

Evidence That α -Synuclein Does Not Inhibit Phospholipase D[†]

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Received October 3, 2008; Revised Manuscript Received December 15, 2008

ABSTRACT: α -Synuclein (α Syn) is a small cytosolic protein of unknown function, which is highly enriched in the brain. It is genetically linked to Parkinson's disease (PD) in that missense mutations or multiplication of the gene encoding α Syn causes early onset familial PD. Furthermore, the neuropathological hallmarks of both sporadic and familial PD, Lewy bodies and Lewy neurites, contain insoluble aggregates of α Syn. Several studies have reported evidence that α Syn can inhibit phospholipase D (PLD), which hydrolyzes phosphatidylcholine to form phosphatidic acid and choline. Although various hypotheses exist regarding the roles of α Syn in health and disease, no other specific biochemical function for this protein has been reported to date. Because PLD inhibition could represent an important function of α Syn, we sought to extend existing reports on this interaction. Using purified proteins, we tested the ability of α Syn to inhibit PLD activity in cell-free assays. We also examined several cell lines and transfection conditions to assess whether α Syn inhibits endogenous or overexpressed PLD in cultured mammalian cells. In yeast, we extended our previous report of an interaction between α Syn and PLD-dependent phenotypes, for which PLD activity is absolutely necessary. Despite testing a range of experimental conditions, including those previously published, we observed no significant inhibition of PLD by α Syn in any of these systems. We propose that the previously reported effects of α Syn on PLD activity could be due to increased endoplasmic reticulum-related stress associated with α Syn overexpression in cells, but are not likely due to a specific and direct interaction between α Syn and PLD.

Parkinson's disease (PD¹) is a debilitating neurodegenerative disorder that affects over one million people in the United States (for review, see ref 1). Although more than 90% of PD cases are believed to occur sporadically, several genes are known to cause familial forms of PD. Among these, point mutations or increased dosage of the α -synuclein (α Syn) gene are known to cause rare cases of early onset familial PD (2–7). The α Syn protein is also the major component of Lewy bodies and Lewy neurites, the insoluble

aggregates that are neuropathological hallmarks of both familial and sporadic PD (8). Thus, dysregulation of α Syn at either the genetic or protein level can contribute to PD-type neurodegeneration.

α Syn is a small, highly conserved cytosolic protein of unknown function. It is enriched in the brain, particularly at presynaptic terminals. Proposed functions of α Syn include lipid binding, regulation of membrane composition, and regulation of neurotransmitter release and/or of the reserve pool of synaptic vesicles (9–15). Given the hypothesis that loss of axonal terminals in the striatum may precede the death of nigral neurons in PD (1, 16), these observations suggest that the dysfunction of α Syn at the synapse could be an early event in the pathogenesis of PD.

Phospholipase D (PLD) is a membrane-associated enzyme that hydrolyzes phosphatidylcholine to form phosphatidic acid (PA) and choline. PA is an essential metabolic intermediate and an intracellular signaling molecule that can be further hydrolyzed into diacylglycerol, another important signaling molecule (for review, see ref 17). Mammalian cells express two PLD isoforms, PLD1 and PLD2. They are regulated by distinct cellular mechanisms but perform a similar hydrolytic reaction. In cell-free systems, PLD1 requires an activator such as Rho or ARF GTPases, while PLD2 is highly active in these systems (18, 19). The precise function of PLD in the cell has not yet been determined, though proposed functions include roles in exocytosis, endocytosis, and intracellular signaling (16, 20, 21).

[†] This work was supported by the Brigham and Women's Hospital Udall Center for Excellence in Parkinson's Disease, NIH/NINDS Grant NS038375 (D.J.S.).

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¹ Abbreviations: α Syn, α -synuclein; β Syn, β -synuclein; BSA, bovine serum albumin; ER, endoplasmic reticulum; hPLD1b, human phospholipase D 1b; hPLD2a, human phospholipase D 2a; PD, Parkinson's disease; PA, phosphatidic acid; PBut, phosphatidylbutanol; PLD, phospholipase D; PMA, phorbol-12-myristate-13-acetate.

