α-Synuclein Interacts with Phospholipase D Isozymes and Inhibits Pervanadate-induced Phospholipase D Activation in Human Embryonic Kidney-293 Cells*

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α-Synuclein has been implicated in the pathogenesis of many neurodegenerative diseases, including Parkinson’s disease and Alzheimer’s disease. Although the function of α-synuclein remains largely unknown, recent studies have demonstrated that this protein can interact with phospholipids. To address the role of α-synuclein in neurodegenerative disease, we have investigated whether it binds phospholipase D (PLD) and affects PLD activity in human embryonic kidney (HEK)-293 cells overexpressing wild type α-synuclein or the mutant forms of α-synuclein (A53T, A30P) associated with Parkinson’s disease. Tyrosine phosphorylation of α-synuclein appears to play a modulatory role in the inhibition of PLD, because mutation of Tyr122 to Phe slightly increases inhibitory effect of α-synuclein on PLD activity. Treatment with pervanadate or phorbol myristate acetate inhibits PLD more in HEK 293 cells overexpressing α-synuclein than in control cells. Binding of α-synuclein to PLD requires phospho and pleckstrin homology domain of PLD and the amphipathic repeat region and non-Aβ component of α-synuclein. Although biologically important, cotransfection studies indicate that the interaction of α-synuclein with PLD does not influence the tendency of α-synuclein to form pathological inclusions. These results suggest that the association of α-synuclein with PLD, and modulation of PLD activity, is biologically important, but PLD does not appear to play an essential role in the pathophysiology of α-synuclein.

α-Synuclein is a small, highly conserved presynaptic protein of unknown function that has been implicated in the development of neurodegenerative diseases, such as Alzheimer’s (AD).1

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3 The abbreviations used are: AD, Alzheimer’s disease; PD, Parkinson’s disease; PLD, phospholipase D; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PKCα, protein kinase C α; DMEM, Dulbecco’s modified Eagle’s medium; HESK cells, human embryonic kidney cells; NAC, non-Aβ component of Alzheimer’s disease plaques; GST, glutathione S-transferase; PBS, phosphate-buffered saline; H & E, hematoxylin and eosin; wt, wild-type.
been described, PLD1 and PLD2 (21–24). Activity of 120-kDa PLD1 is regulated by multiple inputs, including phosphatidyl-
inositol 4,5-bisphosphate, protein kinase C, the Rho family proteins, and ADP-ribosylation factor proteins. PLD2 is a 106-
kDa protein that share 50–55% homology with PLD1. PLD2 is reported to have a much higher basal activity than PLD1 and
appears to be insensitive to further stimulation by the known activators of PLD1. The primary lipid product of PLD, phos-
phatidic acid, exhibits a number of biological activities in vitro
and may be an important mediator of processes controlling vesicular transport and changes in cell morphology (25). In
neuronal cells, PLD activation has been linked to pathways involved in cell growth, differentiation, and neurotransmitter
release (26, 27). Although many studies continue to focus on the functional relationships and the isozyme specificities of
the PLD isozymes, the molecular mechanism of the regulation of the PLDs has not been fully elucidated.

In this regard, the identification of PLD-binding partners may provide clues toward the understanding of complex regu-
ulatory mechanism of PLD in different cells. Since direct cell
cytotoxicity of α-synuclein is still controversial, α-synuclein
might interact with other proteins to cause neurodegeneration.

The investigation of regulation of PLD by α-synuclein is required
to gain insight into the role of these proteins under normal and pathological condition. As a step in this effort, we
now report for the first time that α-synuclein binds to PLD1 and
PLD2 and inhibits pervanadate-induced PLD activation in
human embryonic kidney 293 cells. Moreover, we show that
the state of tyrosine phosphorylation of α-synuclein plays a modu-
lar role in pervanadate-induced PLD activity.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal bo-
vine serum, and LipofectAMINE were purchased from Invitrogen. Pro-
tein A-Sepharose and glutathione-Sepharose 4B were from Amersham Biosciences. Biotech. Hydrogen peroxide and sodium
orthovanadate were from Sigma, and anti-phosphotyrosine antibody (Tyr(P)) (4G10) were from Upstate Biotechnology. The antibody to PKC-α were purchased from Santa Cruz. Mouse monoclonal antibody to α-synuclein was generated using GST-α-synuclein as an antigen by a glutathione-S-acyl
polyclonal antibody that recognizes both PLD1 and PLD2 was generated as de-
scribed previously (28). Phosphatidylbutanol standard was from Avanti Polar Lipid. myo-2-[3H]-Inositol and [9,10-3H]Myristate were purchased from PerkinElmer Life Sciences. Silica gel 60 A thin layer chromatog-
raphy plates were from Whatman. Hors eradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were from Kirkegaard & Perry Laboratory (Gaithersburg, MD). The ECL Western blotting detection kit was from Amersham Biosciences.

