

γ -Synuclein Interacts with Phospholipase C β 2 to Modulate G Protein Activation

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Abstract

Phospholipase C β 2 (PLC β 2) is activated by G proteins and generates calcium signals in cells. PLC β 2 is absent in normal breast tissue, but is highly expressed in breast tumors where its expression is correlated with the progression and migration of the tumor. This pattern of expression parallels the expression of the breast cancer specific gene protein 1 which is also known as γ -synuclein. The cellular function of γ -synuclein and the role it plays in proliferation are unknown. Here, we determined whether γ -synuclein can interact with PLC β 2 and affect its activity. Using co-immunoprecipitation and co-immunofluorescence, we find that in both benign and aggressive breast cancer cell lines γ -synuclein and PLC β 2 are associated. In solution, purified γ -synuclein binds to PLC β 2 with high affinity as measured by fluorescence methods. Protease digestion and mass spectrometry studies show that γ -synuclein binds to a site on the C-terminus of PLC β 2 that overlaps with the G α q binding site. Additionally, γ -synuclein competes for G α q association, but not for activators that bind to the N-terminus (i.e. Rac1 and G $\beta\gamma$). Binding of γ -synuclein reduces the catalytic activity of PLC β 2 by mechanism that involves inhibition of product release without affecting membrane interactions. Since activated G α q binds more strongly to PLC β 2 than γ -synuclein, addition of G α q(GTP γ S) to the γ -synuclein-PLC β 2 complex allows for relief of enzyme inhibition along with concomitant activation. We also find that G $\beta\gamma$ can reverse γ -synuclein inhibition without dissociating the γ -synuclein-PLC β 2 complex. These studies point to a role of γ -synuclein in promoting a more robust G protein activation of PLC β 2.

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Introduction

The synucleins are small (~140 amino acid) proteins, that have a weak homology to 14.3.3 proteins (a typical member of the chaperone protein family (see [1,2,3]). The synucleins are considered to be “natively unfolded” [4] although recent work indicates that in cells α -synuclein folds into a dynamic tetramer [5,6]. There are three members of the synuclein family, α , β and γ that are conserved and found throughout vertebrates. The cellular function(s) of synucleins have not yet been discovered. α -Synuclein, the most notable family member, is associated with neurodegenerative plaques [2].

Although γ -synuclein is found mostly in the peripheral nervous system and in pre-synaptic terminals, its over-expression is associated with cancer progression. γ -Synuclein was identified as the breast cancer specific gene protein 1 (BCSG1) ~10 years ago by screening a breast cancer cDNA library [7]. γ -Synuclein is highly expressed in infiltrating breast cancer [8] but is undetectable in normal or benign breast lesions, and is partially expressed in ductal carcinomas. While the function of γ -synuclein is unknown, it is found in a wide variety of transformed cells and its overexpression leads to a significant increase in proliferation, motility, invasiveness and metastasis [8,9].

Like γ -synuclein, phospholipase C β 2 (PLC β 2) is absent in normal breast tissue, but is highly expressed in transformed tissue where its level of expression is directly related to tumor progression and migration [10,11] presumably through its regulation by small G proteins [10,11]. PLC β 2 is a member of a larger mammalian PLC family that catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PI(4,5)P₂). Cleavage of PI(4,5)P₂ generates the second messengers, diacylglycerol and 1,4,5 inositol trisphosphate (Ins(1,4,5)P₃), which activate protein kinase C (PKC) and cause the release of Ca²⁺ from intracellular stores, respectively. All four isoforms of PLC β are strongly activated by G α_q . Additionally, PLC β 2 and PLC β 3 are activated by G $\beta\gamma$ dimers that can potentially be released upon activation of all G α families. It has also been found that PLC β 2 can be activated by members of the Rho family of monomeric G proteins with the strongest activation by Rac1, which is involved in the cytoskeletal rearrangements that accompany cell mobility [12].

PLC β 2 is a modular protein composed of an N-terminal pleckstrin homology (PH) domain, 4 EF hands, a catalytic domain, a C2 domain and a long C-terminal extension (see [13]). Crystallographic studies have indicated that Rac1 may promote enzyme activity by binding strongly to the PH domain and promoting membrane binding [14]. Alternately, G $\beta\gamma$ activates the

enzyme by simultaneously interacting with both the PH and catalytic domains to change their domain orientation, while G α q activates the enzyme through interactions with the C2 and C-terminal regions of the enzyme (see [15]). Even though PLC β 3 can be simultaneously activated by G α q and G β γ , this does not appear to occur for PLC β 2 [16].

Here, we have tested the idea that γ -synuclein interacts with PLC β 2 to promote cancerous phenotypes. We present data showing that they may associate in breast cancer cells and in solution. The binding of γ -synuclein to PLC β 2 results in inhibition of enzymatic activity that can be overcome by the addition of G protein subunits. This relief of γ -synuclein inhibition along with activation results in a more robust response of the enzyme.

Materials and Methods

Cell culture

MDA MB 231 cells were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 units/mL of penicillin and 50 μ g/mL of streptomycin at 37°C and 5% CO₂. MCF 10 A cells, also purchased from ATCC, were also cultured at 37°C and 5% CO₂ in a medium that consists of 1:1 mixture of Ham's F12 and DMEM media supplemented with 10% FBS, 50 units/mL of penicillin and 50 μ g/mL of streptomycin, 10 μ g/ml bovine insulin, 0.18 μ g/ml hydrocortisone, and 20 ng/ml recombinant human epidermal growth factor.