In 1998, *in vitro* studies suggested that α Syn and its close family member, β -synuclein (β Syn), could act as inhibitors of PLD2 (22). Three other laboratories extended these findings over the next six years, suggesting that α Syn can inhibit PLD activity in mammalian cells (23), in yeast (24), and in cell-free assays (25). Although several hypotheses exist regarding the physiological and pathological roles of α Syn in the brain, no other specific biochemical function for α Syn has been proposed to date. Furthermore, if PLD regulation is a physiological function of α Syn, then PLD dysregulation due to gain or loss of α Syn function could be an early event in the pathogenesis of PD and other synucleinopathies.

Here, we sought to further explore the mechanism of the interaction between α Syn and PLD. We used cell-free and cell-based systems to directly assay PLD activity in the presence or absence of α Syn. In yeast, we extended our previously published genetic findings on α Syn inhibition of PLD. These various approaches did not yield evidence that α Syn significantly inhibits PLD. Based on our data, as well as previous work showing that α Syn expression can induce cytotoxicity associated with endoplasmic reticulum (ER) stress, we suggest that the previously published effects of α Syn on PLD activity may be nonspecific or attributable to general ER stress, rather than a direct and physiological inhibition of PLD by the α Syn protein.

EXPERIMENTAL PROCEDURES

Materials. Chromatographically enriched human PLD1b (hPLD1b) and 6-his-tagged human PLD2a (hPLD2a) were generated as previously described (26, 27) and tested for activity using an exogenous substrate assay (17, 28). Recombinant α Syn protein was generously provided by P. T. Lansbury (29) and by J. M. George (25). Following resuspension in phosphate buffered saline, the protein was filtered through a 0.22 μ m syringe filter followed by a 100,000 MWCO centrifugal filter. The filtered solution was probed by SDS-PAGE and Coomassie stain to confirm the monomeric nature of the recombinant protein preparation. Lipids, including phosphatidylbutanol, were purchased from Avanti Polar Lipids (Alabaster, AL). 3 H-Oleic acid was purchased from PerkinElmer Life Science Products (Newtown, CT). The α Syn expression plasmid was provided by R. Sharon. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell-Based PLD Activity Assay. Endogenous PLD activity was measured as described previously (30). Briefly, 24 h before the assay, HEK 293, HeLa, or undifferentiated PC12 cells were transfected as indicated using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Cells were labeled with 3 H-oleic acid (10 μ Ci/mL) overnight in serum-free medium supplemented with 0.25 mg/mL fatty acid free bovine serum albumin (BSA). On the day of the assay, some cells were preincubated for 5 min in serum-free medium containing the PLD inhibitor VU0155056 (2 μ M) (27). Cells were then stimulated for 30 min with serum-free medium containing 1 μ M phorbol-12-myristate-13-acetate (PMA) with 0.3% butanol and/or VU0155056, as indicated. Lipids were extracted using chloroform:methanol:HCl 0.1 N (1:1:1), then spotted onto silica-coated TLC plates. The plates were developed in chloroform:methanol:acetic acid:acetone:water (50:10:10:20:5),

and the radioactivity was visualized on autoradiographic film with a Kodak Transcreen LE enhancing screen, scanned, and quantified using Quantity One software (BioRad Laboratories, Hercules, CA). A phosphatidylbutanol standard was run on each plate and visualized with sublimed iodine (17).

