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Phospholipase C β Interacts With Cytosolic Partners to Regulate Cell Proliferation

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Abstract

Phospholipase C β (PLC β) is the main effector of the G α q signaling pathway relaying different extracellular sensory information to generate intracellular calcium signals. Besides this classic function, we have found that PLC β plays an important but unknown role in regulating PC12 cell differentiation by interacting with components in the RNA-induced silencing machinery. In trying to understand the role of PLC β in PC12 cell differentiation, we find that over-expressing PLC β reduces PC12 cell proliferation while down-regulating PLC β increases the rate of cell proliferation. However, this behavior is not seen in other cancerous cell lines. To determine the underlying mechanism, we carried out mass spectrometry analysis of PLC β complexes in PC12 cells. We find that in unsynchronized cells, PLC β primarily binds cyclin-dependent kinase (CDK) 16 whose activity plays a key role in cell proliferation. *In vitro* studies show a direct association between the two proteins that result in loss in CKD16 activity. When cells are arrested in the G2/M phase, a large population of PLC β is bound to Ago2 in a complex that contains C3PO and proteins commonly found in stress granules. Additionally, another population of PLC β complexes with CDK18 and cyclin B1. Fluorescence lifetime imaging microscopy (FLIM) confirms cell cycle dependent associations between PLC β and these other protein binding partners. Taken together, our studies suggest that PLC β may play an active role in mediating interactions required to move through the cell cycle.

Keywords

phospholipase C β ; G proteins; cell differentiation; cell cycle; cyclin-dependent kinase; stress granules

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Introduction

PLC β mediates Calcium signals through G α_q

Mammalian inositide-specific phospholipase C β (PLC β) enzymes are the main effectors of the G α_q pathway and respond to hormones and neurotransmitters and other sensory signals (for review see (1)). PLC β 's major role in the cell is to hydrolyze the signaling lipid phosphatidylinositol 4,5 biphosphate (PIP₂) leading to activation of protein kinase C, release of calcium from intracellular stores impacting the activity of a host of calcium-sensitive enzymes. There are 4 known isoforms of PLC β and all are strongly activated by G α_q subunits while two (PLC β 2,3) are additionally activated by G $\beta\gamma$ subunits. Activation of PLC β requires binding to G protein/GPCR complexes on the surface of plasma membranes to undergo conformational changes that allow for catalysis of the hydrolysis of PIP₂. Live cell imaging of PLC β in cultured cells show most of the enzyme resides on the plasma membrane (2). However, PLC β 's will localize in the cytoplasm (e.g. (2, 3)) and two isoforms (PLC β 1,3) display nuclear localization. The role of this latter population has been extensively reviewed (4-6).

Several years ago, we set-out to determine the cellular role of the cytoplasmic population of PLC β 1. Since the activity of PLC β 1 is very low when it is not stimulated, and we could not detect G α_q in the cytoplasm, we initially searched for novel cytosolic PLC β activators. We used the C-terminal region as bait in a yeast two hybrid study since this region is responsible for activation by G α_q (7). These studies identified the nuclease *TRAX* (translin-associated protein X) (8). TRAX is a small helical protein that shuttles between the cytoplasm and nucleus to hydrolyze ssDNA or RNA (for review see (9)). TRAX is routinely found associated with the oligonucleotide binding protein *translin* to form the complex *C3PO* (component 3 of the RNA-induced silencing complex). C3PO has shown to be involved in many functions including RNA-induced gene silencing. Specifically, C3PO has been shown to bind to *RISC* (RNA-induced silencing complex) and promote RNA-induced silencing (10, 11). In cells, PLC β bind to and inhibits C3PO thereby reversing RNA-induced silencing (12, 13). Reversal of siRNA by PLC β was found to be independent of its PIP₂-hydrolyzing activity. Additionally, since PLC β binds to C3PO in the same region as G α_q , we find that over-expressing C3PO inhibits calcium signaling through PLC β while increasing the level of activated G α_q reduces the ability of PLC β to reverse RNA-induced silencing (12, 14). These results suggest that PLC β is in dynamic equilibrium with G α_q on the plasma membrane and C3PO-RISC in the cytoplasm (for review see (15)). Thus, these multiple interactions connect external signals and G protein to post-transcriptional gene regulation (Fig 1).