Immunofluorescence

Cells were fixed using 3.7% formaldehyde and permeabilized with 0.2% nonyl phenoxypolyethoxyethanol (NP40) and incubated with 0.2% NP40 in phosphate buffered saline (PBS) for 5 min and then blocked in PBS containing 4% goat serum for 1 h. The cells were then incubated with the primary antibody (anti-PLC β 2 (Santa Cruz Biochemicals, Inc.) and anti- γ -synuclein (Abcam, Inc.)) diluted to 1:500 overnight at 4°C, followed by incubation with Alexa-labeled secondary antibody for 1.5 hours at room temperature. The cells were washed with tris buffered saline (TBS) buffer after the incubations. Images of the cells were obtained using Olympus Fluoview FV1000 laser scanning confocal microscope, and were analyzed using Olympus (Fluoview) software and Image J (NIH).

Co-immunoprecipitation

MDA MB 231 cells were lysed with 500 μ l of buffer containing 150 mM NaCl, 20 mM HEPES, 2 mM MgCl₂, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin. The lysate was then added to 20 μ l of Protein A beads, which were incubated with 5 μ l of rabbit anti- γ synuclein antibody overnight at 4°C, and the mixture was gently rotated for 4 hours at 4°C. The unbound proteins were separated from the beads, which were then washed twice with the lysis buffer. The bound proteins were then eluted from the beads in sample buffer at 95°C for 3 minutes and were loaded onto a 12% PAGE gel along with an equal volume of the unbound proteins for SDS-PAGE. After western transfer to polyvinylidene difluoride membranes, the membranes were blotted with anti- γ -synuclein and anti-PLC β 2 antibodies.

Protein expression and purification

Human γ -synuclein was expressed and purified using an identical procedure as described for α -synuclein [17] with minor modifications. G β 1 and His₆-G γ 2 were co-expressed with G α q in

Sf9 cells and the heterotrimer was purified on a Ni²⁺-NTA column, and then dissociated by activation with GTP γ S [18]. The purity of proteins was assessed by SDS-PAGE electrophoresis and western blotting. Concentrations were determined by a Bradford assay (BioRad) or on SDS-PAGE gels with known concentrations of BSA for reference. His₆-PLC β 2 was expressed in Sf9 cells using a baculovirus system with minor modifications [17]. A C-terminal truncation mutant of PLC β 2 used for some of the control studies is a chimera of PLC β 2 and PLC δ 1 described in [19]. Rac1 was a generous gift from Dr. Nicolas Nassar (Univ. Cincinnati) and its integrity was verified by SDS-PAGE electrophoresis and mass spectrometry and was prenylated using a kit from Uniprot, Inc.

Digestion studies

μ -Calpain digestion was carried out by adding μ -calpain in 800 μ M calcium to pre-incubated PLC β 2- γ -synuclein complexes, at 100 nM each, for 25°C for 20 minutes. The amount of digestion was assessed by western blot analysis using anti-PLC β 2 (Santa Cruz Biochemicals), or by assessing the loss in enzyme activity as determined by the ability of 20–50 nM enzyme to hydrolyze [³H]PI(4,5)P₂ dispersed on 2 mM sonicated membranes composed of phosphatidyl serine: phosphatidyl ethanolamine: phosphatidyl inositol 4,5 biphosphate (PS: PE: PI(4,5)P₂) at a 2:1:0.5 molar ratio as described previously [20].

Mass spectrometry

PLC β 2 bands alone or subjected to calpain digestion were isolated on SDS PAGE electrophoresis. The bands were cut and removed, digested by trypsin and the peptides were analyzed by LC/MS/MS on a Thermo LTQ XL at the Proteomics Center at Stony Brook University.

Enzyme Activity Studies

Measurements of PI(4,5)P₂ hydrolysis by PLC enzymes were carried out by doing small, unilamellar vesicles composed of 1-palmitoyl -2 -oleoyl phosphatidylethanolamine (PE): 1-palmitoyl-2-oleoyl phosphatidyl serine (PS) :PI(4,5)P₂ at a 66:32:2 molar ratio with enough [³H]PI(4,5)P₂ to achieve reliable signal(see [21]).

Fluorescence labeling and titrations

Proteins were labeled on ice with the thiol-reactive probe 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) or Dabcyl at a probe:protein ratio of 4:1. The reaction was stopped after 60 minutes by adding 10 mM DTT and the protein was purified either by extensive dialysis or using a PD G-25 spin trap column (GE Healthcare).

Fluorescence measurements were performed on an ISS spectrofluorometer (Champaign, IL) using 3 mm quartz cuvettes. Peptide and protein stocks were diluted into 20 mM Hepes (pH 7.2), 160 mM NaCl, 1 mM DTT. The emission spectrum of CPM-labeled protein was measured from 400 to 550 nm (λ_{ex} = 380 nm). The background spectra of unlabeled protein or peptide were subtracted from each spectrum along the titration curve. All of the spectra were corrected for the 10–12% dilution that occurred during the titration.

Results

γ -Synuclein and PLC β 2 associate in breast cancer cells

As an initial step in understanding the relationship between γ -synuclein and PLC β 2 in breast cancer, we determined whether these proteins associate in cells. We first visualized their cellular localization and colocalization in two cultured breast cancer epithelial cell lines, MCF10A representing stage 1 cancer, and

MDA-MB-231 representing stage 4 breast cancer (see [11]). As shown in **Fig. 1a**, the proteins are widely distributed through the plasma membrane and cytoplasm with a low nuclear population. Also, we find a moderate degree of colocalization of the endogenous proteins and a Mander's coefficient of 0.55 ± 0.05 was obtained for the proteins in both cell lines. This value can be compared to a positive control of 0.93 ± 0.01 ($n = 9$) measured for an eGFP-tagged protein that was immunostained with Alexa647, and a negative control of 0.017 ± 0.02 ($n = 7$) measured for an eGFP-tagged protein with only Alexa647-secondary antibody (Calizo and Scarlata, submitted).