Cell-Free PLD Activity Assay. PLD activity was assayed as described previously (28). Briefly, purified hPLD2a (15 nM final concentration) was added to reaction buffer (50 mM HEPES pH 7.5, 80 mM KCl, 3 mM EGTA, 0.1 mM DTT, 4.5 mM MgCl₂, 4.5 mM CaCl₂, 10 μ M GTP γ S) containing lipid vesicles and 3 H-PC (total DPPC:POPE:PI(4,5)P₂:cholesterol at a molar ratio of 10:100:6.2:1.4) in a total volume of 60 μ L per assay tube. Various concentrations of α Syn were added to some reactions as indicated, and the PLD inhibitor VU0155056 (20 μ M) was added to other reactions as a control. Reactions were incubated at 37 °C for 30 min, then terminated with 200 μ L trichloroacetic acid (10% v/v) and 100 μ L BSA (10% w/v). Free 3 H-choline released by hPLD2a activity was measured by scintillation counting. Similar assays were performed using purified hPLD1b.

Yeast Plasmids and Strains. The *sec14-1*, *cki1* Δ , and *sec14-1 cki1* Δ strains were described previously (24). Here, we generated the *sec14-1 cki1* Δ *spo14* Δ strain by replacing *SPO14* with a KanMX cassette by homologous recombination in the *sec14-1 cki1* Δ strain. Colony PCR was used to verify the gene disruption. A *SPO14* entry clone (pDONR221) and the Advanced Gateway destination vectors pAG413GPD-ccdB or pAG416GPD-ccdB (31) were used in a Gateway LR reaction (Invitrogen) to construct pAG413GPD-SPO14 and pAG416GPD-SPO14, respectively. To construct the pAG413GPD- α Syn plasmid, an α Syn entry clone was used in a Gateway LR reaction with the pAG413GPD-ccdB destination vector.

Yeast Transformation and Spotting Assays. Yeast cultures were maintained according to standard protocols (32). We used the PEG/lithium acetate method to transform yeast with DNA (33). Yeast cells carrying the α Syn and/or *SPO14* plasmids were grown overnight at 30 °C in liquid media containing glucose until they reached log or midlog phase. Cultures were then normalized for OD₆₀₀, serially diluted and spotted onto synthetic solid media containing glucose and lacking either uracil (for pAG416GPD plasmids) or histidine (for pAG413GPD plasmids) and were grown at 23 °C or 37 °C for 2–3 days.

Statistical Analyses. For each assay, at least three independent experiments were conducted, each in triplicate. Results were analyzed by one-way ANOVA with Tukey's post-hoc tests, as indicated in the figure legends.

RESULTS

α Syn Does Not Inhibit PLD Activity in Living Cells. A previous report suggested that α Syn can inhibit PLD activity in living cells (23). We sought to repeat and extend these findings to determine the extent to which such inhibition could be relevant to PD pathogenesis. We used two different cell lines: HEK 293 and PC12 cells. Both cell lines express low or undetectable levels of α Syn (Figures 1A and 1B), and both show moderate levels of endogenous PLD activity (Figures 1C and 1D). The HEK 293 cells are similar to the HEK 293 cells that were used in the previously published work (23). Cells were transfected with α Syn and/or EGFP-