Topics

PLC β plays a role in PC12 cell differentiation

Recently, we used PC12 cells as a model system to study neuronal differentiation (16). In their undifferentiated state, PC12 cells have a round morphology and are highly proliferative. Upon treatment with nerve growth factor (NGF), proliferation ceases and the cells begin to sprout neurites and accumulate Ca²⁺ vesicles (17). This process is thought to be complete when the length of the neurites grow to 3-4 times the length of the cell body,

which in our lab is ~36 hrs after NGF treatment. NGF works through growth factor pathways, and hence PLC γ , to change the transcription of proteins associated with neurite growth and synaptic function (see (18, 19)). The onset of differentiation leads to the production of proteins associated with G protein signaling, and notably, members of the G-protein/phospholipase C β signaling pathway.

We followed the changes in the levels of PLC β , TRAX (C3PO) and G α q following NGF treatment. TRAX levels remained constant with differentiation, however, the level of PLC β increased ~4 fold in the first 24 hrs and then declined over the next 48 hrs (Fig 2). On the other hand, increases in G α q lagged behind PLC β reaching a 2-fold increase that peaked at 48 hrs (Fig 2). During the 24 hrs interval when the level of PLC β at a maximum G α q was still low, PLC β accumulated in the cytosol where its association with C3PO increased. However, as G α q accumulated on the plasma membrane, the interaction between PLC β and C3PO diminished, as did the ability of PLC β to inhibit RNA-induced silencing. Taken together, these studies found that although PLC β -C3PO interactions do not initiate PC12 cell differentiation, they are required for its progression through its early phases.

Several years ago, it was reported that over-expression of PLC β 1 does not affect PC12 cell differentiation suggesting that a minimal level of PLC β is required and that excess PLC β does not contribute (20). It is notable that C2C12 cells, differentiation is characterized by a marked increase in nuclear PLC β 1 expression and down-regulation of PLC β 1 inhibits differentiation (21, 22). However, in murine erythroleukemia differentiation, PLC β 1 is down-regulated (23). Importantly, the effect of PLC β 1 on this differentiation process is independent of its catalytic activity.

PLC β inhibits PC12 cell proliferation

In studying the effect of PLC β on PC12 cell differentiation, we found that the level of PLC β greatly effected proliferation. In general, the role of PLC β in proliferation appears to be secondary to PLC γ enzymes, which are regulated by growth factors, which impact transcription through modulation of calcium regulated enzymes (1, 19). We find that in many cultured cancer cell lines, a reduction in the level of PLC β 1 reduces the rate of proliferation (FIG 3). However, in PC12 cells down-regulating PLC β 1 causes an increase in proliferation (FIG 3). This increase is not due to PLC β 1's traditional role in mediating calcium signals since down-regulating G α q did not affect proliferation (FIG 4).

In trying to understand the role of PLC β in cell proliferation, we began searching for unique binding partners that might be involved in cell cycle progression. Cell cycle progression is orchestrated by a series of kinases and phosphatases whose concentrations and/or activities change through the cycle. Cyclins are a family of proteins whose that combine with cyclin dependent kinases (CDKs) to activate their serine/threonine protein kinase activity and move the cell through different phases of the cell cycle. There are several types of CDKs that are activated by different families of cyclins (for background see (24)). Previous studies by Cocco and coworkers have suggested a direct linkage between PLC β 1 in cyclin D (23).

