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Role of Phospholipase C- β in RNA interference

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Abstract

Phospholipase C- (PLC) enzymes are activated by G proteins in response to agents such as hormones and neurotransmitters, and have been implicated in leukemias and neurological disorders. PLC activity causes an increase in intracellular calcium which ultimately leads to profound changes in the cell. PLC localizes to three cellular compartments: the plasma membrane, the cytosol and the nucleus. Under most cell conditions, the majority of PLC localizes to the plasma membrane where it interacts with G proteins. In trying to determine the factors that localize PLC to the cytosol and nucleus, we have recently identified the binding partner, TRAX. TRAX is a nuclease and part of the machinery involved in RNA interference. This review discusses the interaction between PLC and TRAX, and its repercussions in G protein signaling and RNA silencing.

Keywords

Phospholipase C ; G protein signaling; C3PO; RNA interference; TRAX

INTRODUCTION

Overview of the PLC β -G protein signaling system

The PLC signaling pathway is initiated when an extracellular agonist binds to a target G protein coupled receptor (GPCR). GPCRs comprise the largest family of receptors in mammalian cells (for review see (Rebecchi and Pentylana, 2000, Suh et al., 2008)). Their agonists range from light, neurotransmitters, hormones and ions. Agonist binding to the receptor induces a conformational change in the receptor that allows it to catalyze the exchange of GDP for GTP on the α subunit of a heterotrimeric G-protein. Heterotrimeric G-proteins consist of an α subunit that contains the guanine nucleotide binding site and a $\beta\gamma$ subunit (for review, see (Exton, 1997), (Birnbaumer, 2007, Cabrera-Vera et al., 2003)). Activated or GTP-bound α subunits have a greatly reduced affinity for $\beta\gamma$ subunits which allows α and $\beta\gamma$ to interact with a select group of intracellular effector proteins and change their catalytic activity. α subunits have an intrinsic GTPase activity and hydrolysis of GTP to GDP promotes its reassociation to $\beta\gamma$ subunits and cessation of the signal. G subunits have been divided into four subfamilies: G_s , $G_{i/o}$, G_q and G_{12} and each of them are coupled to specific receptors and effectors. This coupling defines the specific pathway a signal will follow, although some cross-over appears to occur in certain

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pathways. Additionally, there are proteins that bind to G GTP and enhance its GTPase activity, called “GAPs” or GTP-activating proteins (Berstein et al., 1992). GAPs proteins allow rapid shut-off of the signal.

The G_q family transduces signals from agents such as angiotensin II, catecholamines, endothelin 1 and prostaglandin F₂. Activation of G_q results in activation of phospholipase C (PLC) (Fig. 1). PLC enzymes catalyze the hydrolysis of the signaling lipid phosphatidylinositol 4,5 bisphosphate (PI(4,5)P₂) to generate the second messengers, diacylglycerol and 1,4,5 inositol trisphosphate (Ins(1,4,5)P₃), which activate protein kinase C (PKC) and causes the release of Ca²⁺ from intracellular stores, respectively (see (Rebecchi and Pentylana, 2000, Suh et al., 2008)). There are four isoforms of PLC that are all activated by G_q. Additionally, PLC 2 and PLC 3 can be activated by G₁₂ dimers. Thus, agonists coupled to other G family members can activate PLC through released G subunits, and this has been observed for activation of G_{i/o}.

PLC (1-4) enzymes vary in their tissue distribution and their ability to be activated by G_q. This review focuses on PLC 1, which is most strongly activated by G_q and is found primarily in neuronal tissue. PLC 1 has been connected with several human diseases such as leukemia (e.g.(Follo et al., 2012)) and Alzheimer’s disease (Albasanz et al., 2005). It is notable that losses of PLC 1 are found in patients with neurological disorders such as epileptic encephalopathies and schizophrenia which are recapitulated in knock-out mice (see (Albert et al., 1997, Koh et al., 2008, Manning et al., 2012)). While the simplest explanation for these disparate effects is that changes in PLC 1 levels is affecting Ca²⁺ signaling, it is possible that PLCb1 perturbs other pathways as well, as discussed below.