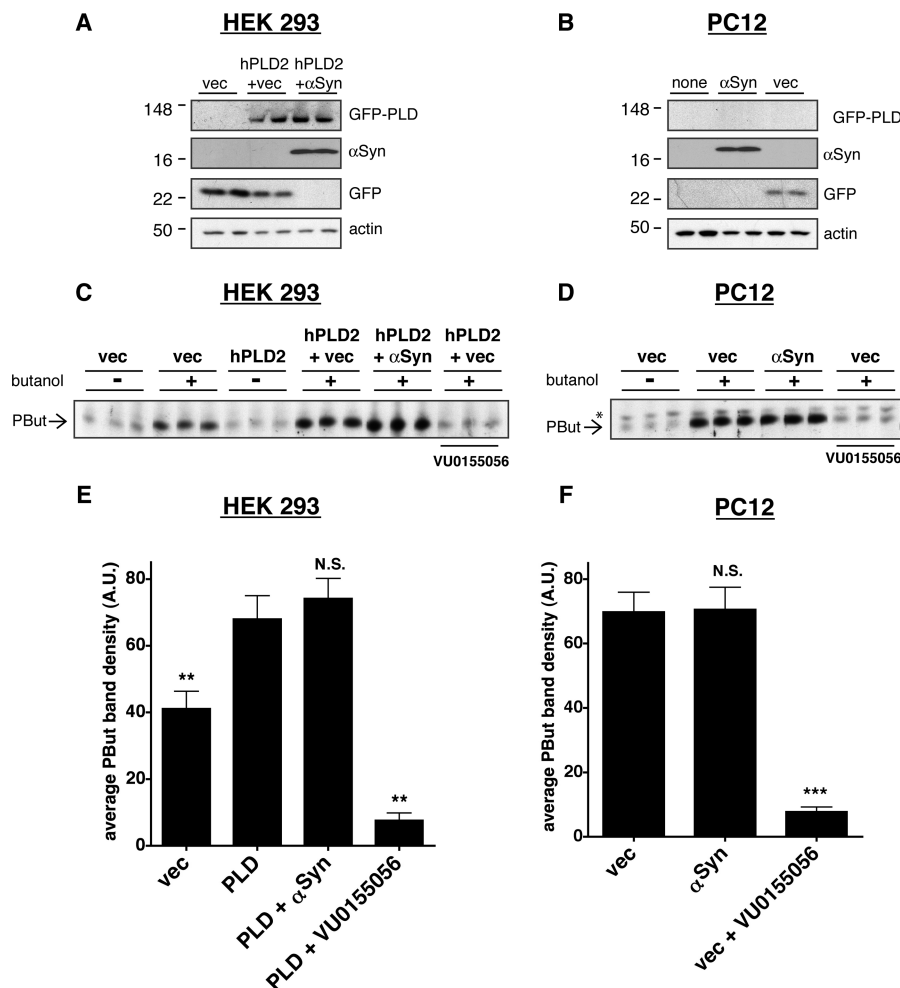


FIGURE 1: αSyn does not inhibit PLD activity in mammalian cells. Cells were transiently transfected with empty EGFP vector as a control (vec), αSyn, or GFP-hPLD2, and PLD activity was then assayed. Some culture wells were used for immunoblots; representative results show αSyn and GFP-hPLD2 protein expression levels in (A) HEK 293 cells and (B) PC12 cells. First panel: GFP-hPLD2 visualized using polyclonal rabbit anti-GFP antibody (Invitrogen). PC12 cells were not transfected with GFP-hPLD2. Second panel: monoclonal mouse anti-αSyn (LB509, Santa Cruz Biotechnology). Third panel: EGFP vector control visualized using anti-GFP antibody. Fourth panel: monoclonal mouse anti-actin (Abcam) was used as a loading control. Twenty-four hours following transient transfection as indicated, PLD activity was assayed. The PLD inhibitor VU0155056 (2 μM (27);) was used as a positive control for inhibition. Representative images are shown from (C) HEK 293 cells and (D) PC12 cells; signal from the PLD product phosphatidylbutanol is indicated with an arrow, and a background band is indicated with an asterisk. Results from three independent experiments, each conducted in triplicate, were quantified and graphed (E and F). Graphs represent means + SEM. Phosphatidylbutanol signal was quantified in each lane, and background-subtracted results were compared by one-way ANOVA with Tukey's post-hoc tests. (E) Quantification of results from HEK 293 cells. **, $p < 0.01$ compared with hPLD2-transfected cells. (F) Quantification of results from PC12 cells. ***, $p < 0.001$ compared with vector-transfected cells.

hPLD2, or with empty EGFP vector as a negative control. PC12 cells were not transfected with EGFP-hPLD2 because these cells are known to have high levels of endogenous PLD2 activity (34). Transfection efficiency for both cell lines was monitored in two ways: under a fluorescent microscope, >50% of the cells in GFP-transfected wells fluoresced green (data not shown), and Western blot results showed high levels of expression (Figures 1A and 1B).

