, Invitrogen, Inc) as previously described to

yield fluorescent proteins with an ~1:1 probe:protein ratio (see (Philip, 2006)). We note that labeling by this method does not affect the activity of PLCβ1 or its ability to be stimulated by Gαq. To prevent protein aggregation, Gαq was labeled in the presence of storage detergents (0.1% lubrol) and was reconstituted into preformed extruded lipid vesicles by simple addition.

Optical Measurements and Data Analysis. CD spectra were obtained on an Olis RSM 1000 CD spectrophotometer (On-line Instrument Systems, Inc. Bogart, GA). TRAX concentration was 20μM in 20 mM Hepes, 0.1 M NaCl, pH 7.4. Binding affinity measurements were performed on an ISS spectrofluorometer (Urbana, IL) using a 3 mm cuvette (see (Philip, 2006)). CPM- labeled proteins were excited at 380 nm and scanned 420 to 560 nm. Binding affinity was determined as a function of the increase in CPM fluorescent intensity as non-fluorescent protein was incrementally added. Samples were corrected using control cuvettes that substituted buffer for non-fluorescent protein. The integrated spectral areas were calculated using ISS Vinci software, and the resulting curves were fit to a bimolecular dissociation constant using Sigma Plot (Jandel, Inc.). Membrane binding studies were carried out by measuring the change in intensity of a 20 μM solution of CPM-PLCβ1 as freshly extruded large unilamellar vesicles were incrementally added. The data were corrected for background by carrying out an identical titration where buffer was substituted for CPM-PLCβ1. The corrected data were then fit to a hyperbolic curve using Sigmaplot (Jandel, Inc.).

Activity Measurements. Measurements were made using full length and truncated His-tagged PLCβ1, $G\alpha_q$, and TRAX and small, unilamellar vesicles consisting of PI(4,5)P2,

POPE, POPS (1:1:1) doped with 3H-PI(4,5)P2 prepared by sonication (see (Runnels, 1999)). Briefly, 2mM of lipid were incubated at 37°C with PLCβ1 in the linear range of the activity as determined for each by running a time course experiment (usually between 2 and 5 minutes). The reaction was initiated by the addition of Ca^{2+}. Activities are reported as the percent of radioactive PI(4,5)P2 hydrolysis that occurred.

Cell Culture. Neuro2A cells (HEK293) were cultured in 50/50 DMEM/F12 media supplemented 10% FBS. C6 glioma cells were cultured in DMEM containing 10% FBS, 100mM sodium pyruvate and 1% PenStrep at 37°C with 5% CO2.

DNA was transfected into cells by electroporation using a protocol adapted from Maniatis (Sambrook, 2001). Cells were grown to near 100% confluence and washed with sterile PBS. The cells were then trypsinized, centrifuged 5 minutes at 1500 x g and resuspended in 10mL of fresh growth medium. 800µl of cells were pipeted into a 0.4cm BioRad cuvette and placed in an electroporator (BioRad Gene Pulser Xcell). Cells were then plated and covered with fresh medium.

Immunofluorescence. Cells were fixed and stained with primary antibodies to TRAX and PLCβ1 (Santa Cruz Biochemicals, Inc.). In certain cases as noted in the text, eYFP-tagged PLCβ1 was transfected via electroporation prior to fixing. Cells were initially washed with PBS + 1mM Ca^{2+} and 2 mM Mg^{2+}. Cells were then fixed with 3.7% formaldehyde in PBS and permeabilized with a solution of 0.2% NP40 in PBS. After, the cells were blocked in 4% goat serum in 1X TBS, washed and a primary antibody added.

Cells were incubated for 1 hour, washed and treated with a secondary antibody. After another wash, the cells were viewed on either a Zeiss Axiovert 200M with an AxioCam MRm camera, or an Olympus Fluoview FV1000 laser confocal microscope. Data were analyzed using either Olympus (Fluoview) software or Image J (NIH).

FRET analysis. A fully functional eYFP-tagged PLCβ1 was cotransfected with TRAX fused with eCFP. Cells were allowed to incubate for 48 hours. Afterwards the live cells were viewed through the CFP, YFP and FRET channels and FRET measurements on regions of interest were made using sensitized emission on an Olympus Fluoview FV1000 laser confocal microscope (for details, see (Golebiewska, 2008)). Analysis was done using Fluoview software (Olympus, Inc.).

RESULTS

Identification of TRAX as a PLCβ1 binding partner. A yeast two-hybrid (Y2H) screen of a rat brain library with the C2 domain-containing COOH-terminus of rat PLCb1 (a.a. 643-1216) was performed to identify novel interacting partners important to the functional regulation of PLCβ1 and uncovered the TRPM7 ion channel as a binding partner (Runnels, 2001 and Runnels, 2002). Among the 69 rat Y2H clones from the screen that were sequenced based on selected growth and β-galactosidase activity, three overlapping clones were identified as translin-associated factor X (TRAX). Since TRAX has a cytosolic and nuclear localization, we carried out further tests to determine whether it is a PLCβ1 binding partner.

Initially, we directly measured the association between purified TRAX and purified PLCβ1 in solution. While the integrity of purified PLCβ1 is readily assessed by its catalytic activity and its ability to be activated by $G\alpha_q$, TRAX does not have a catalytic function. Thus, we assessed its folding by circular dichroism (CD) spectroscopy. In **Fig. 2-1** we show the CD spectrum of a solution of 20 μM TRAX in buffer. We find that the TRAX CD spectrum displays a high degree of secondary structure that corresponds to ~80% helical content (http://www.embl.de/~andrade/k2d). This high helical content is expected from its sequence where it is predicted to have over 50% helical structure with the remainder being loops (www. predictprotein.org)

Fig. 1

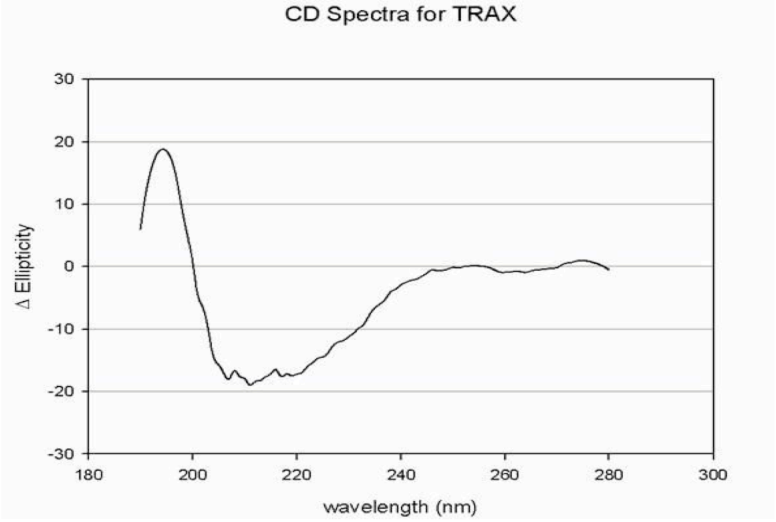

Figure 2: CD spectra for TRAX. Circular dichroism spectrum of 20 μM TRAX in 20 mM Hepes, 160 mM NaCl, pH 7.4. The predicted secondary structure for TRAX is predicted to be mainly alpha helical. Analysis of spectra reveals that TRAX has a high degree of secondary structure that corresponds to approximately 80% helical content with some loop sequences.

We measured the association between TRAX and PLCβ1 using fluorescence methods. In these experiments His6-PLCβ1 was expressed in Sf9 cells and purified, and then labeled with the fluorescent probe CPM (diethylamino-3- (4'-maleimidylphenyl)-4-methylcoumarin). We then monitored changes in the fluorescence spectrum of CPM-PLCβ1 with the addition of purified TRAX (see methods and (Philip, 2006) for details). We find that the fluorescence intensity increased systematically with the addition of TRAX without a significant shift in the emission spectrum. Fitting this increase (an 80% increase relative to control samples that did not contain TRAX) to a bimolecular dissociation curve gives an apparent dissociation constant of K_d = 8 +/- 1 nM (**Fig. 2-2A**). Interestingly, this apparent K_d is only ~10 fold weaker than the one measured for the interaction between PLCβ1 and activated $G\alpha_q$ (Runnels, 1999).

The COOH-terminal region of PLCβ1 was used as bait to identify TRAX in the yeast 2- hybrid screen. Repeating the fluorescence titrations using a COOH-terminal deletion mutant of PLCβ1 (PLCβ1–ΔCT) results in a drastic reduction in its affinity for TRAX (**Fig. 2-2B**). This result confirms that the primary interaction site between TRAX and PLCβ1 is within the C-terminal region.

Fig. 2 TRAX binds strongly to PLCβ1.

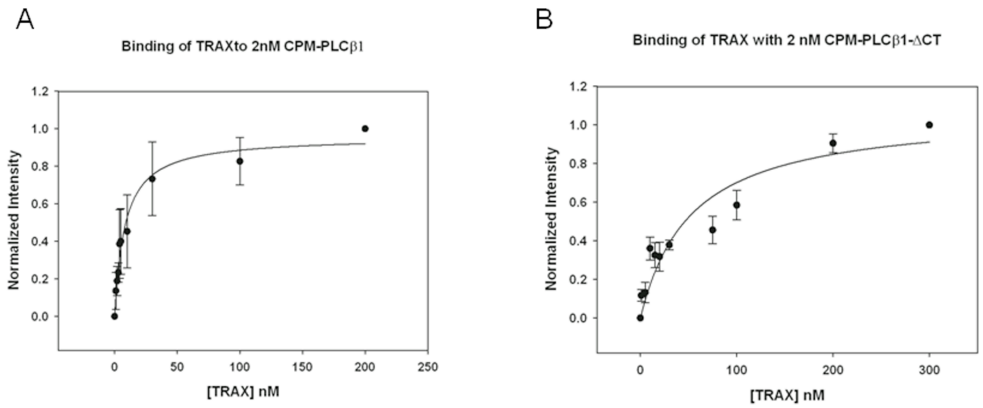

Figure 2 (A) Binding of TRAX to 2 nM CPM-PLCβ1 as monitored by the increase in CPM intensity where the normalized fluorescence intensity is shown as a function of TRAX concentration. In these studies, an 80% increase in intensity was observed as compared to control samples that substituted buffer for TRAX. Also shown is the fitted curve to a bimolecular dissociation constant where *Kd* = 8 + 1 nM (n=6 and S.D. is shown). B – Identical study as 2A except that the COOH-terminal deletion mutant of PLCβ1 (PLCβ1-ΔCT) was used instead of the full-length enzyme (n=3 and S.D. is shown). While a binding curve is shown to guide the eye, the affinity between the proteins was too weak to be accurately fit to a bimolecular dissociation constant. We note that the total change in CPM intensity was also ~80% at the end of the titration.