TOPICS

Cellular localization of PLC β

PLC 1 interacts with G proteins and PI(4,5)P₂ on the plasma membrane, but it also localizes to other cellular compartments (Aisiku et al., 2011, Dowal et al., 2006). These alternate localizations depend on the cell line and the specific state of the cell. For example, we find that in undifferentiated PC12 cells, PLC 1 localizes to the nucleus where it plays a role in nuclear PI signaling (Fig. 2). Upon treatment of the cells with nerve growth factor, PLC 1 moves to the cytosol and plasma membrane. In A10, HEK293 and pancreatic beta cells, we also find a large cytosolic PLC 1 population under both endogenous and over-expressed conditions (*unpublished*). It is interesting to speculate the role of PLC in the cytosol and the nucleus.

The cytosol contains internal vesicles and it is possible that PLC s might serve to keep the level of internal PI(4,5)P₂ in check. Henis and coworkers observed movement of PLC 2 to the plasma membrane upon Rac1 activation suggesting that the cytosolic population serves as a PLC reservoir (Illenberger et al., 2003). However, we found this is not the case for eYFP-PLC 1 where the cytosolic population remained constant during G_q activation (Dowal, Provitera, 2006). Given that PLC 1 can enter the nucleus (see below), then it is possible that the cytosolic population acts as a reservoir for the nuclear PLC.

The function of PLCs in the nucleus remains an intriguing question. It is possible that PLCs modify PI lipids on the nuclear membrane and generate release calcium from nuclear stores independently of the cytoplasmic compartment. PLC 1 and PLC 1 are the most prominent nuclear PLCs. The PH domain of PLC 1 binds strongly to PI(4,5)P₂ (Rebecchi et al., 1992), and PLC 1 is driven into the nuclear by a high level of PI(4,5)P₂ generated by an unknown mechanism (Stallings et al., 2005). This nuclear transit is cell cycle dependent and is needed to move the cell through the G1/S boundary (Stallings et al., 2008). Nuclear PLC 1 may

also serve to drive PKC into the nucleus through generation of diacylglycerol (see (Cocco et al., 2002, Cocco et al., 2006, Martelli et al., 1992)). Nuclear PLCs may cause the release of Ca^{2+} from nuclear stores which may regulate cAMP-response elements and subsequent gene transcription. Additionally, studies show that PLC 1 over-expression is sufficient for increased expression of cyclin D3 and cdk4, hyperphosphorylation of retinoblastoma proteins, and subsequent activation of E2F-1 transcription factor, resulting in progression through the G1/S checkpoint ((O'Carroll et al., 2009)).

Control of the nuclear localization of PLC 1 is not understood. PLC 1 has two alternatively spliced variants ((Bahk et al., 1994)), PLC 1a and PLC 1b, 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. No nuclear export signal (NES) has yet been identified. While it was first thought that PLC 1b was nuclear and PLC 1a was cytosolic based on cell fractionation studies (Bahk et al., 1998), we have found them to be similarly distributed in PC12 and HEK cells by cell imaging (Scarlati et al, *unpublished*).

The basal activity of PLC 1 is low and it is likely that it is activated in the nucleus. However, to this date, G α_q is the only established activator of PLC 1, has not been found in the nucleus (see (Hughes et al., 2001)). There is some evidence that phosphorylation may play a role. Stimulation of Swiss3T3 cells by insulin-like growth factor-1 causes extracellular signal-regulated kinase (ERK) to move to the nucleus where it phosphorylates nuclear PLC 1 on S982 which in some way promotes DNA synthesis (Xu et al., 2001b). Nuclear PLC 1 generates diacylglycerol that causes PKC to move into the nucleus which phosphorylates Ser887 of PLC 1 down-regulating nuclear PLC 1 activity (Xu et al., 2001a). However, neither S982 or S887 phosphorylation alters the activity of purified PLC 1, and neither site is close to the NLS, leading to the idea that phosphorylation of PLC 1 must affect its interaction with a nuclear transporter and/or activator. The simplest idea is that nuclear PLC 1 activity is directly related to its concentration, but how such partitioning might be regulated is completely unknown. Live cell imaging studies show that S887A-PLC 1a has a higher nuclear localization than the analogous S887D point mutant, suggesting that nuclear partitioning is mediated through a binding partner sensitive to phosphorylation (Aisiku, Dowal, 2011).