Because 1-butanol can replace water in the PLD-catalyzed hydrolysis of PC and is favored 1000-fold over water in this reaction, the addition of a small amount of butanol to the culture medium allows for the production of the PLD-specific product phosphatidylbutanol (PBut), which cannot be metabolized by the cells. Quantification of accumulated PBut levels, therefore, serves as a sensitive and specific indicator of the total PLD activity in the cells within the given time frame. Cells were activated with phorbol-12-myristate-13-acetate (PMA), an inducer of the protein kinase C pathway and, indirectly, of PLD (30). PMA was added to the cells in

the presence or absence of 1-butanol, then lipids were extracted in chloroform/methanol and run on a TLC plate to separate the phospholipids. In both cell lines, αSyn did not inhibit the activity of either endogenous or overexpressed PLD (Figure 1). In contrast, the small molecule PLD inhibitor VU0155056 (27) dramatically reduced PLD activity. We conclude that αSyn does not detectably inhibit PLD activity in these cell types.

αSyn Does Not Inhibit PLD Activity in Cell-Free Systems. Previous work suggested that αSyn can inhibit PLD activity in cell-free assays (22, 25). Before extending these findings, we first sought to replicate them. We used protocols similar to those used in the published work to assay the activity of hPLD2a (15 nM) in the presence or absence of αSyn at different concentrations. Based on the published findings, we expected to see near-maximal inhibition of hPLD2a activity with 100nM αSyn (22, 25). However, αSyn did not inhibit hPLD2a in our *in vitro* assays at concentrations up to 1 μM (Figure 2A). For completeness, we also tested the

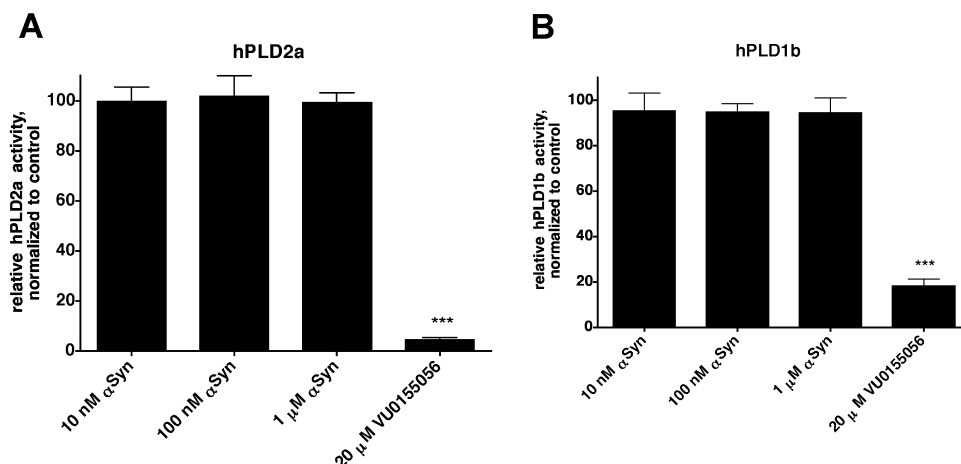


FIGURE 2: α Syn does not inhibit PLD in a cell-free assay. (A) The activity of hPLD2a was assayed in the presence or absence of wild-type human α Syn. Differences between these conditions were not significant, whereas the addition of VU0155056 resulted in a significant decrease in hPLD2a activity. (B) The activity of hPLD1b was assayed with or without wild-type human α Syn. Differences between these conditions were not significant. For all of these experiments, reactions were incubated at 37 °C for 30 min, terminated, and free 3 H-choline release was quantified by scintillation counting (see Experimental Procedures). The small-molecule PLD inhibitor VU0155056 (20 μ M) was used as a control for inhibition in some reactions, as indicated. Background-subtracted values were normalized to the averaged control values and compared by one-way ANOVA with Tukey's post-hoc tests. Graphs represent means \pm SEM from 3 independent experiments, total $n = 9$ per condition. ***, $p < 0.001$ in pairwise comparisons with each α Syn concentration.