To corroborate the colocalization studies suggesting cellular association of γ -synuclein and PLC β 2, we carried out co-immunoprecipitation studies in MDA-MB231 cells. The results, shown in **Fig. 1b**, support the idea that these proteins associate in cells.

In vitro, γ -synuclein binds to PLC β 2 with high affinity

We characterized the interaction between γ -synuclein and PLC β 2 by studying the association of these proteins in solution. For these studies, we covalently attached the fluorescent probe CPM onto purified PLC β 2 (see Methods). CPM is highly sensitive

to the polarity of its environment and binding of a CPM-labeled protein to an unlabeled partner usually results in a large increase in CPM fluorescence. CPM is thiol-reactive and can attach to one of the many Cys side chains distributed throughout the catalytic and C-terminal domains along with one in the EF hand region. We have previously found labeling PLC β 2 with a thiol reactive probe does not affect its activity or G protein activation properties [22]. Addition of binding partners to CPM-PLC β 2 results in a concentration-dependent increase in fluorescence intensity that quantitatively gives identical affinities as FRET and qualitatively by other methods (see [22,23]). We note that CPM is essentially non-fluorescent in its unreacted form, and although addition of excess protein to unreacted probe will increase its fluorescence, its signal never exceeded 1% of the signal obtained for any of the samples used in this study. We added freshly prepared and purified γ -synuclein to a solution of CPM- PLC β 2.

We observed a systematic increase in CPM-PLC β 2 intensity (i.e. $39 \pm 4\%$) when γ -synuclein was incrementally added. No changes in intensity were detected when dialysis buffer was substituted for γ -synuclein. An example of a fluorescence titration curve is shown in **Fig. 2** where the data were corrected for background, which was less than 1% of the signal, and also for

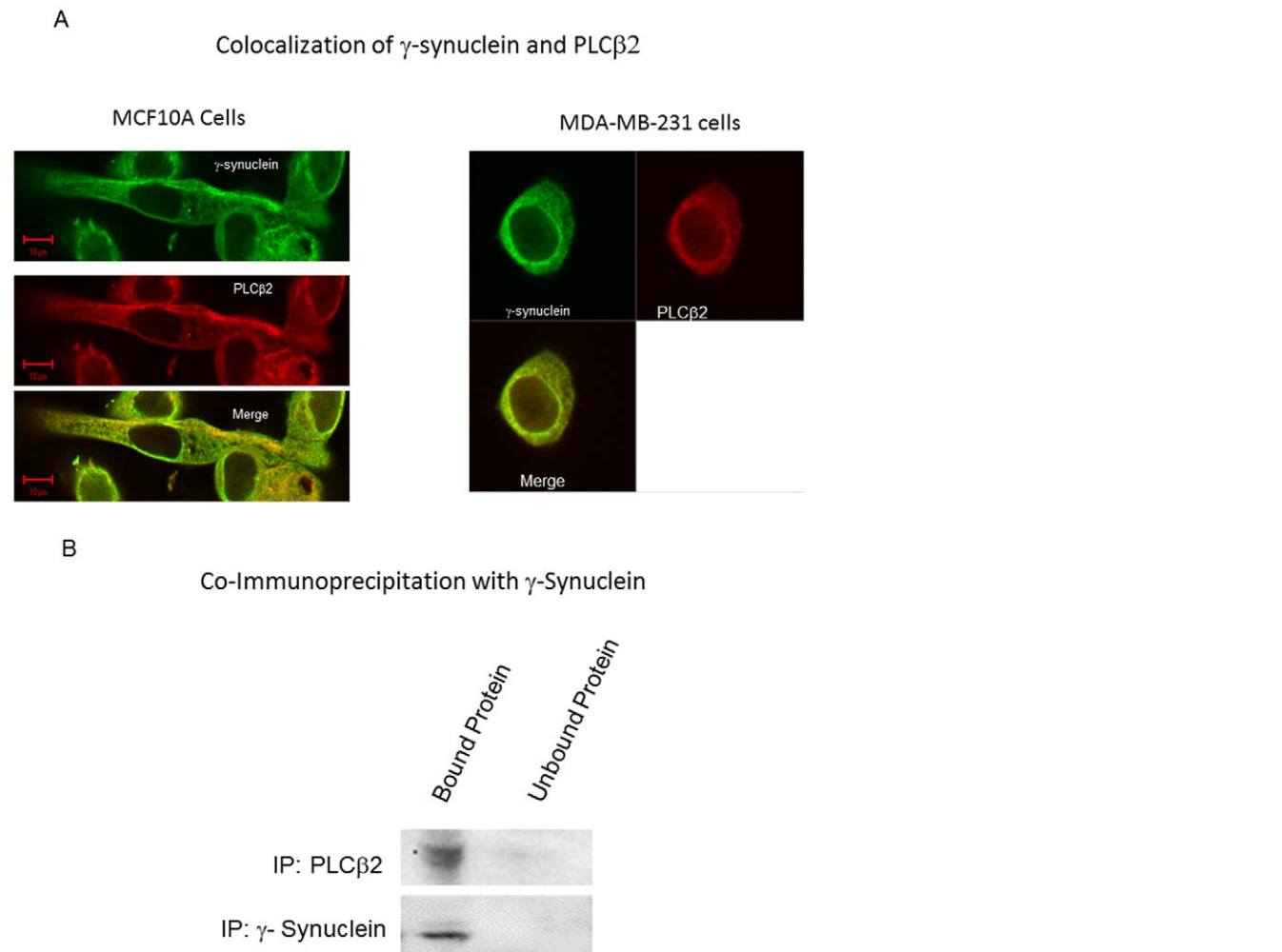


Figure 1. PLC β 2 and γ -synuclein associate in cells. A – Co-immunofluorescence studies showing the colocalization of γ -synuclein (green) and PLC β 2 (red) in two breast cancer cell lines, MCF10A and MDA-MB-231. **B**- Co-immunoprecipitation of endogenous γ -synuclein and PLC β 2 in MDA-MB-231 cells.