We began by identifying binding partners of PLC β 1 in unsynchronized PC12 cells, and thus largely in the G1 phase. We pulled down PLC β 1-protein complexes using a monoclonal

antibody and identified proteins by mass spectrometry (Garwain, Singla, Vallah, Scarlata, *in preparation*). We find that in the G1 phase PLC β 1 primarily associates with cyclin-dependent kinase 16 (CDK16), which is also known as PTCAIRE1. CDK16 is activated by cyclin Y and is present post-mitotically where it has been shown to regulate neurite outgrowth in Neuro2A neuroblastoma cells (25), and is involved in membrane trafficking (26, 27) and targeting presynaptic components to the axons (28). In pre-mitotic cells, CDK16 promotes progression from the G1 to S phase through a signaling network that includes p27 and p53 (29). Live cell imaging confirms the cell cycle association between PLC β 1 and CDK16. Additionally, using purified proteins, we find that PLC β 1 binds to and inhibits CDK16. These results lead to the idea that, by inhibiting CDK16, PLC β 1 helps to maintain cells in the G1 phase.

We repeated the mass spectrometry analysis of PLC β 1 complexes in PC12 cells arrested in the G2/M phase. Here, we find that PLC β 1 now binds to Ago2/C3PO and several stress granule proteins (for review see (30)). Stress granules are large complexes containing mRNA, ribosomes, and many components of the translational machinery. Formation of stress granules leads to loss of protein translation. Our results imply that PLC β 1 may move Ago2 and C3PO from active RISC complexes to stress granules. We find that down-regulation of PLC β increases protein production suggesting that PLC β may either help in the formation of stress granules or interfere with the activity of one or more of the proteins involved in translation.

In addition to Ago2 and C3PO, we find that when cells are arrested in the G2/M phase, PLC β 1 binds to CDK18. CDK18 is expressed in higher eukaryotes and is closely related to cdc2 which is activated by cyclin B1 to move cells from the G2 into the M phase (31). Additionally, CDK18 appears to be linked to neurodegeneration (32). To determine whether PLC β 1 plays a relevant role in progression through the G2/M phase, we first monitored its association with cyclin B1, which is thought to activate CDK18. Live cell FRET imaging supports PLC β 1-cyclin B1 association in G2/M phase but suggests that cyclin B1 is not PLC β 1's primary binding partner.

Concluding Statements

Our studies present a model in which PLC β not only mediates calcium signals by sensory stimulation, but is an active sensor that cues cell to differentiate (15, 16), move from RNA-induced silencing to protein production, and proceed through the cell cycle (Fig 5). These studies may offer novel methods to control key regulatory functions in cell through manipulating PLC β protein interactions.

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Fig 1. Cartoon depicting the shuttling of PLC β 1 from G α q on the membrane to C3PO and the RNA-induced silencing complex (RISC). Factors that affect the dynamics of this equilibria, such as G α q activation or assembly of the RISC complex, will influence the amount of PLC β 1 involved in calcium signaling versus RNA-induced silencing.

Fig 2. From Garwain and Scarlata (16)

(A) PC12 cells 24 hrs after NGF treatment where cells were treated with various reagents as noted 24 hrs before NGF addition. (B) Change in the ratio of neurite to body length for cells under various treatments 24 hrs after NGF treatment where 30 points were taken over 2-5 separate experiments and $p < 0.001$. (C) (Top) Western blot showing the effect of down-regulating Gαq and PLCβ1 by siRNA treatment on the expression of other proteins where the lines note the specific treatment, where $n=4$. (Bottom) Western blot showing the effect of down-regulation of Gαq and TRAX on PLCβ1 levels where $n=5$.

Fig 3.
Results of MTT (cell proliferation) assays for PLC β 1 down-regulation in various cell lines. PLC β 1 down-regulation inhibits proliferation in all cells types but undifferentiated PC12 cells, n=3-8.

Fig 4.

Results of MTT (cell proliferation) assays showing that down-regulation of G α q reverses the increase in PC12 cell proliferation due to PLC β 1 down-regulation. The results suggest that lowering G α q releases more PLC β 1 into the cytoplasm to interact with CDK enzymes.

Fig 5. Working model of PLCβ1's control of cell cycle. In the G1 phase, PLCβ1 interacts with CDK16 inhibiting its kinase activity and its ability to allow progression into the S phase. Also in this phase a small population of PLCβ1 interacts with C3PO/Ago2. In the G2 phase, of PLCβ1 moves into stress granules with C3PO/Ago2 while another population interacts with CDK18; the functional consequences of this interaction are currently unclear.