effect of α Syn on the activity of hPLD1b (3 nM), although the published work exploring the inhibition of PLD1 by α Syn indicated that such inhibition was incomplete at best (22, 23). As with hPLD2a, hPLD1b activity was not affected by α Syn in our cell-free assays (Figure 2B).

Two of the PD-causing mutations in α Syn affect the protein's lipid binding properties and were shown in one report to have differential effects on α Syn inhibition of hPLD2 (25). Specifically, the authors reported that the A53T mutation increased hPLD2 inhibition, while the A30P mutation did not differ significantly from wild-type protein. In our assays, however, neither A30P nor A53T showed significant inhibition of hPLD2a at the concentrations tested (data not shown), consistent with our findings with the wild-type protein. Thus, in our hands, α Syn did not inhibit hPLD1b or hPLD2a in a cell-free system despite our best efforts to replicate the previously published conditions. These results were unanticipated, and possible explanations are explored in the Discussion.

Effect of α Syn on Yeast PLD. Following the unexpected lack of inhibition of PLD by α Syn in mammalian cells and cell-free assays, we returned to the yeast system in which this inhibition has previously been inferred from the results of genetic interactions. In the yeast *Saccharomyces cerevisiae*, the *SPO14* gene encodes PLD activity (35–37). We first investigated the effect of α Syn overexpression in yeast carrying a mutation in the *SEC14* gene, which encodes an essential phosphatidylinositol and phosphatidylcholine transfer protein (38, 39). Yeast cells with a temperature-sensitive mutation in *SEC14* (*sec14-1*) grow normally at the permissive temperature (23 °C) but are unable to grow at the restrictive temperature (37 °C) (40, 41). Occasionally, *sec14-1* cells can acquire “bypass mutations” in one of at least 6 different pathways, conferring the ability to grow at the restrictive temperature (42). In agreement with previously published work (24), expression of low levels of α Syn abolished the *sec14-1* bypass mutants' ability to grow at the restrictive temperature (Figure 3A).

To determine whether the observed inhibition was PLD-dependent, we focused on the well-characterized *sec14* bypass mutant, *cki1* Δ . Deletion of the choline kinase, *CK1I*, allows *sec14-1* mutants to grow at the restrictive temperature (Figure 3B) in a PLD-dependent manner (Figure 3C) (42), and expressing α Syn in these cells blocked this effect (Figure 3B). We hypothesized that if the effect of α Syn on *sec14-1/cki1* Δ cells was caused by PLD inhibition, then expressing saturating levels of ectopic PLD should overcome this growth defect. To this end, we created a plasmid to overexpress *SPO14* (which encodes the yeast PLD homologue) and verified its functionality by virtue of its ability to restore growth to *sec14-1/cki1* $\Delta/spo14$ Δ cells (Figure 3C), which express the *sec14-1* and *cki1* Δ mutations and have an additional genomic deletion of *SPO14*. However, overexpressing this functional *SPO14* in the *sec14-1/cki1* Δ cells was not sufficient to rescue the α Syn-induced growth defect (Figure 3D).

DISCUSSION

To date, the only specific biochemical function that has been proposed for α Syn is a potential inhibition of PLD. Because the normal function of α Syn may be related to its role in PD, we sought to repeat and extend the reported findings regarding this interaction and to explore how it might relate to PD pathogenesis. However, in spite of our efforts to use several independent but complimentary assay systems and to include conditions similar to those previously reported, we were unable to repeat many of the published findings.