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dilution. Fitting the titration curve to a biomolecular dissociation constant gives a $K_d = 6.5 \pm 1$ nM. This same binding constant is obtained when the initial concentration of CPM- PLC β 2 was reduced from 2.0 to 0.5 nM ($K_d = 7.8 \pm 2$ nM) supporting the idea that we are viewing a protein-protein association. Substitution of the full length CPM-labeled enzyme with only its labeled N-terminal PH domain (CPM-PH-PLC β 2) or with CPM-Rac1 (see below) gave a smaller increase in intensity at much higher protein concentrations suggesting that the affinities for these other proteins is at least 50 fold weaker, and indicating that we are indeed observing association of γ -synuclein with proteins and not with the CPM label itself.

Identification of the binding region between γ -synuclein and PLC β 2

We tested whether γ -synuclein would alter the interaction between PLC β 2 and its activators. Since different G protein subunits associate to different regions of the enzyme, we first set out to identify the PLC β 2 domain where γ -synuclein binds. Rac1 and G $\beta\gamma$ bind to the N-terminal PH domain of PLC β 2, and so we measured the association of γ -synuclein to a PLC β 2 construct that is missing the long \sim 400 amino acid C-terminal region (see [19]). We find that γ -synuclein binds to this truncated enzyme at a far lower affinity than the whole enzyme (**Fig. 3 top**). This result, along with the fluorescence titrations described above, suggests that γ -synuclein interacts primarily with the C-terminal region of the protein.

PLC β 2 has a calpain cleavage site that releases the entire C-terminal tail region and truncation can be seen by the shift in the 133.7 KDa enzyme electrophoresis band to \sim 80 and \sim 45 KDa bands (see [24]). In **Fig. 3 bottom** we show that γ -synuclein protects PLC β 2 from calpain digestion. We extracted the 100 KDa band obtained from calpain-digested PLC β 2 and analyzed this product by mass spectrometry. Comparing this fragment to the undigested and γ -synuclein – PLC β 2 complex, we find that γ -synuclein binding protects PLC β 2 from calpain

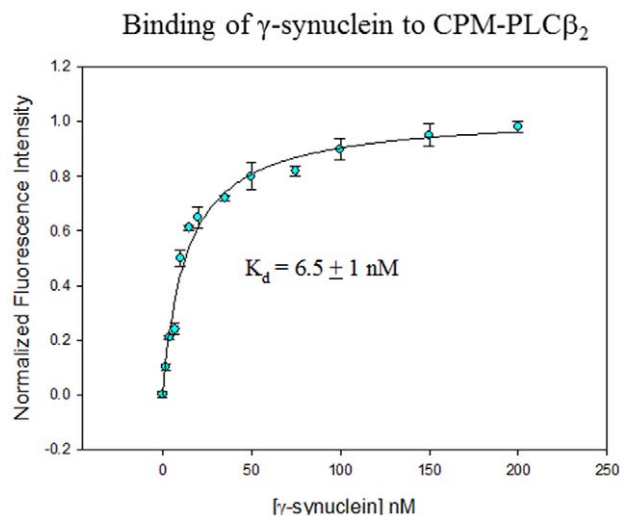


Figure 2. PLC β 2 and γ -synuclein associate in solution. Normalized change in fluorescence intensity of 2 nM CPM- PLC β 2 in solution as purified γ -synuclein is added where the total intensity increase was $39 \pm 4\%$. The data shown are corrected for dilution and background, which was less than 1% of the signal, and are an average of 3 sets of measurements. doi:10.1371/journal.pone.0041067.g002

Binding of γ -synuclein to truncated PLC β 2

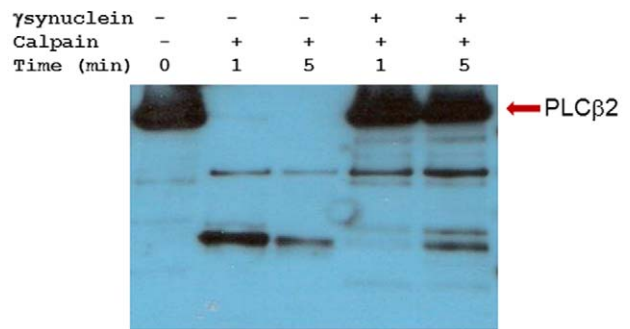
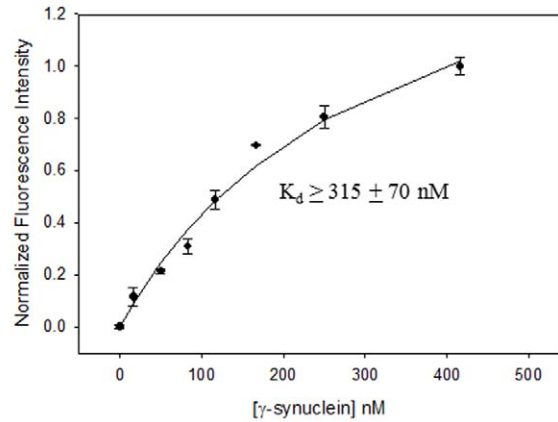


Figure 3. The C-terminus of PLC β 2 is required for strong γ -synuclein binding. **Top** – Association of γ -synuclein to a C-terminal truncated PLC β 2 construct under identical conditions as in **Fig. 2**. While the affinity cannot be accurately obtained since the titration curve did not plateau, the estimated minimum apparent dissociation constant is given. **Bottom** – Calpain digestion of PLC β 2 showing cleavage of the \sim 134 kDa enzyme into \sim 80 and \sim 45 kDa fragments where identical amounts of PLC β 2 were loaded onto each lane. Note the protection of enzyme digestion in the presence of γ -synuclein. doi:10.1371/journal.pone.0041067.g003

cleavage at residue 753. This residue lies in a region that connects the C2 domain with the C-terminal tail.