Cell Culture and Transfection—HEK 293 cells were maintained in
Hepes-buffered DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum under 5% CO2. The cells were transfected with the indi-
cated plasmid DNA and LipofectAMINE according to man-
ufacturer’s instructions. G418 (500 μg/ml) was used for selection.

Co-immunoprecipitation and Immunoblot—HEK 293 cells were lyses
with a buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 1% sodium
decyloxylate, 0.2% SDS, 150 mM NaCl, 1 mM NaVO4, 1 mM NaF, 10%
glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenethyl-
sulfonylfuoride) and precleared with preimmune IgG and protein
A-Sepharose for 30 min at 4°C with rocking. Protein concentrations were determined using the Bio-Rad Protein Assay with bovine serum
albumin as a standard. Equal protein aliquots of precleared cell lysates
(1 mg) were incubated with the indicated antibodies and 30 μl of a 1:1
slurry of protein A-Sepharose beads for 4 h at 4°C. The immune
complexes were collected by centrifugation and washed five times with a
buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM Na2VO4, 10% glycerol, and 1% Nonidet P-40) and resuspended in
sample loading buffer and subjected to SDS-PAGE and Western blot analysis using the indicated antibody. The protein bands
were visualized using ECL (Amersham Biosciences).

Construction and Preparation of GST Fusion Proteins—The full-
length cDNA of human PLD1 or PLD2 was digested into fragments containing specific domains. These individual PLD1 or PLD2 fragments were then ligated into the EcoRI or SmaI site of the pGEX4T1 vector.

Subcloning and the polymerase chain reaction (PCR) were used to
produce the expression vectors encoding the respective GST fusion
proteins. Escherichia coli BL21 cells were transformed with individual
expression vectors encoding the GST fusion proteins, and after har-
vesting the cells, the GST fusion protein expressed were purified by stand-
ard Bio-Rad chromatography methods (29) using glutathione-Sepharose 4B (Amersham Biosciences).

Preparation of Rat Brain Extract—Rat brain (2 g) was homogenized
in lysis buffer using a polytron homogenizer. After centrifugation at 100,000 × g for 1 h at 4°C, the resulting supernatant was used to
investigate potential α-synuclein or PLD binding domains. Protein con-
centration measurements were determined using the methods developed by Bradford (30).

In Vitro Binding Experiment—Clariified lysates (1 mg) of rat brain
were incubated with 3 μg of GST fusion proteins immobilized on glu-
thione-Sepharose beads in a final volume of 500 μl of lysis buffer for
1.5 h at 4°C. Protein complexes were collected by centrifugation and
washed four times with washing buffer (1% Triton X-100, 150 mM NaCl,
20 mM Tris-HCl, pH 8.0, 20 mM NaF, 2 mM sodium orthovanadate, 1 mM
PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Associated protein
complexes were resolved by SDS-PAGE and transferred to a nitrocel-
lulose membrane. Immunoreactivity was detected using the indicated
antibodies, horseradish peroxidase-conjugated secondary antibodies, and
ECL according to the manufacturer’s instructions.