Identification of a novel PLC β binding partner TRAX

The observation of a significant cytosolic population of PLC under a variety of cellular conditions at first was not surprising since could be interpreted to serve as a 'reservoir' to be used during G protein signaling. However, we could not detect movement to the plasma membrane when G α_q was activated (Dowal, Provitera, 2006) (We note that exogenous, fluorescent-tagged G α_q remains on the plasma membrane during the signaling process, (Dowal, Provitera, 2006, Hughes, Zhang, 2001, Philip et al., 2007)). The idea that this population of PLC may modulate the PIP $_2$ levels in internal membranes is probably not the case since its basal activity is so low. We then set out to determine alternate functions of this enzyme by first seeking unknown binding partners.

As mentioned, PLC enzymes are distinguished by a long (~400 aa) C-terminal tail which contains the primary binding site of G α_q , two phosphorylation sites, a calpain cleavage site, and a nuclear localization signal (see (Rebecchi and Pentylana, 2000)). Using this region as bait, we identified two nuclear binding partners by an unbiased yeast-2-hybrid study: translin-associated factor X (TRAX) and RBM4 (Aisiku et al., 2010).

TRAX, or translin-associated factor X, is a 33kDa protein that forms a DNA and RNA binding complex with translin (26 kDa), which is its only known binding partner to date (for

review see (Jaendling and McFarlane, 2010)). Both proteins are essential for normal cell proliferation. Translin is a DNA and RNA-binding protein with proposed functions in chromosomal translocations in lymphoid cells and mRNA transport and storage in brain and testis. Translin was implicated as having a role in chromosomal translocation and its name is based on this function. *In vitro*, translin binds to chromosomal DNA breakpoint junctions as an octameric ring and recognizes breaks at genomic hotspots. It has been suggested that the function of TRAX is to modulate the localization of translin. TRAX has a nuclear localization signal while translin has a nuclear export signal. Under cellular conditions where the level of TRAX is higher than translin, the nuclear localization signal of TRAX is exposed and complex localizes in the nucleus. However, if translin is in excess, then the complex moves to the cytosol (Cho et al., 2004). The stability of TRAX depends on the presence of translin. When translin is knocked out, TRAX is significantly reduced despite a normal level in mRNA suggesting that the interdependence is at a protein level (Cho, Chennathukuzhi, 2004). In cells, the level of TRAX closely parallel the level of translin throughout the cell cycle and is elevated in the proliferative state. Translin shares homology with the switch regions conserved of most GTPases that are critical in GTP/GDP exchange, and although translin has been reported to bind RNA and DNA in a GTP-dependent manner, which may parallel the GTP-dependent binding of PLC 1 to G α_q (Wang et al., 2004) we have found this not to be the case (Philip, *unpublished*).

TRAX binds to PLC β and competes for G α_q activation

We verified the association between TRAX and PLC 1 in solution and in cells. Using fluorescence spectroscopy, we measured the association between purified TRAX and PLC 1 in solution (Fig. 3a), and found that the proteins interact strongly with a dissociation constant that is only somewhat weaker than between PLC and G α_q (Aisiku, Runnels, 2010). Removing the C-terminus of PLC 1 greatly reduces its affinity for TRAX which is consistent with the use of this region as bait in the yeast two hybrid study.

In solution, PLC binds strongly to model membranes where it can encounter G proteins and substrates (Runnels et al., 1996). This binding energy comes mainly from its N-terminal PH domain and to a lesser extent through residues in the C-terminal domain. Since TRAX binds to this latter region, we determined whether TRAX could bind to membranes, or would interfere with membrane binding of PLC 1. We could not detect membrane binding of TRAX and found that it does not interfere with PLC binding (Aisiku, Runnels, 2010).