TRAX competes with Gαq-GTP for binding to PLCβ1. PLCβ1 strongly associates with $G\alpha_q$-GTP on membrane surfaces (K_d = 0.67 nM) through sites in its C2 and COOH- terminal regions (Park, 1993 and Wang, 1999). Since both TRAX and $G\alpha_q$ bind to the COOH-terminal region, we asked whether TRAX would compete with binding of $G\alpha_q$ to PLCβ1. For these studies, purified $G\alpha_q$ activated with non-hydrolyzable GTPγS was labeled with CPM and reconstituted onto large unilamellar

vesicles (LUVs) composed of POPC:POPS:POPE (1:1:1). We measured the dissociation constant for PLCβ1 and $G\alpha_q$-GTPγS in the absence and presence of excess TRAX (300nM). The presence of TRAX resulted in a 3-fold reduction in PLCβ1-$G\alpha_q$-GTPγS binding affinity. While this is a modest reduction there is some competition between TRAX and $G\alpha_q$ for binding to PLCβ1 (**Fig 2-3A**).

PLCβ1 binds strongly to lipid membranes where it associates with $G\alpha_q$ and accesses its substrate. Membrane binding of PLCβ1 has been found to be primarily mediated through the N- terminal PH domain of PLCβ1 and to a lesser extent, its C-terminal region (Runnels, 1996). Since TRAX might affect the association between PLCβ1 and $G\alpha_q$ by altering its membrane binding affinity, we determined whether TRAX would interfere with PLCβ1's association with membranes. We find it does not (**Fig. 2-3C**), which correlates well with the finding that TRAX associates with the C-terminal region of PLCβ1.

We repeated the above study substituting unactivated $G\alpha_q$(GDP) for activated $G\alpha_q$ (GTPγS). It is worth noting that in the deactivated state, the affinity between $G\alpha_q$ and PLCβ1 is reduced by a factor of ~50 (Runnels, 1999). We find that TRAX no longer competes with deactivated $G\alpha_q$ for binding to PLCβ1. This result suggests that deactivation of $G\alpha_q$(GDP) alters its binding interaction with PLCβ1, consistent with previous work (Dowal, 2006), to a site that is less competitive for TRAX association (**Fig. 2-3B)**.

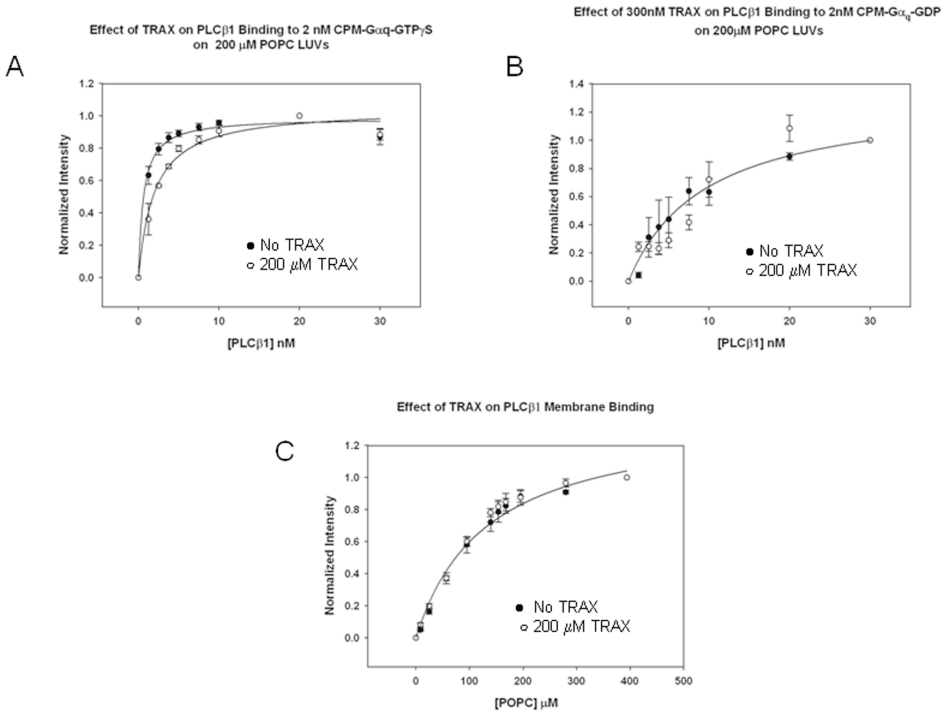

Figure 2 A-Binding of PLCβ1 to 2 nM activated CPM-Gαq(GTγS) in the absence (•) and presence (○) 200 nM TRAX right panel showing the loss in Gαq affinity when TRAX is present, where n=6 and S.D. is shown. We note that an ~20% increase in CPM intensity was seen both without and with TRAX. B- Binding of PLCβ1 to PC:PS:PE (1:1:1) large, unilamellar vesicles in the absence (Kp = 132μM) and presence (Kp = 120μM) of 200 nM TRAX as measured by the increase in CPM intensity as LUVs are titrated into the solution, where n=3 and S.D. is shown. C- Binding of PLCβ1 to 2 nM deactivated CPM-Gαq (GDP) in the absence (•) and presence (○) of 200 nM TRAX, where n=3 and S.D. is shown.

TRAX interferes with the activation of PLCβ1 by $G\alpha_q$ subunits. We next determined whether TRAX has the ability to modulate the enzymatic activity of PLCβ1 or its activation by $G\alpha_q$. These studies were carried out by monitoring the amount of PI(4,5)P2 hydrolysis catalyzed by 2 nM PLCβ1 in the presence and absence of TRAX. We find that a 300 molar excess of TRAX does not greatly affect the initial velocity of the reaction catalyzed by PLCβ1, but reduces the velocity at later times, suggesting that

TRAX causes a small reduction in the maximum rate (**Fig. 2-4A**). We then tested whether TRAX could block PLCβ1 activation by $G\alpha_q$. We find that TRAX prevents the activation of PLCβ1 by $G\alpha_q$ (**Fig. 2-4B**), which is consistent with the ability of TRAX to disrupt the association between PLCβ1and $G\alpha_q$(GTPγS) (**Fig. 2-3A**).

Fig. 4

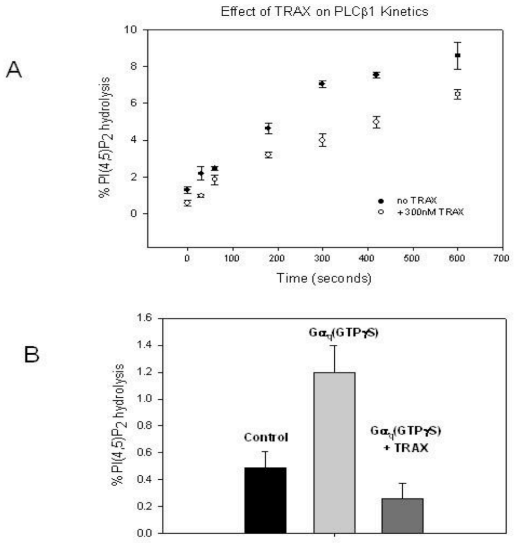

Figure 2 (A) The effect of 300 nM TRAX on the rate of PI (4,5) P2 hydrolysis catalyzed by 25nM PLCβ1 (n=3 and S.D. is shown). As can be seen, TRAX does not affect the initial velocity of the curve. B- Prevention of activation of 5 nM PLCβ1 by 5nM Gαq by 300 nM TRAX, where n=8 and S.D. is shown.

TRAX and PLCβ1 are associated in cultured cells. We verified that TRAX and PLCβ1 associate in cells using fluorescence microscopy. First, we used immunofluorescence to determine whether endogenous TRAX and endogenous PLCβ1 are colocalized in C6 glia cells. The images in **Fig. 2-5** show almost complete colocalization of the proteins throughout the cell suggesting association between the two proteins.

Since colocalization will only indicate whether the proteins reside in the same region of the cell, we measured the physical association of TRAX and PLCβ1 by Förster resonance energy transfer (FRET) using attached "donor" and "acceptor" fluorescent probes. For these studies, we linked enhanced yellow fluorescence proteins (eYFP) to PLCβ1 and enhanced cyano fluorescence protein (eCFP) to TRAX. The observation of FRET from eCYP donors to eYFP acceptors indicates that the probes are at least within 30 Å of each other (Patterson, 2000), implying that the two labeled proteins are physically associated.

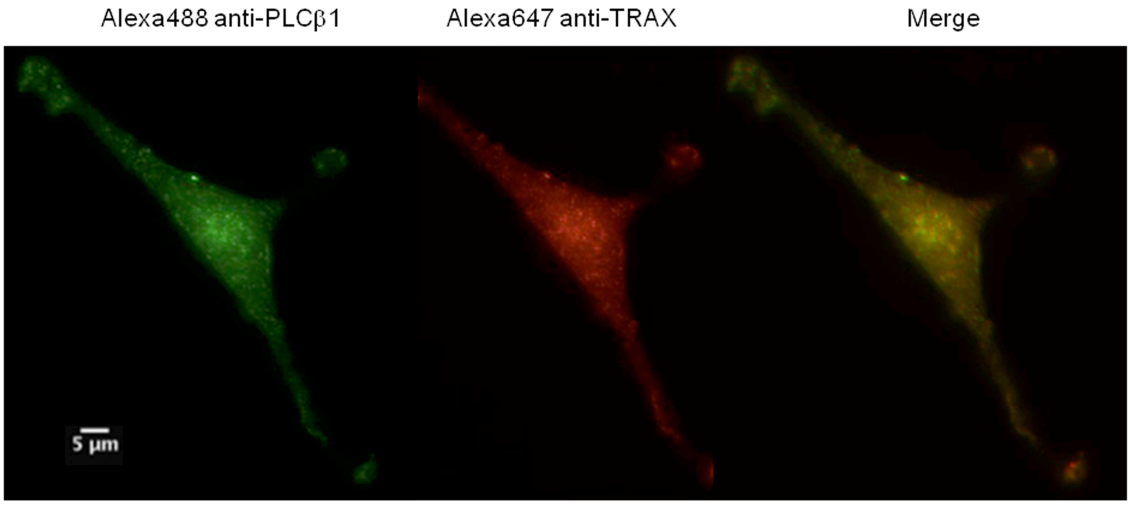

Figure 2-5: Example of a co-immunofluorescence study of endogenous PLCβ1 (*left panel*) as visualized by Alexa488-labeled antibody, TRAX (*middle panel*) visualized by Alexa647-labeled antibody and the resulting merged image (*right panel*) in C6 glial cells. The scale bar is 5 µm.