γ -Synuclein inhibits G α q but not G $\beta\gamma$ association to PLC β 2

The studies above show that γ -synuclein binds to the region of PLC β 2 that is close to the binding region of G α q as indicated by the recent crystal structure of a truncated PLC β 3 construct complexed with G α q [25] and overlaps with the region required for G α q activation [26]. Thus, we tested whether γ -synuclein could compete with G α q for PLC β 2 association by two complementary types of fluorescence titrations (**Fig. 4A**). In the first, we measured the association between CPM-G α q(GDP) and PLC β 2 in the absence and presence of a ten-fold excess of γ -synuclein. In the second, we added PLC β 2 labeled with a non-fluorescent FRET acceptor (Dabcyl) to CPM- G α q(GDP) and measured their association by the decrease in CPM donor fluorescence in the presence and absence of γ -synuclein. Both studies show a clear inhibition of G α q(GDP) – PLC β 2 binding when γ -synuclein is present (**Fig. 4A**). Activation of G α q results in an \sim 20 fold increase in affinity for PLC β 2 [22], and we find that a ten-fold

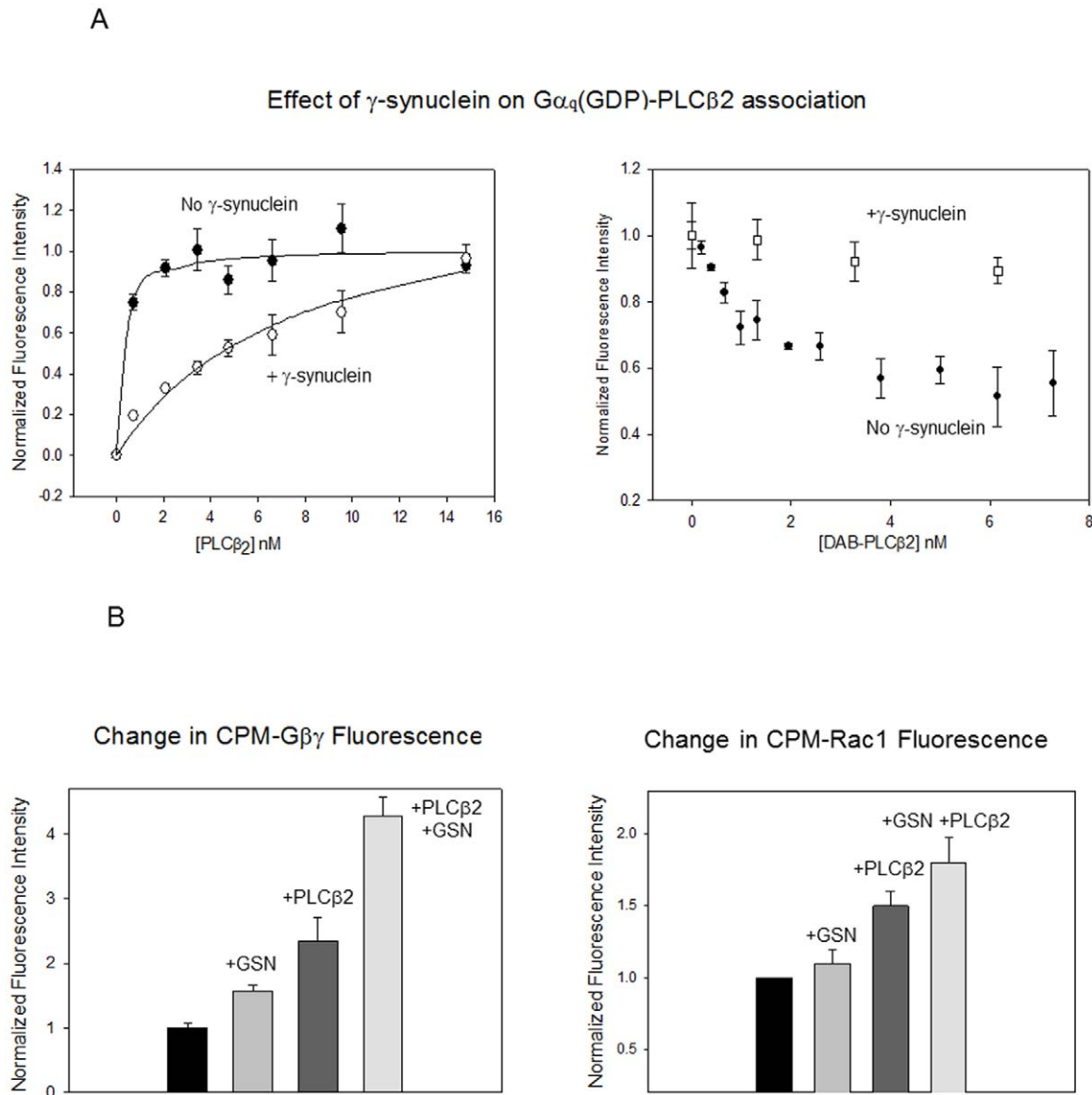


Figure 4. γ -Synuclein inhibits the binding of $G\alpha_q$ but not $G\beta\gamma$ or $Rac1$, to PLC β 2. **A** – (left) Fluorescence titrations showing that 20 nM γ -synuclein inhibits the association of PLC β 2 to 2 nM CPM- $G\alpha_q$ (GDP) as seen by the $27 \pm 3\%$ increase in CPM- $G\alpha_q$ fluorescence as PLC β 2 is added ($n=4$) and (right) by the ability of 20 nM γ -synuclein to prevent FRET when PLC β 2 with a non-fluorescent energy transfer acceptor (DAB) is added to CPM- $G\alpha_q$ (GDP), $n=3$. **B**– (left) Change in the intensity of 10 nM CPM- $G\beta\gamma$ when 100 nM γ -synuclein is added, when 10 nM PLC β 2 is added, and when 100 nM γ -synuclein and 10 nM PLC β 2 are added, $n=2$. (right) Change in the intensity of 2 nM CPM-Rac1(GDP) when 5 nM PLC β 2 is added, when 20 nM γ -synuclein is added and when 5 nM PLC β 2 and 20 nM γ -synuclein were added. doi:10.1371/journal.pone.0041067.g004

excess γ -synuclein has little, if any, effect on the association between activated $G\alpha_q$ and PLC β 2, ($K_d = 0.3 \pm 0.2$ nM without γ -synuclein and 0.9 ± 0.3 with γ -synuclein, $p = 0.049$).