PLC interacts with G α_q through regions close to the C2 domain and through the C-terminus (Lyon et al., 2011, Wang et al., 1999). We determined whether the presence of TRAX would interfere with G α_q binding. We found that this is the case. TRAX effectively blocked G α_q binding and activation of PLC without affecting the basal activity of the enzyme (Fig. 3b) (Aisiku, Runnels, 2010).

Association between PLC and TRAX is also seen in cultured cells. Studies using co-immunofluorescence, immunoprecipitation and Förster resonance energy transfer all show a significant population of TRAX-PLC complexes in the cytosol, with a much lower amount in the nucleus in several cell lines (Aisiku, Runnels, 2010, Philip et al., 2012). TRAX is never seen on the plasma membrane consistent with its lack of binding to model membranes. Additionally, the plasma membrane population of PLC 1 is strongly associated to G α_q , whose affinity is somewhat higher than its affinity for TRAX. The distribution of PLC 1 between the plasma membrane and cytosolic compartments may reflect differences in the available G α_q and TRAX binding sites. Taken together, the above studies support the idea that TRAX is a novel binding partner of PLC 1.

C3PO, a complex of TRAX and Translin functions in RNA interference

It was recently discovered that the TRAX-translin complex plays a key role in RNA interference (Liu et al., 2009). RNA interference is a type of post transcriptional gene regulation in which target mRNA is degraded by the slicer activity of Argonaute protein (Ago) with the help of complementary small, interfering RNA (siRNA) (see (Hammond et al., 2001)). In this pathway, duplex RNAs are processed into 21-22 bp duplex siRNAs by Dicer2 which is then loaded onto RNA Induced Silencing Complex (RISC) whose main component is Ago2 (see Fig. 4). The two strands of duplex siRNA are the guide strand that targets the RNA-induced silencing complex (RISC) to the complementary RNA for degradation, and the passenger strand that has to be removed in order to activate RISC. Liu and coworkers identified a TRAX:translin complex named C3PO or Component 3 Promoter of RISC that enhances RISC activity in vitro as well as in *drosophila* ovary extracts (Liu, Ye, 2009). They found that C3PO promotes RISC activity by removing the passenger strand once it has been nicked by Ago2. Various RNA cleavage experiments show that C3PO is a Mg^{2+} -dependent endoribonuclease in which TRAX is responsible for nuclease activity while translin is thought to aid in binding.

While C3PO enhances RISC activity but is not necessary in *drosophila*, Ye and coworkers found that C3PO is absolutely necessary for duplex siRNA mediated RISC activity in humans (Ye et al., 2011). In this study, they solved the crystal structure of full length human C3PO, and found that similar to other species (Liu, Ye, 2009, Tian et al., 2011), C3PO is an octamer consisting of two heterodimers of TRAX:translin and two homodimers of translin (Fig. 5). Parizotto et al obtained the crystal structure of a C3PO like protein from *A. fulgidus* (AfC3PO) and found a very similar octameric arrangement of eight subunits that form two catalytic centers similar to C3PO of other species (Parizotto et al., 2013).

PLC β binds to C3PO and affects its activity

The studies described above suggest that C3PO is the functional form of TRAX/translin complexes in the cells, and we verified that purified PLC β 1 binds to purified C3PO in solution using fluorescence methods (Sahu et al., *unpublished*). Interestingly, the affinity between PLC β 1 and C3PO is similar to PLC β 1 and TRAX. Subsequent native gel and fluorescence studies showing a much weaker affinity between PLC β 1 and translin, and that one PLC β 1 binds to a one C3PO (Sahu et al., *unpublished*), suggests that a single PLC β 1 binds to an exposure TRAX molecule in the C3PO complex.

In C3PO, translin is responsible for specific binding of RNAs while TRAX has nuclease activity. We determined whether PLC β 1 affects the activity of C3PO using a short model ssRNA (whose sequence is based on previous work ((Liu, Ye, 2009, Tian, Simanshu, 2011)). For these studies, we labeled the 5' end with a fluorescent probe (FAM), and the 3' end with a strong fluorescence quenching group (black hole quencher 1 or BHQ). RNase activity is seen by the increase in fluorescence as the quenching group separates from the fluorophore (Philip et al., *unpublished*). We find that the presence of PLC β 1 significantly reduces RNase activity (Fig. 6) suggesting that PLC β 1 might affect its cellular function.