We transfected Neuro2A cells with eYFP-PLCβ1 and eCFP-TRAX and measured the amount of FRET by sensitized emission (see methods) using a confocal microscope. FRET values were compared to a positive control consisting of eYFP-X12-eCFP and a negative control consisting of free eYFP and eCFP (see (Dowal, 2006)). An example of a

FRET study is shown in **Fig. 2-6**. We find that a large degree of FRET occurs between the proteins in the cytosol while only a small amount is seen in the nucleus and none on the plasma membrane compartments (**Fig. 2-6**). Averaging the amount of FRET over the entire cell gives a value of 40.1 + 0.6 % (*n=5*). The lack of FRET in the nucleus contrasts with the coimmunofluorescence results in C6 glia cells and can be explained by a lack of PLCβ1 in the nuclear compartment. Additionally, the lack of FRET on the plasma membrane reflects an absence of TRAX. Thus, these studies show that TRAX and PLCβ1 associate in the cytosol of cells.

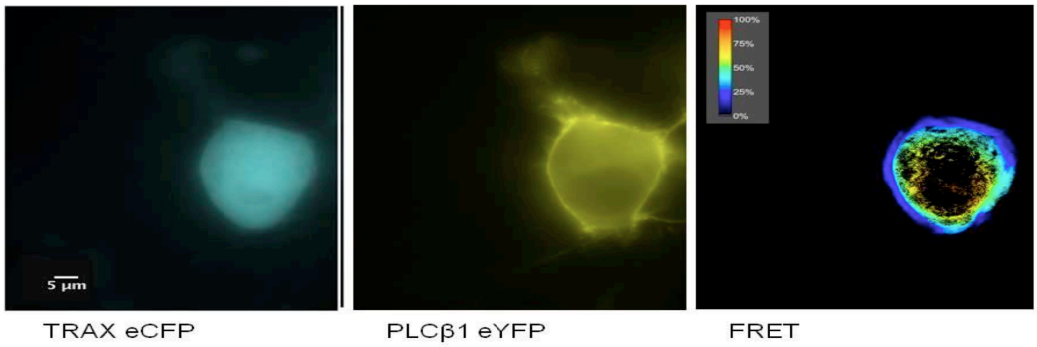

Figure 2-6: Example of a FRET study showing the raw images of eYFP-PLCβ1 (*left panel*), eCFP-TRAX (*middle panel*) and the normalized FRET image (*right panel*) in transfected Neuro2A cells where amount of FRET is determined by the sensitized emission (see (*6*). The scale bar is 5 µm.

DISCUSSION

In this study, we have identified TRAX as a novel cellular binding partner of PLCβ1. The potential interaction between PLCβ1 and TRAX was found in a yeast 2-hybrid experiment using the 574 residue C2-domain containing COOH-terminal region of PLCβ1 as bait, and subsequent binding studies between the purified proteins verified this interaction.

The COOH-terminal region of PLCβ enzymes distinguishes this family from other

PLCs. This region contains the only identified nuclear localization signal of PLCβ1 (a nuclear export signal on PLCβ1 has never been identified), as well as mitogen-activation protein kinase and protein kinase C phosphorylation sites (we note that unpublished studies by our group suggest that PKC phosphorylation does not affect the interaction between TRAX and PLCβ1: Aisiku, Dowal & Scarlata, *unpublished*). Importantly, the COOH-terminal region of PLCβ1 is necessary for Gαq activation, and has GTPase-promoting activity (Park, 1993 and Berstein, 1992). Therefore, it is not surprising that TRAX competes with Gαq for binding to PLCβ1. The crystal structure of the isolated COOH-terminal region of PLCβ1 has been solved and is found to be an intertwined helical dimer (Singer, 2002), although it is not clear whether PLCβ1 itself is dimeric. While it is impossible to speculate the mode of interaction between TRAX and PLCβ1, it is notable that translin, TRAX's known binding partner, is comprised of a network of helixes (Sugiura, 2004). CD data and structure prediction programs also suggest that TRAX itself is comprised of a network of alpha helixes.

We assessed the association between endogenous TRAX and PLCβ1 in C6 glial cells, which express these proteins at high levels. We speculate that the high level of expression of PLCβ1 in these cells is responsible for the low plasma membrane localization relative to other compartments. We find that the two proteins co-localize throughout the cell. We then visualized the associated proteins using FRET by co-expressing fluorescent-tagged proteins in a cell line where their endogenous expression is more limited (i.e. Neuro2A cells). We find that the protein complexes largely reside in the cytosol, since little PLCβ1 localizes to the nucleus and since TRAX is not found on the

plasma membrane in these cells. Interestingly, the FRET values that have been reported for PLCβ1 and Gαq (Dowal, 2006) are close to the FRET values between PLCβ1 and TRAX observed here. PLCβ1 is known to have a high plasma membrane population due to its strong interaction with Gαq both in the basal and stimulated states (Dowal, 2006). The ability of TRAX to hold PLCβ1 in the cytosol despite the higher affinity of the enzyme for Gαq and the concentrating effect of the membrane in promoting protein association correlates well with the strong association between TRAX and PLCβ1. It is interesting to note that recent biochemical studies suggest that Gαq and PLCβ1 bind to a protein scaffold on the plasma membrane, which stabilizes their interactions (Garcia, 2010).

While the majority of PLCβ1 resides on the plasma membrane, a significant amount is also seen in the cytosol. The role of PLCβ1 in the cytosol is unclear especially since its activity is too low to be expected to impact the level of PI(4,5)P2 levels in internal membranes without being activated. We find that the activity of PLCβ1 is not significantly affected by TRAX. However, this low activity might be sufficient to keep internal PI(4,5)P2 at basal levels.

In C6 glia cells, TRAX and PLPLCβ1 appear to be co-localized in the nucleus as well as the cytosol. Since both proteins have a nuclear localization sequence, it is just as likely that either partner could be responsible for nuclear transit. Nuclear transit might occur through exposure of either nuclear localization signal upon protein association or upon saturation of PLCβ1's plasma membrane and cytosolic sites. Although PLCβ1 is not considered a nuclear protein, it has been found in the nucleus under some conditions

(see (Manzoli, 1999 and Cocco, 2000)). We observe large amount of the enzyme in undifferentiated PC12 cells (Dowal & Scarlata, *unpublished*). Cocco and colleagues find PLCβ1 travels to the nucleus in Swiss 3T3 cells upon stimulation with insulin-like growth factor (Manzoli, 1997). The role of PLCβ1 in the nucleus is unknown, but is assumed to be involved in the nuclear phosphatidylinositol signaling pathway (Cocco, 2006). It is notable that PLCβ1 does not appear to be associated with the nuclear membrane.

The identification of TRAX as a potential binding partner of PLCβ1 is surprising since TRAX is not known to be associated with inositol phosphate signaling. One study did report a link between TRAX and activation of a G protein coupled receptor linked to cAMP (Sun, 2006) giving rise to the possibility that TRAX may modulate additional aspects of G protein signaling. To date, TRAX has only been known to bind to translin and modulate its cellular localization (Cho, 2004). It has recently been found that TRAX and translin are part of the RNA-induced silencing complex, and their presence helps to guide RNA insertion into the complex to induce gene silencing (Liu, 2009). PLCβ1 has been shown to reside in nuclear speckles (Cocco, 2006 and Cocco, 2002), which are storage deposits for splicing factors and other transcription machinery (for review see (Lamond, 2003)). The localization of PLCβ1 in speckles suggests a possible role in RNA splicing and/or in mRNA processing.

While the strong binding constant and large FRET value measured for cytosolic PLCβ1 and TRAX suggest a specific cellular role, the functional consequence of these proteins' association is not yet clear. One possibility is that TRAX buffers PLCβ1 in the

cytosol until Gαq becomes activated to high enough levels to displace TRAX from PLCβ1. TRAX may also regulate the entry of PLCβ1 into the nucleus so that it may participate in RNA processing. Alternately, PLCβ1 may regulate entry of TRAX into the nucleus or its association to translin. These functional studies are presently underway.

ACKNOWLEDGEMENTS

The authors are grateful for funding from NIH GM53132, to Dr. Steven Smith (Stony Brook University) for the use of his circular dichroism instrument, and to Drs. Guo and Rebecchi (Stony Brook University) for their helpful comments.

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Chapter 3: Phospholipase Cβ1 is Linked to RNA interference through specific binding to Translin-Associated Factor X

(Colocalization between overexpressed eYFP-PLCβ1 and TRAX was performed by Finly Philip. The GAPDH siRNA experiment was performed by Yuanjian Gao. The yeast two-hybrid experiment was carried out by Loren Runnels. All other work was performed by the first author).

Phospholipase Cβ1 is Linked to RNA interference through specific binding to Translin-Associated Factor X

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ABSTRACT

Mammalian inositol-specific phospholipase Cβ1 (PLCβ1) is activated by $G\alpha_q$ subunits on the plasma membrane where it catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate. PLCβ1 is distributed on the plasma membrane, as well as in the cytosol and nucleus. However, the functions of PLCβ1 in the latter compartments are unknown. We recently identified a novel binding partner of PLCβ1, translin-associated factor X (TRAX) and found that TRAX competes with $G\alpha_q$ for PLCβ1 binding. TRAX is a cytosolic protein that can partition into the nucleus where it participates in RNA-induced silencing. In HEK293 cells, PLCβ1 interacts strongly with TRAX in the cytosol. Activation of $G\alpha_q$ by carbachol stimulation results in movement of TRAX into the nucleus concurrent with activation of PLCβ1. Increasing the level of PLCβ1 in the cytosol causes movement of TRAX out of the nucleus suggesting that PLCβ1 impacts the cellular localization of TRAX. Movement of TRAX out of the nucleus due to an increase in the level of cytosolic PLCβ1 is associated with reversal of small interfering RNA-induced down-regulation of a housekeeping protein, glyceraldehye-3-phosphate

dehydrogenase. Our studies suggest a novel a link between $G\alpha_q$ activation, PLCβ1 localization and siRNA activity through the control of TRAX localization.

INTRODUCTION

Phospholipase Cβ (PLCβ) enzymes are the main effectors of the $G\alpha_q$ family of G proteins (for reviews see (Illenberger, 2003; Boggon, T.J., 1999;)). This family of enzymes catalyze the hydrolysis of the signaling lipid phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) to produce the second messengers inositol 1,4,5 trisphosphate and diacylglycerol that stimulate the release of Ca^{2+} from intracellular stores and activate protein kinase C, respectively. In PC12 cells, PLCβ1 is the major isoform in neuronal cell lines where it resides mainly on the plasma membrane in association with $G\alpha_q$ (Dessy, 2000). Also, a significant population of PLCβ1 is localized in the cytosol where it may regulate internal $PI(4,5)P_2$ stores. Additionally, PLCβ1 can be found in the nucleus. The nuclear localization of PLCβ1 depends on the state of cellular differentiation, cell cycle and external conditions (see (Feng, 2001; Cocco, 2002)). The partitioning of PLCβ1 in the nucleus may result from exposure of PLCβ1's nuclear localization signal (NLS) or changes in phosphorylation of S888 by MAPK or dephosphorylation of S887 by nuclear protein kinase Cα(Cocco, 2002). The regulation and function of PLCβ1 in these alternate compartments is unknown.