We also determined whether γ -synuclein could affect $G\beta\gamma$ - PLC β 2 interactions. Although we could not detect changes in $G\beta\gamma$ -PLC β 2 association in the presence of excess γ -synuclein, we found evidence that γ -synuclein weakly associated to the $G\beta\gamma$ -PLC β 2 complex. When γ -synuclein was added to CPM- $G\beta\gamma$, an increase in intensity was observed and a further increase occurred upon the addition of PLC β 2 (**Fig. 4B left**). An increase in intensity was also seen when γ -synuclein was added to the $G\beta\gamma$ -PLC β 2 complex. These results indicate that γ -synuclein may form a ternary complex with $G\beta\gamma$ - PLC β 2. Identical results were obtained with deactivated or activated Rac1 (**Fig. 4B right**).

Thus, γ -synuclein may form ternary complexes with $G\beta\gamma$ -PLC β 2 and Rac1-PLC β 2 but not with $G\alpha_q$ -PLC β 2.

γ -Synuclein inhibits the activity of PLC β 2, but not its activation by G proteins

We determined the impact of γ -synuclein on the activity of PLC β 2. These studies were carried out by measuring the changes in PLC β 2-catalyzed hydrolysis of 3H -PI(4,5) P_3 . We find that the presence of γ -synuclein decreases PLC β 2 activity approximately 4 fold (**Fig. 5**). This decrease is more pronounced than the decrease seen using α -synuclein [27].

Rac1 has been reported to activate PLC β 2 by increasing its affinity for lipid membranes [12] although this does not appear to be the case in purified systems (Golebiewska & Scarlata,

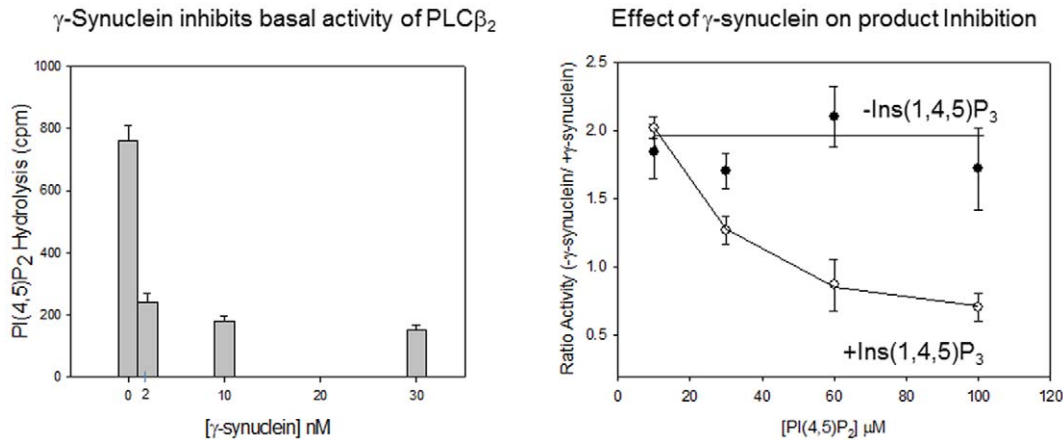


Figure 5. γ -Synuclein inhibits the enzymatic activity PLC β 2. (left) Change in 20 nM PLC β 2 activity with increasing amounts of γ -synuclein. (right) Ratio of PLC β 2 activity with increasing substrate in the presence and absence of 10 mM Ins(1,4,5)P₃ showing that γ -synuclein promotes product inhibition.

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unpublished). Nevertheless, we determined whether γ -synuclein affected PLC β 2 activity by altering its membrane interactions. These studies were done by measuring changes in binding of PLC β 2 to large, unilamellar vesicles composed of PC/PS/PE at a 1:1:1 molar ratio in the presence and absence of excess γ -synuclein. Membrane binding was monitored by the large increase in fluorescence intensity of CPM- PLC β 2 that occurs when the enzyme binds to membranes (see [28]). We find that a fourfold excess of γ -synuclein (100 nM) does not affect the partition coefficient for membrane binding of PLC β 2 ($45 \pm 10 \mu$ M, $n = 4$ without γ -synuclein versus $39 \pm 18 \mu$ M, $n = 3$ with γ -synuclein). These data suggest that PLC β 2 binds to membranes with a similar affinity as the PLC β 2- γ -synuclein complex.