Functional significance of C3PO-PLC β interactions in cells

Given that C3PO and PLC β 1 can mutually affect each other's activation properties in solution, we determined whether they also affect each other's cellular function. To this end, we found that when we over-expressed TRAX in HEK293 cells, we could ablate carbachol-induced Ca^{2+} signals mediated through G q-PLC β , suggesting that excess TRAX can compete with G q for PLC β binding.

We then determined whether PLC β could interfere with C3PO function through its interaction with TRAX. Using HEK293 cells that can be induced to over-express PLC β 1 by tetracycline, we knocked-down the housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH). Knock-down of GAPDH not only resulted in a loss of this enzyme but also a significant amount of cell death. We then determined whether over-production of PLC β 1 would reverse GAPDH down-regulation. Surprisingly, increased levels of PLC β 1 not only prevented cell death but reversed siRNA(GAPDH) activity as seen by a return to basal levels of protein (**Fig.7**) and mRNA transcript (Philip, Guo, 2012). The reversal of siRNA(GAPDH) by PLC β 1 can be ablated by over-expression of TRAX. Additionally, down-regulation of PLC β 1 either in HEK29 cells or HeLa allowed for a more robust down-regulation of GAPDH by siRNA treatment.

While these siRNA(GAPDH) studies suggested that PLC β 1 plays an active role in siRNA regulation through its interaction with TRAX, further studies showed that this picture was much too simple. Although we found that over-expression of PLC β 1 reverses siRNA down-regulation of lactate dehydrogenase (LDH) similar to GAPDH, we found that PLC β has little effect on the down-regulation of other proteins (translin, Hsp90 and cyclophilin A), (Philip, Guo, 2012). Thus, the effect of PLC β on RNA interference is specific for specific genes. This finding suggests either that cells contain different RISC complexes that have varying affinities to PLC β , or that RISC processes RNAs differently depending on their structure/sequence which are differentially affected by PLC β .

To address this question, we carried out solution studies where we measured the effect of purified PLC β on the activity of purified C3PO towards labeled siRNA(GAPDH) and siRNA(Hsp90) using the fluorescence dequenching method described above. We find that C3PO hydrolyzes a double stranded form siRNAs(GAPDH) at a much faster rate than double stranded siRNA(Hsp90), and PLC β selectively reduces the rate of siRNA(GAPDH) hydrolysis but not siRNA(Hsp90) (Philip et al., *unpublished*). These results provide a molecular basis for the effect seen in cells.

Interestingly, GAPDH and LDH also have a moonlighting function by forming a complex that allows for histone H2B transcription, which precedes progression of the cell into the S phase (Dai et al., 2008, Zheng et al., 2003). We find that down-regulation of LDH or GAPDH results in a loss of H2B and cell death, which are reversed by PLC β expression (Philip, Guo, 2012). These observations prompted us to determine the effect of PLC β on the levels of various genes and miRNAs. We find that PLC β expression is closely linked to genes for H2B, for proteins involved in maintaining the redox state of the cell, and for proteins that positively regulate cell death (Caso et al., *unpublished*). We also surveyed changes in microRNAs (miRNAs) with PLC β expression. miRNAs that are significantly down-regulated when PLC β 1 is over-expressed in HEK29 cells are closely linked to various leukemias and lymphomas, and PLC β 1 expression is directly linked to these diseases (Table 1) (e.g. (Follo, Faenza, 2012)). Additionally, PLC β 1 impacts the levels of specific miRNAs associated with differentiation. These results suggest that PLC β 1 may, in some way, be linked to its roles in G protein signaling and gene expression.