In mammalian cells, we observed no significant inhibition of PLD activity by overexpression of human α Syn. We used both the neuronal rat PC12 cell line and the non-neuronal human HEK 293 cell line in order to ensure that any effects we observed were not specific to one particular cell line or species. Furthermore, the HEK 293 cells are similar to the cells used in the previously published work showing α Syn inhibition of cellular PLD (23). Similar to those experiments, we overexpressed both α Syn and hPLD2 in these cells by

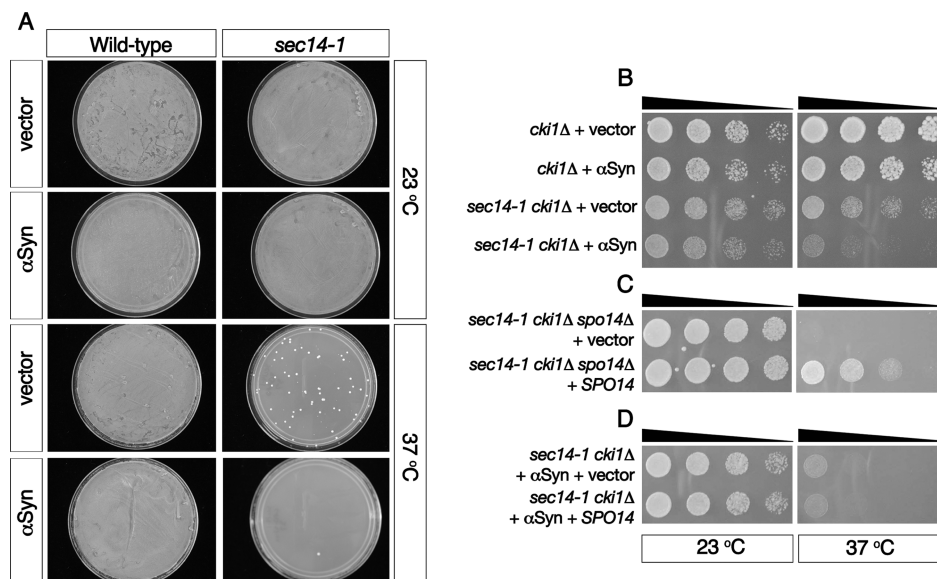


FIGURE 3: α Syn effects on yeast growth are not rescued by overexpression of PLD. (A) Yeast cells with a temperature-sensitive mutation in *SEC14* (*sec14-1*) grow normally at 23 °C, but are unable to grow at 37 °C, unless they acquire various “bypass mutations” (*sec14-1*, vector, 37 °C). As reported previously (24), α Syn expression prevents the ability of the *sec14-1* bypass mutants to grow at the restrictive temperature (*sec14-1*, α Syn, 37 °C). (B) Deletion of the choline kinase 1 gene (*cki1Δ*), allows the *sec14-1* mutants to grow at the restrictive temperature in a PLD-dependent manner, and α Syn expression in these cells blocks this rescue effect. (C) Deletion of *SPO14* (*spo14Δ*) in the *sec14-1/cki1Δ* mutants abolishes their ability to grow at the restrictive temperature. Overexpression of functional *SPO14* restores growth to *sec14-1/cki1Δ/spo14Δ* cells (C) but is unable to rescue the α Syn-induced growth defect (D) ($n = 3$).

transient transfection. However, unlike the earlier work, we controlled for possible titration effects by coexpressing two plasmids in all of the experimental conditions. Thus, cells were transfected with α Syn and GFP, or GFP-hPLD2 and GFP, or α Syn and GFP-hPLD2. However, α Syn did not inhibit PLD activity (Figure 1E) in these experiments. We also overexpressed α Syn in PC12 and HeLa cells to determine whether the protein could inhibit the activity of the highly expressed endogenous PLD in these two cell types. As with the HEK 293 cells, we observed no inhibition of PLD by α Syn in either PC12 (Figure 1F) or HeLa cells (data not shown).