Preparation of Phosphatidylbutanol—PLC—Cells were plated into 60-mm dishes at 5 × 105 cells per dish and grown for 1 day. The cells were then labeled with myo-[3H]-Inositol (2 μCi/ml) in inos-
itol-free DMEM for 20 h. Subsequently, the labeled cells were washed
and pretreated with 20 mM LiCl for 15 min in DMEM containing 20 mM
Hepes, pH 7.2, and 1 mg/ml bovine serum albumin. Stimulation was
initiated by the addition of pervanadate for 50 min and terminated by
the addition of ice-cold 5% HClO4. After 30 min in an ice bath, extracts
were centrifuged, diluted with distilled water, and applied to Bio-Rad
Dowex AG 1-X8 anion exchanger column. The column was then washed
with 10 ml of distilled water followed by 10 ml of 60 mM ammonium
formate containing 5 mM of sodium tetraborate. Total inositol phos-
phates were eluted with a solution containing 1 mM ammonium formate and
0.1 M formic acid.

Phospholipase D Assay—PLD activity was assessed by measuring
the formation of [3H]phosphatidylbutanol, the product of transphos-
phatidylhydrolysis in the presence of 1-butanol. HEK 293 cells were subcul-
tured in six-well plates at 2 × 106 cells/well. The cells were serum-
starved in DMEM for 24 h before the start of the assay. For the final
20 h of serum starvation, the cells were labeled with 1 μCi/ml [9,10-
3H]Myristate for 20 h at 4°C. The resulting supernatant was used to
the sections were incubated with a mixture of fluorescein isothiocya-
late (FITC) and Texas Red (TRX) conjugates of secondary antibodies,
and ECL according to the method of Bligh and Dyer (31). The lower phase was dried under N2, resuspended in 30 μl of
chloroform/methanol (2:1), and spotted onto silica gel 60A thin layer chromatography plate (Whatman). The plates were developed in
the upper phase of the solvent system of ethyl acetate/iso-octane/H2O/acetic
acid (55:25:50:10) and stained with iodine. A phosphatidylbutanol
standard (Avanti Polar Lipids) was used to locate the bands, which
were scraped into scintillation mixture. Radioactivity incorporated into
total phospholipids was measured, and the results were presented as
percentage of total lipid counts/min incorporated into phosphatidylbutanol
to normalize the results.

Immunofluorescent Staining—To examine the co-localization of
α-synuclein with PLD1, a double-immunofluorescent staining technique
was used. Three male Sprague-Dawley rats (3–6 months old) were deeply
anesthetized with 4% chloral hydrate (1 ml/100 mg) and were sacrificed
transcardial perfusion with a fixative containing 4% paraformalde-
hyde in 0.1 M phosphate buffer, pH 7.4. Cryostat coronal sections (25
μm thick) were cut throughout the mesencephalon and were processed
for double-immunofluorescence histochemistry. Free floating sections were preincubated in 10% normal serum in phosphate-buffered saline
(PBS) 1 h. Incubation with primary antibodies was performed with
a mouse monoclonal antibody against α-synuclein and an affinity-
purified anti-PLD1 antibody overnight at 4°C. After washing in PBS,
the sections were incubated with a mixture of fluorescein isothiocya-
nate-conjugated goat anti-mouse IgG (Jackson Immunoresearch, di-
luted at 1:100) and Cy3-conjugated goat anti-rabbit IgG (Jackson Im-
munosciences, diluted at 1:1000).
Regulation of PLD by α-Synuclein

RESULTS

α-Synuclein Inhibits Pervanadate or PMA-induced PLD Activity in HEK 293 Cells—To investigate whether α-synuclein might affect PLD activity in human cell lines, we generated a line of 293 HEK cells overexpressing wild-type (wt), A30P, or A53T α-synuclein, as well as a vector transfected control cell line (Vec). Pervanadate, a complex of vanadium and hydrogen peroxide, is a competitive inhibitor of protein-tyrosine phosphatase that works by irreversible oxidation and functions on intact cells because of its cell permeability (33). Pervanadate is also known to stimulate PLD activity (34–36). Pervanadate stimulated PLD activity less in wild-type, A30P, or A53T α-synuclein cell lines than in the control cell line (Fig. 1A). Inhibition of pervanadate-stimulated PLD activity in α-synuclein HEK 293 cells occurred in a time dependent manner, compared with that of control cells (Fig. 1B). We also examined an effect of α-synuclein on another activator of PLD, PMA. PMA-induced PLD activation in cells expressing α-synuclein also was more reduced, compared with that of control cells (Fig. 1C). To examine the relative contribution of PLD protein to PLD activity in HEK cells, we investigated the expression level of PLD isozymes. By immunoprecipitation and Western blot analysis using anti-PLD antibody, cells overexpressing wild-type α-synuclein or vector-transfected cell line were found to express similar levels of PLD1 (Fig. 2). However, PLD2 was not detected in either cells (data not shown). Similar results were observed in HEK 293 cells stably transfected with A30P or A53T (data not shown). To further demonstrate the inhibitory effect of α-synuclein on the PLD activity, we transiently co-transfected α-synuclein and PLD1 or PLD2 into HEK 293 cells. Transfected cells were labeled with [3H]myristate and then either left untreated or stimulated with pervanadate (100 μM sodium orthovanadate and 0.5 mM H2O2). Cells co-transfected with α-synuclein and PLD1 or PLD2 showed less pervanadate-induced PLD activation than cells transfected with PLD and vector (Fig. 4). These results demonstrate that α-synuclein inhibits both PLD1 and PLD2 activation induced by pervanadate in mammalian cells.