We have recently identified the protein TRAX (translin-associated factor X) as a cellular PLCβ1 binding partner. TRAX has been shown to form strong complexes with translin (Cocco, 2006); a single-stranded DNA and RNA-binding protein with proposed

functions in chromosomal translocations in lymphoid cells and mRNA transport and storage in brain and testis (Lamond, 2003). Recently it was found that translin/TRAX are novel activators of the RNA-induced silencing complex (RISC) in flies where their endonuclease activity promotes the removal of siRNA passenger strand cleavage products (Bahk, 1994).

TRAX binds translin in the cytosol which allows the complex to partition into the nucleus through TRAX's nuclear localization signal (Peruzzi, 2002). It is possible that TRAX may similarly modulate the localization of other cellular proteins, such as PLCβ1. In this study, we show that PLCβ1 binds to TRAX in the cytosol, and that TRAX can be released from PLCβ1 upon carbachol stimulation of $G\alpha_q$ allowing TRAX to move into the nucleus where it can form RISC. Reciprocally, raising the level of PLCβ1 in the cytosol causes movement of TRAX out of the nucleus resulting in a reversal of siRNA activity. This study directly links $G\alpha_q$ and PLCβ to RNA silencing through TRAX.

MATERIALS AND METHODS

Cell Culture. Stably transfected human embryonic kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1mM sodium pyruvate and incubated at 37° C with 5% CO_2. C6 glioma cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate and 1% PenStrep at 37° C with 5% CO_2.

DNA was transfected into cells by electroporation using a protocol adapted from Maniatis. Cells were grown to near 100% confluence and washed with sterile PBS. The

cells were then trypsinized, centrifuged 5 minutes at 1500 x g and resuspended in 10mL of fresh growth medium. 800µl of cells were pipeted into a 0.4cm BioRad cuvette and placed in an electroporator (BioRad Gene Pulser Xcell). Cells were then plated and covered with fresh medium.

Immunofluorescence. Cells were fixed and stained with primary antibodies to TRAX (Santa Cruz Biochemicals, Inc.) and PLCβ1 (Santa Cruz Biochemicals, Inc.). In certain cases as noted in the text, eYFP-tagged PLCβ1 was transfected via electroporation prior to fixing. Cells were initially washed with PBS + Ca^{2+}, Mg^{2+} (1mM and 2mM respectively). Cells were then fixed with 3.7% formaldehyde in PBS and permeabilized with a solution of 0.2% NP40 in PBS. After, the cells were blocked in 4% goat serum in 1X TBS, washed and a primary antibody added. Cells were incubated for 1 hour, washed and treated with a secondary antibody. After another wash, the cells were viewed on either a Zeiss Axiovert 200M with an AxioCam MRm camera, or an Olympus Fluoview FV1000 laser confocal microscope. Data were analyzed using either Olympus (Fluoview) or Image J software.

FRET analysis. A fully functional eYFP-tagged PLCβ1 was cotransfected with TRAX fused with eCFP. Cells were allowed to incubate for 48 hours. Afterwards the live cells were viewed and FRET measurements on regions of interest were made using sensitized emission on an Olympus Fluoview FV1000 laser confocal microscope. Analysis was done using Fluoview software.

Preparation of nuclei. HEK293 cells were grown and harvested by centrifugation at 600g for 5 minutes. The cells were resuspended in 5 volumes of prechilled (4°C) PBS

and centrifuged for minutes as in step 1. The washed pellet was resuspended in 10 volumes of a hypotonic buffer solution (Active Motif Inc., Carlsbad, CA) and allowed to sit on ice for 10 minutes. Cells were checked for swelling under a phase contrast microscope. Once 90% of the cells had swollen they were pipetted into a prechilled Dounce-type homogenizer. They were subjected to 10-12 quick upward strokes of the pestle. The cells were examined again and once 90% of the cells consisted of free nuclei they were taken to the next step. The suspension was centrifuged at 1000g for 3 minutes to pellet the nuclei. The pellet was resuspended in 10 volumes of the hypotonic buffer solution. Centrifugation and subsequent resuspension was repeated twice and at this point individual nuclei were present.

siRNA studies. For the following studies GAPDH siRNA was purchased from Ambion Inc. PLCβ1 siRNA was purchased from Dharmacon Inc. A 2μM siRNA solution in 1X siRNA buffer was prepared. In separate tubes, the appropriate volume of 2μM siRNA plus serum-free medium (Tube 1) and the appropriate volume DharmaFECT (Dharmacon Inc.) transfection reagent plus serum-free medium (Tube 2) was prepared. Each individual tube was mixed and incubated for 5 minutes at room temperature. The content from Tube 1 was added to Tube 2 and allowed to incubate for 20 minutes at room temperature. A sufficient volume of antibiotic-free complete medium was added to the mix in the preceding step for the desired volume of transfection medium. Culture medium was removed from the cells followed by addition of the transfection medium. The cells were incubated at 37°C in 5% CO_2 for 72 hours.

RESULTS

PLCβ1 and TRAX are associated in the cytosol of HEK293 cells. Since the endogenous level of PLCβ1 is low, we transfected the cells with eYFP-PLCβ1 and determined its colocalization with endogenous TRAX using a fluorescent-tagged polyclonal TRAX antibody in fixed cells (**Fig. 3-1**). In accord with previous studies [3], PLCβ1 localizes primarily to the plasma membrane and has a significant cytosolic population. Very little enzyme is seen in the nucleus. Alternately, TRAX is found in the cytosol and nucleus but not on the plasma membrane. The absence of TRAX from the plasma membrane is thought to be due to a lack of intrinsic membrane affinity as well as competition for PLCβ1 binding sites by $G\alpha_q$ (*see Chapter 2*).

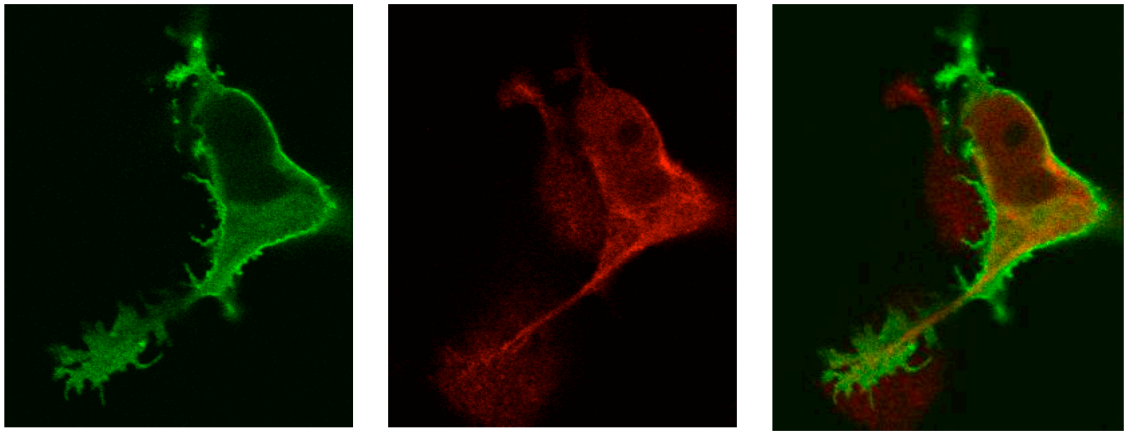
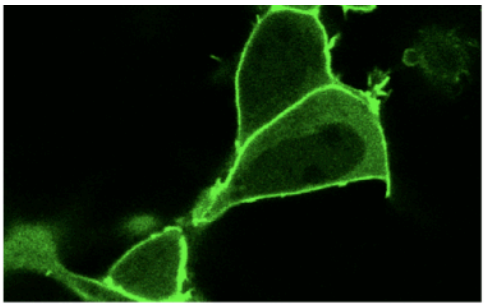


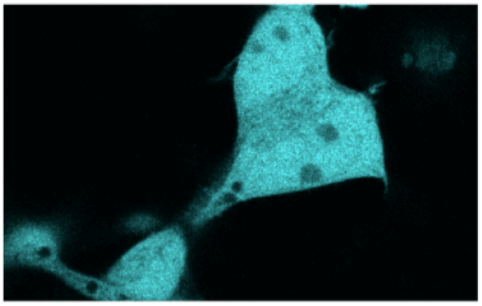
Figure 3-1 : ***Colocalization of PLCβ1 and TRAX.*** **Images of HEK293 cells expressing eYFP-PLCβ1** ***(left)*****, and immunostained with anti-TRAX visualized by Alexa647 secondary antibody** ***(middle).*** **No bleed-through between the YFP and Alexa5657 channels was detected. Merging the two images shows a high degree of colocalization of the two proteins both in the cytosol and nucleus** ***(right).***

To determine whether TRAX and PLCβ1 are physically associated in cells, we measured their interaction by Förster resonance energy transfer (FRET). These studies were carried out by cotransfecting cells with eYFP-PLCβ1 and eCFP-TRAX. The extent of FRET is highly dependent on the distance between the fluorophores and for the eCFP/eYFP pair, will only occur when the probes are within 25-35Å (Baek, 1996). Given the size of the proteins and the placement of the probes (the N-terminus and C-terminus for PLCβ1 and TRAX, respectively), the appearance of FRET would indicate that the proteins are complexed.

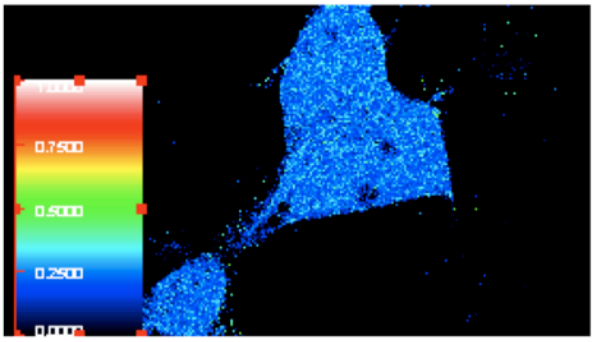
In **Fig. 3-2** we show an example of an eYFP-PLCβ1 / eCFP-TRAX FRET study. As can be seen, a large amount of FRET occurs throughout the cell except for the plasma membrane. Even though little PLCβ1 is found in the nucleus, the same proportion of it is associated with TRAX as the cytosolic population. If we compare the FRET values seen in these two compartments with the positive control, eCFP-X_{12}-eYFP (FRET= 0.38 $\pm$ 0.002) and a negative control using free CFP and free YFP (FRET = 0.001 $\pm$ 0.001), then a normalized value (normalized to CFP-X_{12}-eYFP as 100%) of 0.78$\pm$0.02 is obtained for the cytosolic population and 0.58$\pm$0.12 is obtained for the nuclear population. These results suggest that a large fraction of PLCβ1 and TRAX are physically associated. The association of endogenous PLCβ1 and TRAX the proteins was also verified in a pull-down experiment (**see Fig. 3-3**).