Based on one report of a possible direct interaction between α Syn and PLD (23), we attempted to co-immunoprecipitate these two proteins from HeLa cells using antibodies against α Syn or the HA tag on our PLD2 construct. However, we were unable to obtain evidence of co-immunoprecipitation of these proteins (data not shown). In all of our experiments we included a small-molecule PLD inhibitor, VU0155056, to establish the maximal level of inhibition that we could expect to see in these assays. While the PLD inhibitor consistently reduced PLD activity in our assays, overexpression of α Syn did not.

In cell-free assays, we did not observe inhibition of PLD activity upon the addition of recombinant α Syn protein, although we achieved dramatic inhibition with the small-molecule inhibitor VU0155056. Thus, the activity of our hPLD1b and hPLD2a enzymes was sensitive to inhibition under these conditions, in spite of the failure of recombinant α Syn to affect activity levels. We used up to 67-fold molar excess of α Syn compared with our PLD isoenzymes. Based on the published data, we expected this excess of α Syn to yield at least 50% inhibition of hPLD2a in the cell-free assays. However, in our hands α Syn had no effect on hPLD2a or hPLD1b activity. Furthermore, an inhibition that requires greater molar excess would not appear to be

physiologically relevant, nor can it be immediately understood with regard to protein–protein interactions in a living cell.

We then turned to experiments in yeast as a tool to explore the physiological relevance of the reported inhibition of PLD by α Syn. Using the *sec14-1* temperature-sensitive mutant yeast strain, we showed that α Syn prevented the acquisition of bypass mutations at the restrictive temperature, as previously published (24). Such bypass mutations can be acquired through 6 different pathways (42), among which the well-characterized *CKI1* pathway is PLD-dependent (43). Inhibition by α Syn overexpression of all possible bypass mutation pathways implies that this effect is not likely to depend on PLD activity. In the *sec14-1 cki1Δ* double mutant strain in which bypass mutations are acquired in a PLD-dependent manner, α Syn prevented growth at the restrictive temperature but this deficit could not be rescued by coexpression of functional Spo14. This result further strengthens our conclusion that α Syn does not directly affect PLD in this system.

In separate work, some of us (A.D.G., S.L.) have recently reported that α Syn accumulation causes endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) secondary to a severe block in vesicular trafficking between the ER and Golgi (44). Intriguingly, it has been found that *sec14* bypass mutants are particularly sensitive to ER stress and require an intact UPR for survival (45). Inducing ER stress, either genetically (by mutating the UPR pathway components *IRE1* or *HAC1*) or chemically (by treatment with the *N*-linked glycosylation inhibitor tunicamycin), abolishes the ability of *sec14-1/cki1Δ* cells to grow at the restrictive temperature (45), and this deleterious effect is strictly independent of PLD activity. Thus, we propose a new interpretation for the confirmed ability of α Syn to inhibit *sec14* bypass mutant growth (24). Rather than a direct inhibition of PLD activity by α Syn, the increased ER stress present in α Syn-expressing cells may

be sufficient to prevent the *sec14* bypass mutants from growing. This interpretation is consistent with our failure to suppress the effect by overexpressing *SPO14* (i.e., PLD).

In light of the variety of assays and conditions we used, we conclude that α Syn is not a robust inhibitor of PLD. From the experiments in cell-free systems we conclude that α Syn does not directly inhibit PLD activity, and from the experiments in yeast and mammalian cells we conclude that α Syn does not inhibit PLD through either a direct or indirect interaction. It may be that previous findings implicating α Syn in PLD inhibition in cells could instead be interpreted as effects of general α Syn cytotoxicity, because overexpression of PLD in these systems does not rescue the effects of α Syn. We further conclude that dysregulation of PLD inhibition is not likely to be involved in the role of α Syn in PD pathogenesis.

ACKNOWLEDGMENT

We thank Michelle Armstrong, Matthew Hemming, and Tracy Young-Pearse for their advice and assistance.

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BI801871H