α-Synuclein Is Tyrosine-phosphorylated by Pervanadate in HEK 293 Cells—Protein-tyrosine phosphorylation plays a pivotal role for the functional properties of numerous proteins. α-Synuclein appears to contain four tyrosine residues, which are consensus sequences for tyrosine kinase-mediated phosphorylation. Recently, it was reported that α-synuclein is ty-
Fig. 3. Pervanadate-induced tyrosine phosphorylation of α-synuclein in cells. HEK cells overexpressing wild-type α-synuclein were treated with pervanadate (PV) (100 μM Na3VO4 and 0.5 mM H2O2) for 20 min. Cells were immunoprecipitated with anti-α-synuclein or anti-Tyr(P) antibody. Resulting immunoprecipitants were separated in duplicates by SDS-PAGE and transferred to nitrocellulose membranes using anti-Tyr(P) or α-synuclein (Syn) antibodies. The results shown are representative of three separate experiments.

Tyrosine-phosphorylated by the Src family of protein-tyrosine kinases such as c-Src and Fyn (37). We investigated tyrosine phosphorylation of α-synuclein in transfected cells. Immunoprecipitated α-synuclein from either pervanadate-treated stably transfected HEK 293 cells expressing human α-synuclein was separated by SDS-PAGE in duplicate. Western blot analysis was then performed with phosphotyrosine specific antibody (4G10), or α-synuclein antibody, which specifically recognizes α-synuclein (Fig. 3). The cell lysates were also immunoprecipitated with anti-phosphotyrosine antibody and then analyzed by immunoblotting with anti-α-synuclein antibody. Pervanadate (100 μM sodium orthovanadate and 0.5 mM H2O2) induced tyrosine phosphorylation of α-synuclein.

Effect of Tyrosine Phosphorylation State of α-Synuclein on Pervanadate-induced PLD Activation—We investigated the effect of the tyrosine phosphorylation state of α-synuclein on pervanadate-induced PLD activity by mutating each of the tyrosines in α-synuclein. Mutation of Tyr125 to Phe was the only tyrosine mutation to alter the activity of α-synuclein. This reduced tyrosine phosphorylation to −5% of the wild-type control, whereas other single amino acid changes do not change tyrosine phosphorylation significantly. Next, we examined the effect of tyrosine phosphorylation state on the ability of α-synuclein to inhibit PLD activity. The cells were co-transfected with PLD1 or PLD2 along with the Y125F mutant construct or wild-type control α-synuclein. After co-transfection, cells were treated with or without pervanadate (100 μM Na3VO4 and 500 μM H2O2). Mutation of Tyr125 to Phe slightly increased the inhibitory effect of α-synuclein on pervanadate-induced PLD activity. (Fig. 4A). The expression level of PLDs or α-synuclein was similar in these transfection experiment (Fig. 4B). Thus, the tyrosine phosphorylation state of α-synuclein appears to modulate PLD activity.