eYFP-PLCβ1



TRAX-eCFP



FRET efficiency

Figure 3-2: ***Association between PLCβ1 and TRAX as determined by FRET.*** **A. HEK293 cells were cotransfected with eYFP-PLCβ1 and eCFP-TRAX. FRET was detected by sensitized emission and measured on an Olympus Fluoview confocal microscope as previously described (Guo et al. in press.**

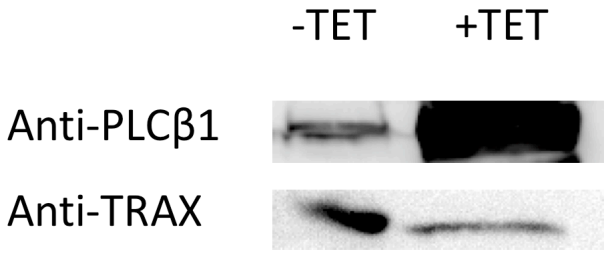


Figure 3-3: **<u>Pull-down assay:</u> After induction with tetracycline, PLCβ1, which has been modified to contain both streptavidin and calmodulin binding sites, is expressed. TRAX and PLCβ1 were copurified using beads coated with streptavidin. Both proteins were eluted in a 50mM Tris-HCl buffer containing 150mM KCl, 2mM EDTA, 0.1% Igepal CA-530, 5mM 2-mercaptoethanol, 0.1mg/ml BSA and 2mM D-biotin.**

The amount of PLCβ1 in the nucleus varies depending on the cell type, growth conditions and expression level (Feng, 2001; Cocco, 2002). It has been found that phosphorylation of S887 of PLCβ1 by nuclear protein kinase Cα promotes its exit from the nucleus (Cocco, 2002). We determined whether phosphorylation of S887 could play a role in the cellular interaction between PLCβ1 and TRAX by repeating the FRET study with two PLCβ1 point mutants; one that cannot be phosphorylated (eYFP-PLCβ1 S887A) and a phosphorylation mimic (eYFP-PLCβ1 S887D). Z-stack FRET images confirm association of the proteins in both the nucleus and cytosol (**see Fig. 3-4**). We find that the amount of FRET between eCFP-TRAX and the two mutants, eYFP-PLCβ1 S887A and S887D, are similar to the wild type in the cytosol and nucleus (**see Fig. 3-5**). These results suggest that TRAX-PLCβ1 interaction is not regulated by PKC phosphorylation.

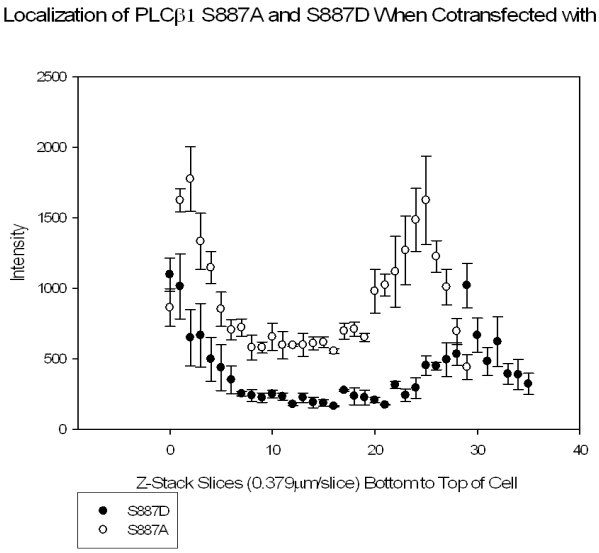


Figure 3-4 - Comparison of Z stack data for cells cotransfected with both TRAX and eYFP-PLCb1S887A or eYFP-PLCb1S887D

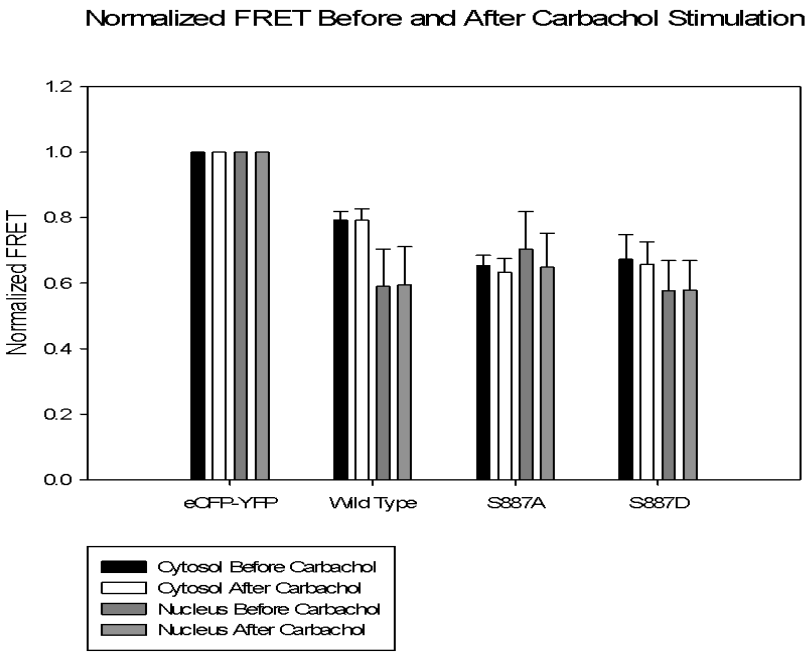
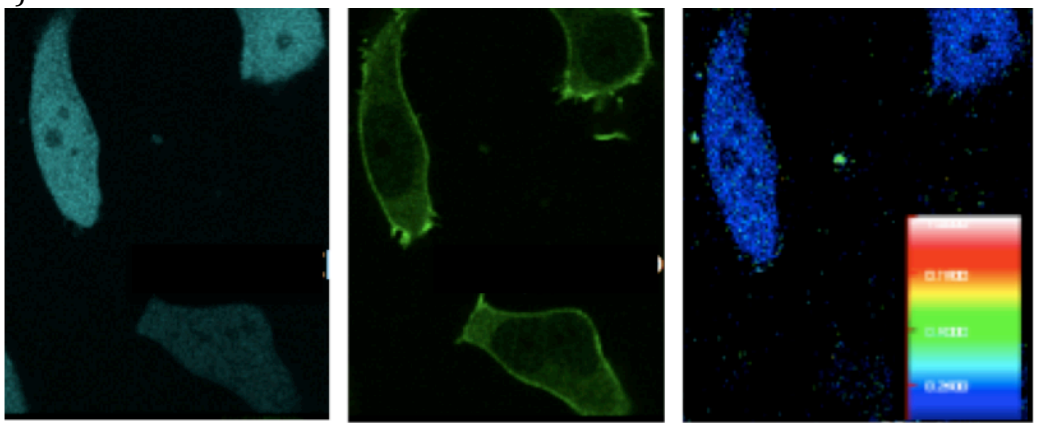


Figure 3-5: HEK293 cells were cotransfected with TRAX-eCFP and eYFP-PLCβ1. After 48 hours, FRET was monitored before and after stimulation with 1μM carbachol as described in the text. Data for the wild type of PLCβ1 are a compilation of 3 measurements and the SD is shown. Data for the mutants are a compilation of 4 measurements and the SD is shown.

$G\alpha_q$ stimulation promotes the localization of TRAX to the nucleus. In previous studies using purified proteins, we found that TRAX competes with $G\alpha_q$ for PLCβ1 binding (see Chapter 2). Because the affinity between PLCβ1 and $G\alpha_q$ is greatly enhanced when $G\alpha_q$ is in the activated state, we determined whether activation of would disrupt TRAX-PLCβ1 complexes. We initially monitored changes in FRET between eYFP- PLCβ1 and eCFP-TRAX in live HEK293 cells with carbachol stimulation. However, no significant changes in FRET were detected (**see Fig. 3-6**). Additionally, no visible change in localization of either induced eYFP-PLCβ1 or endogenous TRAX was observed in cells that were transfected with a constitutively active $G\alpha_q$ mutant ($G\alpha_q$RC12) (**see Fig. 3-7).**

A)



B)

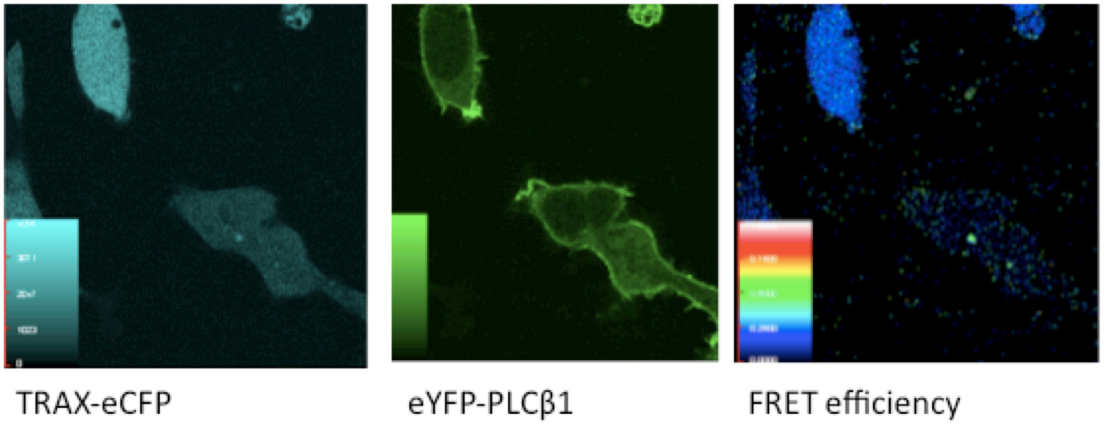


Figure 3-6: Two examples of images of cells expressing TRAX-eCFP and eYFP-PLCβ1. (A) FRET between TRAX-eCFP and eYFP-PLCβ1 before carbachol stimulation. (B) FRET between TRAX-eCFP and eYFP-PLCβ1 after carbachol The FRET images have been corrected for bleed-through. Imaging conditions are described in the text.

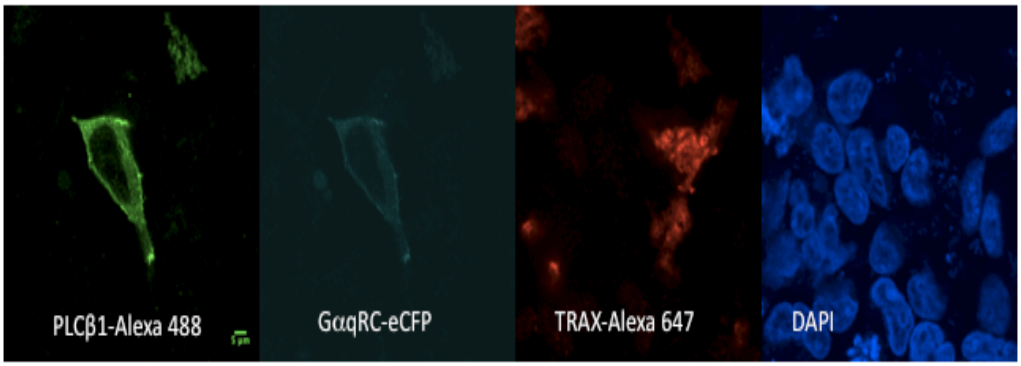


Figure 3-7: HEK293 cells were transfected with GαqRC and the localization of TRAX was observed. We found that activation of Gαq, either by transfection with GαqRC or by stimulation by carbachol causes a loss in exposure of TRAX epitope as detected by polyclonal or monoclonal antibody. Imaging was carried out as described in the text.