We have previously found that G $\beta\gamma$ activates PLC β 2 by increasing the rate of release of Ins(1,4,5)P₃ product [21]. With this in mind, we determined whether γ -synuclein inhibits PLC β 2 by inhibiting the release of product. We measured product inhibition of PLC β 2 and the PLC β 2- γ -synuclein complex as a function of substrate (PI(4,5)P₂) concentration. In the absence of product, γ -synuclein did not affect the change in PLC β 2 with increasing amount of substrate. However, in the presence of γ -synuclein, we find that Ins(1,4,5)P₃ reduces the ability of PI(4,5)P₂ to promote activity (Fig. 5B). Our interpretation of these results is that γ -synuclein stabilizes an early or intermediate conformation of the enzyme where product is bound more strongly in the active site.

G proteins reverse γ -synuclein inhibition of PLC β 2

Our binding studies predict that γ -synuclein should effect activation of PLC β 2 by G α_q by direct competition, but not by G $\beta\gamma$ or Rac1. We first tested this idea by measuring the ability of γ -synuclein to diminish activation of PLC β 2 by G α_q . We found that addition of an 80 fold excess of γ -synuclein did not reduce the activity of the PLC β 2-G α_q complex, due to the very strong affinity between PLC β 2 and activated G α_q . However, because γ -synuclein inhibits PLC β 2, the ratio of G α_q /PLC β 2/ γ -synuclein activity versus γ -synuclein -PLC β 2 shows an apparent activation (Fig. 6). This apparent activation is due to the ability of G α_q (GTP γ S) to displace γ -synuclein from PLC β 2 and cause a reversal in γ -synuclein inhibition as well as activation.

We also determined whether γ -synuclein could affect activation of PLC β 2 by G $\beta\gamma$ and Rac1. We found that even though γ -synuclein inhibits isolated PLC β 2, it does not inhibit the PLC β 2-

G $\beta\gamma$ complex. In fact, the addition of G $\beta\gamma$ to PLC β 2 overcomes γ -synuclein inhibition so that when the ratios of PLC β 2- γ -synuclein/PLC β 2 as a function of γ -synuclein are plotted, an apparent activation is seen (Fig. 6). In contrast, γ -synuclein similarly inhibits PLC β 2 and PLC β 2-Rac1 in accord with the idea that Rac1 may enhance activity by promoting membrane interactions of the enzyme [14].

Discussion

In this study, we have established a link between γ -synuclein expression and PLC β 2 activation in cultured cells. While the function of PLC β 2 is to transform G protein signals into calcium responses [29,30], little is known about the cellular function of γ -synuclein in neuronal cells, where it is highly expressed, or in certain transformed cells (but see <http://www.disprot.org/protein.php?id=DP00630>). A large over-production of γ -synuclein in breast cancer was first noted by Shi and coworkers who identified it as the breast cancer specific gene protein 1, which was later found to be γ -synuclein [7]. Anticancer agents that target γ -synuclein have been designed [31]. Over-production of PLC β 2 correlates with the severity of breast cancer [10,11]. Since PLC β 2 mediates mitogenic, proliferative and migratory events through its interactions with heterotrimeric and monomeric G proteins, we reasoned that γ -synuclein might not only bind PLC β 2 and affect its basal activity, but also affect G protein activation.

γ -Synuclein is classified as an unstructured protein and has several potential binding partners (see [32]). Thus, we first determined whether PLC β 2 is a natural binding partner of γ -synuclein in cultured cells. In these studies, we visualized the two proteins by immunofluorescence in MCF 10A and MDA MB231 cells that mimic stage 1 and 4 breast carcinoma, respectively. We find a high degree of colocalization in both cell lines, and additionally, the protein coimmunoprecipitate. Thus, γ -synuclein appears to be a cellular binding partner of PLC β 2.

We have previously carried out studies that investigated the interaction between another PLC β family member, PLC β 1, and α -synuclein [24]. Both proteins are highly expressed in neural tissue. We found that the region of interaction between these proteins is the same and that their cellular association prevents calcium-stimulated degradation of the enzyme by the protease calpain. While it is unclear whether calpain degradation plays an important role in cells expressing γ -synuclein, it is probable that γ -