α-Synuclein Associates with PLD1 and PKCα—To explore the significance of this inhibition, we examined whether α-synuclein interacts with PLD isozymes. HEK 293 cells overexpressing α-synuclein were treated for 20 min with or without pervanadate. The lysates were immunoprecipitated with anti-PLD antibody, and the precipitates were probed with monoclonal anti-α-synuclein antibody (Fig. 5A). The presence of α-synuclein in the PLD immune complex was apparent. In a reciprocal experiment, α-synuclein was immunoprecipitated by antibody to α-synuclein. Subsequent immunoblotting with the anti-PLD1 revealed PLD1 expression (Fig. 5A). Interestingly, α-synuclein was constitutively associated with PLD1. Furthermore, both α-synuclein and PLD1 proteins were associated with PKCα in cells overexpressing α-synuclein (Fig. 5B and C). α-Synuclein was also associated with PLD2 in co-transfection experiments (Fig. 5D). Interestingly, pervanadate did not alter the interaction of α-synuclein with PKCα or PLD. To examine whether the effect of α-synuclein on PLD is specific, we investigated the effect of α-synuclein on the activity of other lipid modifying enzyme and their interaction (Fig. 6). Pervanadate induced an increase in PLC activity in the HEK 293 cells, but α-synuclein did not affect PLC activity (Fig. 6A) and did not associate with PLC-β1 (Fig. 6B) in cells overexpressing α-synuclein, suggesting that the effect of α-synuclein on PLD is specific.

Amphipathic Repeat Region and NAC Domain of α-Synuclein Is Involved in the Interaction with PLD1—To map the region on
the α-synuclein protein that is responsible for the interaction with PLD1, we prepared GST-fused human α-synuclein protein, GST-Syn-whole (amino acid residues 1–140), GST-Syn-N (amino acid residues 1–60), GST-Syn-NAC (amino acid residues 61–95), and GST-Syn-C (amino acid residues 96–140) and used them in in vitro binding experiments. As shown in Fig. 7, the GST-Syn-N and GST-Syn-NAC containing the amphipathic repeat region and the NAC domain, respectively, precipitated PLD1 from rat brain lysates. Thus, these data suggest that the amphipathic repeat region and the NAC domain might be involved in the interaction with PLD1.

FIG. 5. Interaction of α-synuclein with PLD and PKCα. A–C, HEK 293 cells expressing α-synuclein were stimulated without or with pervanadate (PV) (100 μM Na3VO4 and 0.5 mM H2O2) for 20 min. The cells were then lysed, and immunoprecipitates (IP) were prepared using anti-PLD, anti-α-synuclein (Syn), or anti-PKCα antibody. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer of proteins to nitrocellulose membranes and Western blotting (Blot) with anti-PLD, anti-α-synuclein (Syn), or anti-PKCα antibodies. D, HEK 293 cells were transiently transfected with the empty vector (lane 1), PLD1 and α-synuclein (lane 2), or PLD2 and α-synuclein (lane 3). After immunoprecipitation with the anti-PLD or anti-α-synuclein antibody, the immunoprecipitates were then immunoblotted with anti-α-synuclein or anti-PLD antibody. "Mock" represents a mock-precipitated control. The blots shown are representative of three independent experiments.

FIG. 6. α-Synuclein does neither affect PLC activity nor interact with the enzyme. A, subconfluent, quiescent cells were labeled with 2 μCi/ml myo-[2-3H]inositol in inositol-free DMEM for 20 h and then stimulated with pervanadate (100 μM Na3VO4, and 0.5 mM H2O2) for 50 min in the presence of 20 mM LiCl, and total inositol phosphates were measured as described under "Experimental Procedures." Results are the means ± S.D of three independent experiments. B, the cells were then lysed, and immunoprecipitates (IP) were prepared using anti-PLCβ1 or anti-α-synuclein (Syn) antibody. The immunoprecipitates were then subjected to Western blotting (Blot) with anti-PLCβ1 or anti-α-synuclein (Syn). The blots shown are representative of three independent experiments.