Because FRET studies require protein overexpression, it is possible that changes in smaller portions of the protein populations may be masked. To determine whether this is the case, we attempted to follow changes in localization of endogenous TRAX with $G\alpha_q$ stimulation. Interestingly, we observed a large reduction in intensity of Anti-TRAX-Alexa647 with carbachol stimulation or when transfected with a $G\alpha_q$RC suggesting occlusion of the epitope using a monoclonal antibody (**see Fig. 3-7**). Repeating these studies with a polyclonal antibody gave similar results. Thus, either carbachol stimulation is increasing the turnover of TRAX, which does not seem to be case as shown below, or stimulation is changing the environment of TRAX so as to occlude the antibody epitope. A similar result is seen when the TRAX polyclonal antibody is used. It is also

possible that the epitope is occluded by the formation of TRAX-translin complex.

Since imaging the proteins was not reliable, we followed changes in the endogenous localization of the proteins with $G\alpha_q$ activation by cell fractionation and SDS-PAGE and western blotting. These studies were carried out by stimulating HEK293 cells with carbachol for 5 minutes and determining changes in the amount of TRAX in the cytosolic and nuclear fractions by western blotting. The results from these studies show a pronounced shift in TRAX localization from the cytosol to the nucleus with no significant movement of PLCβ1 (**see Fig. 3-8**). These results suggest that activation disrupts a portion of PLCβ1-TRAX complexes allowing TRAX to move into the nucleus.

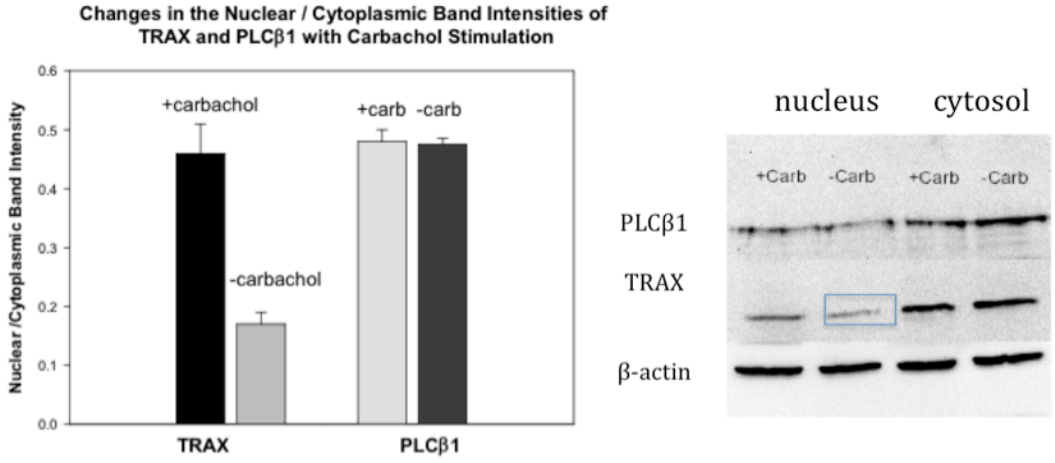


Figure 3-8 - ***Movement of TRAX to the nucleus with cell stimulation*****. Western blot analysis of nuclear and cytosolic fractions of TRAX and PLCβ1 from HEK293 cells untreated or treated with 1μM carbachol and incubated for 5 minutes before lysing and fractionating. The graph is a composition of four independent experiments and was carried out by digitizing the band density and normalizing each band to actin loading controls.**

PLCβ1 overexpression reverses siRNA activity. We carried out a series of studies to determine whether TRAX could regulate the cellular localization of PLCβ1, or

vice versa. Since TRAX is part of the RISC complex, we could not successfully down-regulate its protein expression, and so we overexpressed TRAX by transient transfection. We found that increased TRAX expression did not have any significant effect on the localization of PLCβ1 (**see Fig. 3-9**).

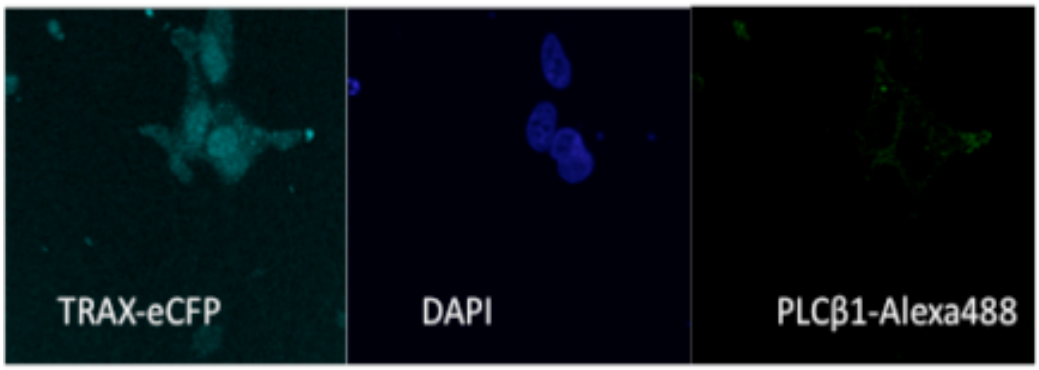


Figure 3-9: HEK293 cells were transfected with DNA expressing TRAX-eCFP and the localization of endogenous PLCβ1 was observed. We did not observe any changes in the localization of PLCβ1.

We then determined whether PLCβ1 could affect the cellular localization of TRAX. At endogenous levels of PLCβ1, TRAX localizes to the cytosol and nucleus. However, when expression of PLCβ1 is low, TRAX localizes almost entirely to the nucleus (**Fig. 3-10a**). This result implies that lowering the amount of PLCβ1 in the cytosol allows TRAX to move into the nucleus. Conversely, when cells are induced to express higher levels of PLCβ1 (via induction with tetracycline), TRAX moves from the nucleus into the cytosol (**Fig. 3-10b**).

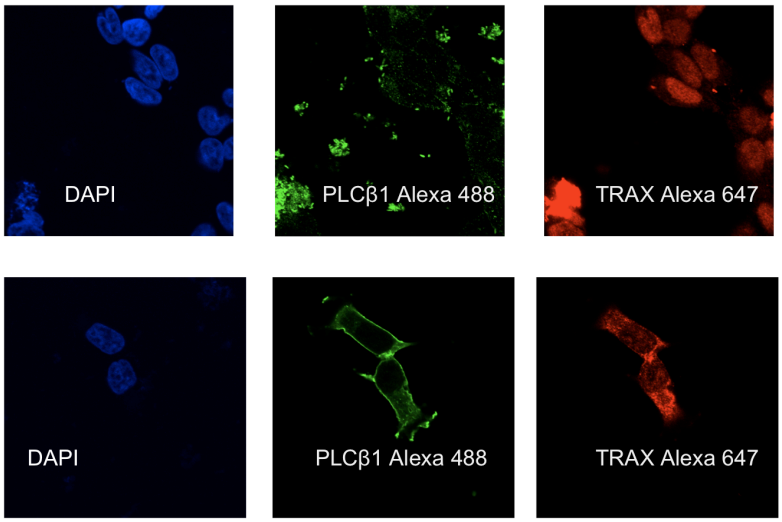


Figure 3-10 – *PLCβ1 regulates TRAX localization.* A. Images of fixed HEK283 cells expressing a low amount of eYFP-PLCb1 (left panel). This image shows a single cell expressing eYFP-PLCβ1. This cell is the only one that shows a significant population of TRAX in the cytosol as visualized by immunostaining as opposed to a nuclear population (right panel). B. Images of fixed cells showing the movement of TRAX from the nucleus to the cytosol when HEK293 cells are induced to overexpress PLCβ1 by treatment with tetracycline.

TRAX has been shown to be an activator of RISC in mediating RNA silencing. Since a relatively high amount of PLCβ1in the cytosol causes movement of TRAX out of the nucleus, we determined whether the changes in TRAX localization by PLCβ1 forces its removal from RISC resulting in a reduction of RISC activity. To test this idea, we down-regulated the housekeeping enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by siRNA in 293-TAP-PLCβ1 cells, which allows expression of PLCβ1 when tetracycline is added to the culture media. We confirmed that siRNA (GAPDH) does not affect our ability to induce PLCβ1 expression. We noted that cells treated with siRNA (GAPDH) grew more slowly than controls, however, this behavior was reversed when the cells were simultaneously treated with both siRNA (GAPDH) and tetracycline.

Importantly, we found that the level of GAPDH in the siRNA-treated cells was restored when PLCβ1 was induced (**Fig. 3-11**). This result suggests that PLCβ1 causes dissociation of TRAX from RISC attenuating siRNA activity.

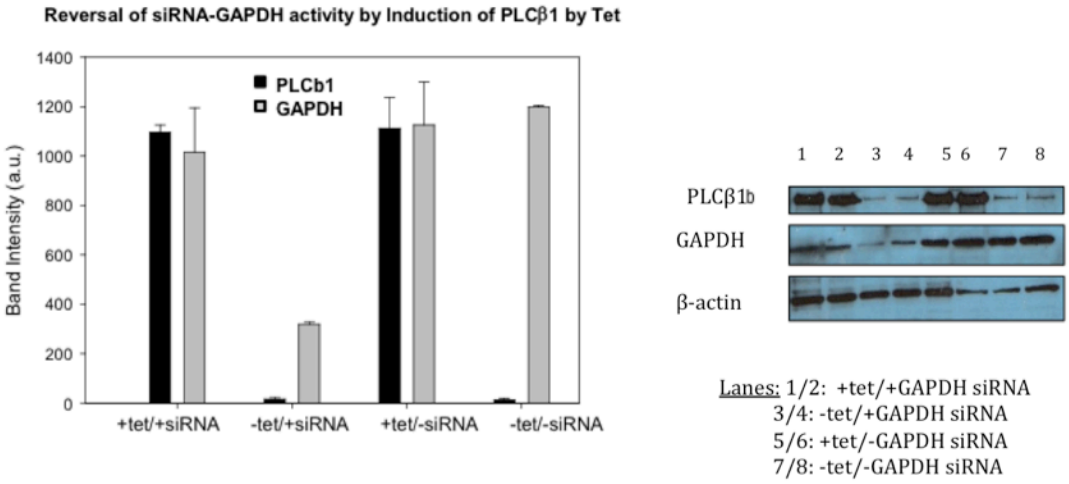


Figure 3-11 – ***Increased expression of PLCβ1 abolishes siRNA activity*****. HEK293-TAP-PLCβ1 cells were treated with GAPDH siRNA and/or tetracycline (tet) to induce PLCβ1 expression. Cells treated only GAPDH-siRNA had diminished protein expression, which was reversed in cells, also treated with tetracycline. All bands were normalized according to the actin loading control, n=3.**

DISCUSSION

In this study, we have shown that the presence of PLCβ1 in the cytosol may function as a localization factor that regulates TRAX entry into the nucleus to participate in RNA silencing. This unexpected function of cytosolic PLCβ1 connects G protein signaling with siRNA activity.