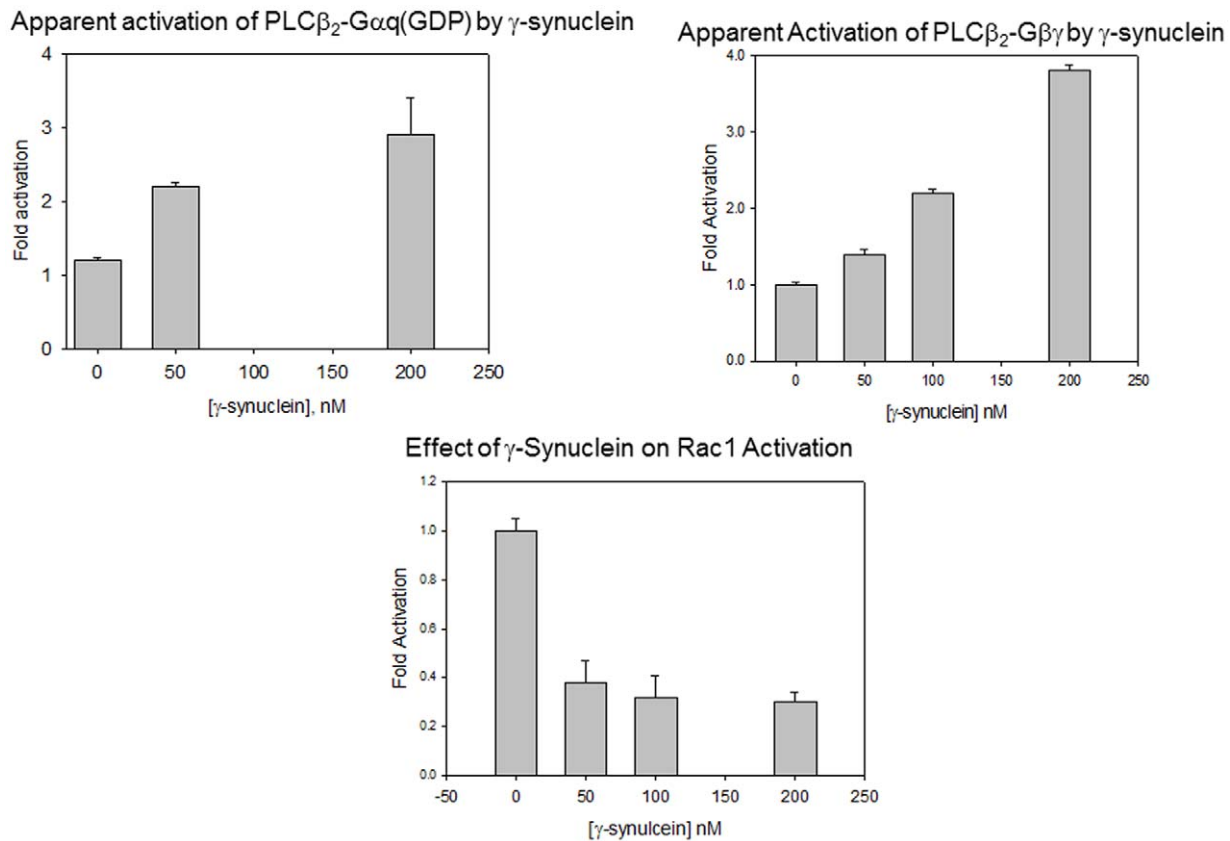


Figure 6. γ -Synuclein binding to PLC β 2 results in an apparent increase in G protein activation. *Top panels* – Apparent activation of 20 nM PLC β 2, as calculated by the ratio of the activity of PLC β 2 complexed with G α _q(left) or G $\beta\gamma$ (right) over PLC β 2 alone, due to reversal of γ -synuclein by G protein subunits. *Bottom* – Activity studies showing that Rac1 does not interfere with inhibition of PLC β 2. doi:10.1371/journal.pone.0041067.g006

synuclein will also protect PLC β 2 from cleavage. This stabilization could underlie the correlation between elevated PLC β 2 levels with increased γ -synuclein expression making it difficult to quantify the decrease in calcium release through PLC β with increased cellular γ -synuclein (Yerramilli et al., unpublished).

We characterized the affinity of γ -synuclein-PLC β 2 association using purified proteins. Fluorescence studies show that the two proteins associate strongly in solution with an affinity that is slightly stronger than G α _q(GDP), G $\beta\gamma$ and Rac1 but much weaker than activated G α _q. Interestingly, the γ -synuclein binding site suggested by the calpain digestion studies overlaps with the G α _q binding site. We speculate that when γ -synuclein expression is very high, as in many carcinomas, PLC β 2- γ -synuclein may disrupt preformed PLC β 2-G α _q-receptor complexes in the basal state (see [33,34]) or perturb the cellular localization of the enzyme. However, upon activation, it is likely that G α _q can displace γ -synuclein from PLC β 2 allowing γ -synuclein to homo- or hetero-oligomerize with other proteins. Importantly, activation of PLC β 2 by G proteins to promote mobility and migration is preserved in the presence of γ -synuclein.

Our studies suggest that γ -synuclein binding decreases the basal activity of PLC β 2 through a mechanism that appears to involve product release rather than access to substrate by inhibition of membrane interactions. Since G α _q binds to the same region of the PLC β 2 as γ -synuclein, it is not surprising that it reverses γ -synuclein inhibition by direct displacement. In contrast, Rac1 associates to the extreme N-terminus [35] and we find that this association does not perturb γ -synuclein binding or inhibition. In

contrast, G $\beta\gamma$ interacts with both the N-terminal pleckstrin homology domain and the catalytic domain (see [13]) and can reverse inhibition of PLC β 2 by γ -synuclein. This reversal is not due to competition of γ -synuclein binding by G $\beta\gamma$ since γ -synuclein binds well to both PLC β 2 and PLC β 2-G $\beta\gamma$. Instead, our data suggest that ternary complexes can form (Fig. 4). We hypothesize that the mechanism through which G $\beta\gamma$ activates the enzyme does not allow for γ -synuclein inhibition. Since G $\beta\gamma$ and γ -synuclein inversely affect product release, (Fig. 5 and [36]), it is possible that the conformational changes associated with product release which are promoted by G $\beta\gamma$ are preserved in the presence of γ -synuclein, and that displacement of γ -synuclein may not be required for reversal of inhibition. Thus, G $\beta\gamma$ is a more potent activator of PLC β 2- γ -synuclein than isolated PLC β 2. More studies are needed to understand the conformational changes associated with product release. Regardless of the mechanism, inhibition of PLC β 2 by γ -synuclein may not have significant cellular effects since the basal activity of PLC β 2 is low. However, the ability of G α _q and G $\beta\gamma$ to activate PLC β 2 while simultaneously reversing inhibition may lead to an apparently more robust calcium signals.

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Author Contributions

Conceived and designed the experiments: UG SS. Performed the experiments: YG NK CZ VSY. Analyzed the data: UG YG NK CZ

VSYS. Contributed reagents/materials/analysis tools: UG CZ YG. Wrote the paper: UG SS.

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