FIG. 7. Interaction of α-synuclein with PLD and PKCα in Rat Brain—As with HEK 293 cells overexpressing α-synuclein, we found that PLD1 co-precipitates with endogenous α-synuclein by using a co-immunoprecipitation method on rat brain (Fig. 9A). We tried to further check whether PLD1 co-localized with α-synuclein. Recently, we reported endogeneous PLD1 expression in neurons of the substantia nigra par compact (28), which are affected in PD. Expression of α-synuclein has been also reported in these neurons (38, 39). Therefore, rat brain sections were double-immunolabeled with anti-PLD1 and anti-α-synuclein antibodies to identify the co-localization of residues 61–95, and GST-Syn-C (amino acid residues 96–140) and used them in in vitro binding experiments. As shown in Fig. 7, the GST-Syn-N and GST-Syn-NAC containing the amphipathic repeat region and the NAC domain, respectively, precipitated PLD1 from rat brain lysates. Thus, these data suggest that the amphipathic repeat region and the NAC domain might be involved in the interaction with PLD1.

α-Synuclein Associates with a Region (Amino Acids 1–331) of PLD1—Next, we analyzed the interaction site(s) of PLD1 with α-synuclein. To identify the PLD1 sequence involved in the α-synuclein binding, we constructed the GST fusion proteins shown in Fig. 8A. The lysates from rat brain were precipitated with GST-PLD1 fragments (Fig. 8B) and analyzed by Western blotting with anti-α-synuclein antibody. GST-PLD1 (amino acid 1–331) was found to be the region that most potently bound to α-synuclein. It appears, therefore, that the region between amino acids 1 and 331 containing the phox and pleckstrin homology domains may be important for the interaction with α-synuclein.

Interaction of PLD1 with the α-Synuclein in Rat Brain—As with HEK 293 cells overexpressing α-synuclein, we found that in rat brain tissues, PLD1 co-precipitates with endogeneous α-synuclein by using a co-immunoprecipitation method on rat brain (Fig. 9A). We tried to further check whether PLD1 co-localized with α-synuclein. Recently, we reported endogeneous PLD1 expression in neurons of the substantia nigra par compact (28), which are affected in PD. Expression of α-synuclein has been also reported in these neurons (38, 39). Therefore, rat brain sections were double-immunolabeled with anti-PLD1 and anti-α-synuclein antibodies to identify the co-localization of...
The results shown are representative of three separate experiments. Western blotting using anti-GST antibody (lower panel) visualized the precipitated proteins. The precipitated proteins were subjected to immunoblot analysis using antibodies against PLD1 (upper panel). The amount of the GST fusion protein was visualized by Western blotting using anti-GST antibody (lower panel). The results shown are representative of three separate experiments.

**FIG. 7.** Amphipathic repeat region and NAC domain of α-synuclein interact with PLD1. α-Synuclein was fragmented into individual domains consisting of NH2 terminus (1–60), NAC (61–95), COOH terminus (96–140). The fragments and whole protein were cloned as GST fusion proteins, expressed in E. coli, and purified using glutathione-Sepharose beads. Equal amounts (1 mg) of GST or GST fusion proteins (GST-Syn fragment) were incubated with rat brain extract as described under “Experimental Procedures.” The precipitated proteins were subjected to immunoblot analysis using antibody against PLD1 (upper panel). The amount of the GST fusion protein was visualized by Western blotting using anti-GST antibody (lower panel). The results shown are representative of three separate experiments.

**FIG. 8.** α-Synuclein associates with phox and pleckstrin homology domains of PLD1. A, a schematic representation of the structure of PLD1 is shown on the top. The possible functions of each box have been proposed or demonstrated in Ref. 24. Boxes are the regions of highly conserved sequences in PLD. PX, phox domain; PH, pleckstrin homology-like domain; I–IV, conserved regions in the PLD family (24). loop, loop region, CT, COOH terminus. B, equal amounts (1 mg) of GST or GST fusion proteins (GST-PLD1 fragments, F1–F7) were incubated with rat brain extract as described under “Experimental Procedures.” The precipitated proteins were subjected to immunoblot analysis using antibody against α-synuclein (upper panel). The amount of the GST fusion protein was visualized by Western blotting using anti-GST antibody (lower panel). The results shown are representative of three separate experiments.

α-synuclein with PLD1 in the same neuron. Examination with confocal microscopy revealed that all α-synuclein immunoreactive neurons in the substantia nigra pars compacta co-localized with PLD1 immunoreactivity (Fig. 9B). Taken together, these results suggest that regulation of PLD by α-synuclein might occur through in vivo interaction.