We have previously found that TRAX is a novel binding partner of PLCβ1. In studies using purified proteins, we showed that PLCβ1 interacts strongly and specifically with TRAX and TRAX competes with $G\alpha_q$ for PLCβ1, (Aisiku, Runnels and Scarlata, *submitted*). The competition between membrane-localized $G\alpha_q$ and TRAX for PLCβ1

may explain the lack of visible TRAX on the plasma membrane.

We consistently observe a significant population of PLCβ1 in the cytosol. The function of cytsolic PLCβ1 is unclear since its basal activity is low and $G\alpha_q$ is only found on the plasma membrane (see (Dessy, 2000; Cocco, 2001; Cocco, 2003)). It is possible that cytosolic PLCβ1 serves as a reservoir for excess $G\alpha_q$ upon cell stimulation. However, in accord with previous FRET studies(Dessy, 2000), our studies here do not show a significant shift in the cytosolic population (after carbachol stimulation) as detected by western blot analysis of endogenous protein or immunostaining and imaging of transfected enzyme. The lack of detectable movement of PLCβ1 to the plasma membrane with stimulation is surprising when one considers the large increase in affinity between $G\alpha_q$ and PLCβ1 with activation and the higher cellular concentration of $G\alpha_q$ relative to PLCβ1, both of which should promote dissociation from cytosolic TRAX. Thus, cytosolic PLCβ1 may serve some other role besides a reservoir for activated $G\alpha_q$.

The role of PLCβ1 in the nucleus is also mysterious. The nucleus has a phosphoinositol lipid signaling pathway that is independent of the one found on the plasma membrane (see Kim, 1996). PLCβ1 is one of the two major PLCs in the nucleus. The second, PLCδ1, which binds strongly and specifically to $PI(4,5)P_2$ in contrast to PLCβ1, is driven into the nucleus upon elevation $PI(4,5)P_2$ levels in a cell cycle specific manner (Yang, 2004; Wang, 2004). It has been found that cell stimulation by integrin growth factor results in the nuclear transit of PLCβ1 through phosphorylation by mitogen-activated protein kinase, which promotes nuclear entry, and phosphorylation by protein kinase C-α which promotes nuclear exit (Cocco, 2002). We tested whether the

PKCα phosphorylation site alters the interaction of the enzyme with TRAX using two point mutants that mimic the unphosphorylated or phosphorylated enzyme (i.e S887A and S887D, respectively) and found that TRAX associates equally well to these enzymes both in the cytosol and nucleus. This result suggests that TRAX is not involved in the pathway that involves acute $PI(4,5)P_2$ nuclear processing by PLCβ1.

Nuclear PLCβ1 has been shown to reside in speckles (Feng, 2001; Kasai, 1997), which are storage deposits for splicing factors and other transcription machinery (for review see Ishida, 2002). The localization of PLCβ1 in speckles suggests a possible role in RNA splicing and/or in mRNA processing. It is tempting to speculate that PLCβ1, through its interaction with TRAX, may serve a functional role in the nucleus. This idea is presently being tested.

Until recently, TRAX has only been reported to bind to the long C-terminal of A2A adenosine receptors where it can to help modulate neurite outgrowth [19], and this interaction may also help localize TRAX in the cytosol. TRAX's other known binding partner, translin, has been well established. Translin is a helical protein that catalyzes the cleavage of single stranded DNA and RNA in vitro (Lamond, 2003). In cells, the expression of TRAX and translin are closely coupled, and both are required for cell proliferation (Cocco, 2006; Peruzzi, 2003). It has been shown that TRAX must be expressed at a sufficient level to move translin into the nucleus to from the multimeric complex that activates RISC (Bahk, 1994). The endonuclease activity of this complex, called C3PO (component 3 promoter of RISC) facilitates the removal of the siRNA passenger strand cleavage products promoting complex loading and activation.

Even though TRAX does not appear to affect the cellular localization of PLCβ1, we find that PLCβ1 has a pronounced effect on TRAX localization. Our data show that decreasing the level of cytosolic PLCβ1 allows TRAX to move into the nucleus, and we speculate that, in contrast to TRAX-translin association, PLCβ1 might occlude the nuclear localization signal of TRAX.

The most significant result of this study is that elevating the level of PLCβ1 in the cytosol disrupts siRNA activity presumably by removing TRAX from RISC. Thus, any factor that reduces the amount of PLCβ1 in the cytosol or disrupts PLCβ1-TRAX complexes, such as $G\alpha_q$ stimulation may reduce RNA silencing. This finding gives an unexpected link between G protein activation and gene silencing. Much more work is required to fully understand the role of TRAX and PLCβ1 in the nucleus.

ACKNOWLEDGEMENTS - This work was supported by NIH GM53132.

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Chapter 4: Summary and Conclusion

The signaling of phospholipase C β1 at the plasma membrane is well understood. However, PLCβ1 exists in other compartments of the cell. The goal of this project was to understand the role of PLCβ1 in these other compartments. A binding partner for PLCβ1 called translin-associated factor-X (TRAX) has been found. We sought to characterize the relationship between PLCβ1 and TRAX using a combination of biochemical and biophysical techniques. The project was separated into two parts: in part one, the in vitro and in vivo binding characteristics were studied, and in part two, a functional study was carried out to determine the consequence of their relationship. The results of these experiments open up possibilities for future work that will be explained at the end of this chapter.

We searched for alternate protein partners of PLCβ1 in a yeast two-hybrid approach carried out by Loren Runnels using the 574 residue C2-domain containing COOH-terminal region of PLCβ1 as a bait. Using this technique we identified a protein called TRAX. Up to this point the only known binding partner for TRAX was a protein called translin that forms a strong complex with TRAX. Early evidence showed that the primary function of TRAX is to carry translin into the nucleus (Yang, et al., 2004). As a result, we initially proposed that TRAX functioned in a similar manner with PLCβ1.

The first aim of this project was to characterize the binding between TRAX and PLCβ1 in solution and in live cells. The initial step was to determine the binding between TRAX and PLCβ1 by performing fluorometer-based experiments in solution. It was found that both proteins bind strongly in solution. In a similar binding experiment using the truncated form of PLCβ1, it was discovered that the –COOH terminal end of PLCβ1 was crucial for binding, which correlates well with the pull-down and yeast two-hybrid

experiment used to identify TRAX as a binding partner.

Since the –COOH terminal region of PLCβ1 appears to be critical for the binding of TRAX, we tested the ability of TRAX to disrupt the binding between PLCβ1 and activated $G\alpha_q$ as well as the ability of PLCβ1 to be stimulated by $G\alpha_q$. Given the importance of the –COOH terminal region in the binding between TRAX and PLCβ1 it was not surprising to see that TRAX competes with $G\alpha_q$ (only in the activated state) for binding to PLCβ1. TRAX also disrupts the activation of PLCβ1 by $G\alpha_q$. Interestingly, TRAX does not seem to disrupt the initial activation of PLCβ1 in its basal state.

The crystal structure of the isolated –COOH-terminal region of PLCβ1 has been solved and is found to be an intertwined helical dimer (Fig. 4-1; 23 from Ch.2), although it is not clear whether full length PLCβ1 itself forms a dimer. At this point, it is impossible to speculate the mode of interaction between TRAX and PLCβ1, but it is worth noting that translin (Fig. 4-2), TRAX's putative binding partner, is comprised of a network of helixes (see 24 from Ch. 2). CD data and structure prediction programs also suggest that TRAX is comprised of a network of alpha helixes.

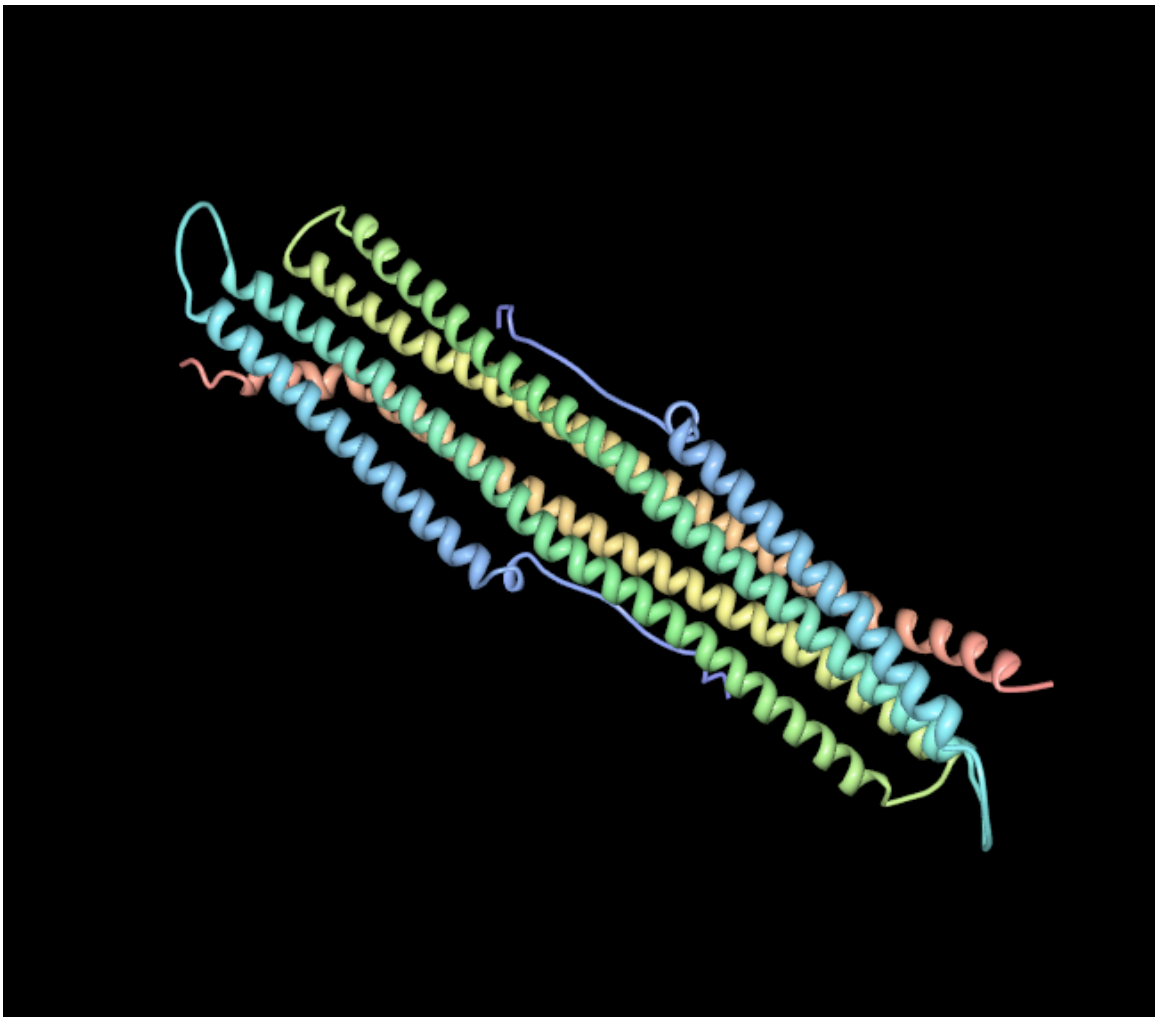

Figure 4-1 - Crystal structure of the -COOH terminal domain of PLCb1. The C-terminal region is comprised of intertwined helixes.