Future Directions

While the interaction between PLC β 1 and TRAX can potentially link G protein regulation with gene expression, at the present time, these links are tenuous. Our knowledge of the genetic targets of C3PO, and how they change with the state of the cell are not understood. More genetic information regarding which miRNAs are altered during periods of elevated Ca^{2+} due to sustained hormonal or neuronal stimulation, as well as differentiation may help to connect these processes. Additionally, our studies only focused on PLC β 1 and it is unclear whether other PLC β isoforms can similarly regulate C3PO, especially considering

that PLC β 3 is additionally regulated by G α , and PLC β 2 by G α and small G proteins (Illenberger, Walliser, 2003, Park et al., 1993). The role of PLC β in the nucleus is also unclear. We have not detected a significant amount of FRET between PLC β 1 and TRAX in the nucleus (Aisiku, Dowal, 2011), and it is possible that nuclear PLC β functions to regulate PIP $_2$ levels or interact with other, unknown nucleases. Studies delineating these two PLC functions in the nucleus will be very interesting.

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Fig. 1.
Cartoon of the phospholipase C -G protein signaling pathway.

Localization of eYFP-PLC β 1 in PC12 Cells

Colocalization of PLC β 1 and G α_q in the nucleus of undifferentiated PC12 cells

G α_q -eCFP

YFP-PLC β 1

Merge

Fig. 2.

Top – Localization of eYFP-PLC β 1 in undifferentiated (*left*) and differentiated (*center* and *right*) PC12 cells showing nuclear, cytosolic and plasma membrane localization of the enzyme. **Bottom** –Colocalization of eCFP-G α_q and eYFP-PLC β 1 in undifferentiated PC12 cells.

a - Binding of TRAX to 2nM CPM-PLC β 1 **b- TRAX reverses G α_q activation of PLC β 1** **Fig. 3.**

From (Aisiku, Dowal, 2011); **a** – Binding of purified TRAX to PLC 1 labeled with the fluorescent probe CPM, where binding is detected by the change in CPM fluorescence (~18% increase in total). The dissociation constant derived from this studies is $K_d = 8\text{nM}$. **b**- Study showing that a 50 fold excess of TRAX can reverse the activation of PLC 1 by G α_q where PLC activity is followed by the extent of PIP₂ hydrolysis.

Fig. 4. Schematic showing the siRNA mediated RNA interference pathway and the proposed role of C3PO.

Figure 5. Crystal structure of octameric human C3PO (Ye, Huang, 2011) consisting of two TRAX subunits (blue) and six translin subunits (pink). Adopted from PDB ID 3PJA

Fig. 6.

Cleavage of a 12 base ssRNA of GU repeat by C3PO in the presence (green) and absence of PLC (brown) as indicated by the increase in fluorescence intensity is shown. PLC 1 reduces the rate of cleavage by C3PO 4-5 fold and PLC 1 alone does not change the fluorescence.

Table 1
miRNAs down-regulated by more than 2-fold following overexpression of PLC 1 (in HEK293 cells)

miRNA	Fold change
miR-30c*	-5.4
miR-19-1*	-4.7
miR-4539	-4.1
miR-181c*	-3.8
miR-30e*	-3.4
miR-1263	-3.1
miR-181a*	-3.1
miR-100	-3.1
miR-4529-3p	-2.8
miR-16-2*	-2.7
miR-200b*	-2.7
miR-let-7g	-2.5
miR-4498	-2.5
miR-1910	-2.4
miR-371b-5p	-2.4
miR-3200-3p	-2.3
miR-605	-2.3
miR-3935	-2.3
miR-363*	-2.2
miR-4274	-2.2
miR-4688	-2.2
miR-3648	-2.2
miR-509-3-5p	-2.1
miR-4776-5p	-2.1
miR-4654	-2.1
miR-181a-2*	-2.1
miR-4742-5p	-2.1
miR-216b	-2.1
miR-34a*	-2.1
miR-4286	-2.1
miR-221*	-2.0
miR-4440	-2.0
miR-202	-2.0
miR-4667-5p	-2.0
miR-1323	-2.0
miR-191*	-2.0
miR-7-1*	-2.0
miR-3663-3p	-2.0

miRNA	Fold change
miR-25*	-2.0
miR-589	-2.0