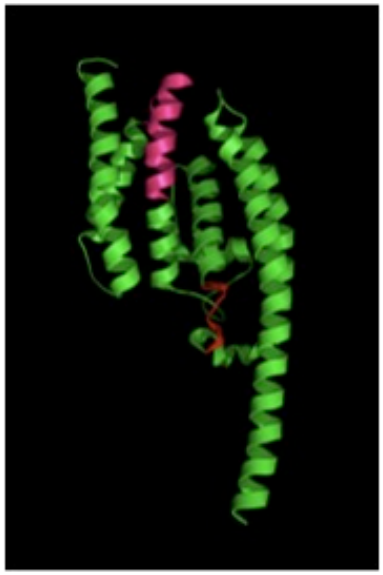

Figure 4-2 - (a) Translin monomer.

Another key feature of PLCβ1 is its pleckstrin homology (PH) domain that is critical for its ability to bind to membranes. We sought to determine if the interaction between TRAX and PLCβ1could interfere with the membrane binding ability of PLCβ1. Our results indicate that TRAX binding does not affect the ability of PLCβ1 to bind to artificial lipid bilayers.

The next step was to look at the association between PLCβ1 and TRAX in cells. In C6 glial cells both proteins are expressed at high levels and both proteins colocalize throughout the cell. It is noteworthy that PLCβ1 in C6 glial cells has low plasma membrane localization relative to other compartments presumably due to the high level of expression of PLCβ1. Both proteins were also shown to interact using FRET. TRAX and PLCβ1 were co-expressed using fluorescent-tagged proteins in a cell line where their endogenous expression is more limited (i.e. Neuro2A cells). The majority of the interaction occurs in the cytosol as

little PLCβ1 is found in the nucleus and TRAX is not found at the plasma membrane in these cells. FRET values previously reported for PLCβ1 and $G\alpha_q$ are similar to the FRET values between PLCβ1 and TRAX observed in this report (Dowal et al., 2006).

The strong binding constant and large FRET value measured for the cytosolic PLCβ1 and TRAX suggests that there is a specific cellular role for the complex. The second part of this project sought to determine this cellular role. Recent evidence has demonstrated that both translin and TRAX are part of the RNA-induced silencing complex (RISC) where they help guide double stranded RNA into the silencing machinery (Liu et al., 2009). While TRAX primarily binds to translin there is evidence that TRAX binds to other proteins. For example, TRAX has been shown to interact with the long C-terminal tails of A2A adenosine receptors reversing impairment of neurite growth by p53 in PC12 cells. As a result it was initially postulated that TRAX might similarly modulate the localization of other cellular proteins, such as PLCβ1.

When we first started the functional study, we initially wanted to see whether the expression level of TRAX could influence the localization of PLCβ1. To test this we overexpressed TRAX by transfecting eCFP-labeled TRAX protein and immunostained for PLCβ1. Immunofluorescence showed that overexpressing TRAX did not change the localization of PLCβ1.

In the second part of this work, we obtained evidence suggesting that the cytosolic population of PLCβ1 might regulate the entry and subsequent localization of TRAX to the nucleus to participate in RNA silencing. While not every cell has a significant nuclear population of PLCβ1, there does appear to be a significant cytosolic PLCβ1 population. The role of this population is still unknown as its activator, $G\alpha_q$, is only found on the plasma

membrane. Previous reports fail to show any detectable movement of PLCβ1 to the plasma membrane with stimulation. It is possible that the cytosolic population of PLCβ1 serves as a pool for other as yet unidentified proteins, presumably TRAX.

Immunohistochemistry of endogenous TRAX and PLCβ1 provided some clues as to what the biological endpoint might be. It was observed that cells expressing low amounts of PLCβ1 also had a high amount of TRAX present in the nucleus. When the cells expressed a higher amount of PLCβ1 there was less TRAX expressed in the nucleus. Next, we wanted to see if PLCβ1 could disrupt siRNA activity by preventing the entry of TRAX into the nucleus. Using a tetracycline driven expression system we were able to increase the level of PLCβ1 expression. In cells that were induced to express PLCβ1 we noticed a reduction in siRNA activity. This result implies that any factors that could reduce the amount of PLCβ1 available in the cytosol, such as the stimulation of Gaq might increase RNA silencing. We observe accumulation of TRAX into the nucleus with carbachol stimulation and so this might just be the case.

Future Directions

While we know that PLCβ1 and TRAX bind in solution we still do not know the key residues involved in the binding between the two proteins. Several experiments need to be carried out to address this question. Crystallization of TRAX would prove to be very valuable in determining what key residues are involved in the interaction between PLCβ1 and TRAX. Mutation of potential residues might provide answers. Furthermore, it is already known that TRAX and translin bind strongly. It remains to be seen if the interaction between PLCβ1 and TRAX can affect the relationship between TRAX and translin. Studies should be

carried out in solution and in cultured cells.

Functional studies have begun to determine what consequence the interaction between TRAX and PLCβ1 have in living cells. While TRAX does not appear to affect the localization of PLCβ1 as initially suspected, we have discovered that PLCβ1 might affect the localization of TRAX. Interestingly, TRAX has been reported to bind to the C-terminus of A2A adenosine receptors where it can help modulate neurite outgrowth in PC12 cells (Sun et al., 2006). This interaction may also help to localize TRAX in the plasma membrane. This association, along with the expression of PLCβ1 in PC12 cells warrants further investigation. Another key determinant of localization is the cell cycle. Detailed studies need to be carried out to determine the role this might play in the interaction between TRAX and PLCβ1.

We have shown that there may be a link between PLCβ1 and G protein signaling to the RNAi pathway. TRAX and translin's basic function is to bind to nucleic acids. Previous gel-shift assays have shown the ability of translin to bind to nucleic acids (Sengupta, 2006). Additionally, nuclear PLCβ1 has been shown to reside in speckles, which are deposits for splicing factors and other transcription machinery (Feng et al., 2001; Kasai et al., 1997; Ishida et al., 2002). This would suggest a possible role for PLCβ1 in the nucleus. It would be worthwhile to design experiments to test the ability of PLCβ1 to affect the ability of TRAX and/or translin to bind to nucleic acids. Much work remains to explore this unexpected link between PLCβ1 and RNAi.

Significance of Research Project Moving Forward

The association between TRAX and PLCβ1 has several possible implications that could have a significant biological impact. It has been well established that PLCβ1 is

involved in cell growth and proliferation. TRAX has been implicated to have a role in cell proliferation, genome stability, human genetic disease and RNA interference (Jaendling, 2010).

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide. It accounts for 7.4 million deaths, which is around 13% of all deaths in 2004. TRAX was first identified as a binding partner for translin, a protein that binds to breakpoint junctions in lymphoid neoplasms (Jaendling, 2010; Atlas, 1998; Kanoe, 1999; Xiang, 2008). This was significant because it suggested that the two might play a role in carcinogenesis or genome stability regulation. Translin binding sites have been found in a range of human cancer-related chromosome translocation breakpoints. There are conflicting reports as to how significant a role TRAX and translin play in carcinogenesis and genome stability. Some groups report that in mice, *Drosophila*, and S. *pombe* translin-null mutants do not exhibit any obvious defects in processes in which recombination is involved, including DNA damage recovery (Jaendling, 2008; Chennathukuzhi, 2003). It remains to be tested whether or not translin and/or TRAX play a role in the redundant DNA repair/recombination pathway. Translin has been found in the nucleus of haemopoietic cell lines, whereas it is predominantly cytoplasmic in many other tissues (Aoki, 1995). Furthermore, it translocates from the cytosol to the nucleus of HeLa cells in response to DNA-damaging agents mitomycin C and etoposide (Kasai, 1997).

Additionally, TRAX and translin appear to have a role in cell proliferation. For example, translin levels appear to be reduced following irradiation-induced cell-cycle arrest, and expression of the translin gene appears to exhibit some cell-cycle dependent periodicity (Ishida, 2002). Translin overexpression has led to in apparent increase in cell proliferation.

In mice suffering lung cancer an increase in translin expression occurred and was linked with an invasive phenotype. This would suggest that translin might have a role in tumor progression (Sargent, 2008). Translin-deficient mice also show a lower rate of cell proliferation.

PLCβ1 is highly expressed in brain tissue and it appears that TRAX and translin function in the brain as well. It has been demonstrated that TRAX and translin are required for the intracellular targeting of mRNA splice variants of the mammalian BDNF (brain-derived neurotrophic factor) gene(Chiaruttini, 2008). Binding sites for TRAX and translin exist on BDNF. Any defect in the binding region has been linked with human neurological and psychiatric disorders, including reducing hippocampal dendritic complexity and volume, memory defects and mood disorders (Bath, 2006). The TRAX gene has also been genetically linked to human psychological disorders, supporting the proposal that mRNA processing by translin/TRAX is important for brain function in humans (Okuda, 2010). TRAX has also been identified as a binding partner of the A_{2A}-R (A_{2A} adenosine receptor), which is involved in regulation of neuronal plasticity and development.

Finally, TRAX has been identified as a key player in RNAi pathways. In *Drosophila*, it has been proposed that TRAX along with translin are required for activation of the RISC complex. TRAX and translin, termed C3PO, are involved in delivering siRNAs to RISC via the RISC-loading complex (Liu, 2009). Furthermore, TRAX alone was proposed to have RNase activity (Liu, 2009). It was here in our research that we found that expression of PLCβ1 appears to affect the localization of TRAX. We propose that interaction of PLCβ1 with TRAX affects the ability of TRAX to participate in the RISC-loading complex. Obviously, further testing is required to prove this but the mere possibility is tantalizing.

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Chapter 4:

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