**FIG. 9.** Interaction of PLD1 with α-synuclein in rat brain. A, α-synuclein and PLD was co-immunoprecipitated from rat brain lysates using antibody to PLD or α-synuclein. The resulting immunoprecipitates were immunoblotted with antibodies to α-synuclein or PLD. Mock represents a mock-precipitated control. B, Cy3-labeled, PLD1 immunoreactive cells and fluorescein isothiocyanate-labeled, α-synuclein immunoreactive cells in the neuron of the substantia nigra pars compacta were visualized with confocal microscopy. Superimposed images display the co-localization of, respectively, PLD1-labeled and α-synuclein-labeled neurons in the substantia nigra pars compacta. Scale bars: 50 μm. The results shown are representative of two separate experiments.

Quantification of Eosinophilic Inclusions—Since α-synuclein was observed to interact with both PLD1 and PLD2, we assessed their functional significance in terms of the formation of intracellular cytoplasmic inclusions, which is pathological characteristics of PD (8, 9, 40, 41). The morphological composition of cells was determined by hematoxylin and eosin (H & E) staining to evaluate the formation of inclusion bodies in cells transfected with both PLD and α-synuclein proteins (Fig. 10A). Consistent with the previous result, we observed that ~6% cells out of total cell numbers had eosinophilic inclusion bodies in cytoplasm when HEK 293 cells were co-transfected with both PLD and α-synuclein proteins (Fig. 10A). The percentage of cells that develop inclusion bodies is ~10–20%, out of cells expressing both NAC and synphilin-1 based on a transfection efficiency of 30–40%, which was calculated using a green fluorescent protein reporter plasmid. In contrast, cells co-transfected constructs encoding NAC and PLD1 or NAC and PLD2 exhibited only ~1–2% of eosinophilic inclusion bodies (Fig. 10B). In addition, cells co-expressing α-synuclein and PLD1 or PLD2 revealed same effect that was observed in cells co-expressing NAC and PLDs (data not shown). This is similar to the results that were obtained from cells expressing any protein alone, such as NAC, α-synuclein, PLD1, or PLD2 protein. These results suggest that the specific interaction between PLDs and α-synuclein might not be an essential feature in the formation of inclusion bodies in mammalian cells.

**DISCUSSION**

We demonstrate for the first time that α-synuclein binds to both PLD1 and PLD2 and inhibits its enzymatic activity in...
human cell lines. α-Synuclein has been implicated in Parkinson’s and Alzheimer’s disease. It is of great interest to determine the specific function(s) of α-synuclein because of its potential importance in the pathogenesis of these diseases. The link between α-synuclein and PLD appears to be particularly intriguing, because α-synuclein inhibits PLD2 activity in vitro (20), and PLD activity was significantly increased in AD brain tissues as compared with control tissues (42). Recently, we confirmed the increase in PLD1 protein in AD brain and also observed that PLD co-localized with α-synuclein in AD brain tissues as compared with control tissues (15). Taken together, these results suggest that modulation of PLD by α-synuclein might play a role in some aspects of the pathophysiology of neurodegenerative diseases. Further investigations of the regulation of PLD by α-synuclein could provide valuable insights into the role of these proteins play in normal and pathological conditions.

To investigate this issue, we examined the regulation of PLD by α-synuclein in HEK 293 cells overexpressing α-synuclein. Pervanadate or PMA-stimulated PLD activation was decreased in cells overexpressing α-synuclein, compared with that of vector-transfected cells. Cells overexpressing Ala<sup>53</sup> → Thr (A53T) and Ala<sup>30</sup> → Pro (A30P) mutant α-synuclein, which have been associated with familial forms of PD, showed greater inhibition of PLD than vector-transfected cells. By immunoprecipitation and Western blot analysis using an anti-PLD antibody, the HEK 293 cells were found to express similar levels of 110-kDa PLD1 protein. However, the 105-kDa PLD2 protein was not detected in any of the cell lines, indicating that the PLD activity shown in these cells is due to mainly to PLD1. Using in vitro studies, α-synuclein has been reported to inhibit PLD2 activity more potently than PLD1 activity (20). However, we found that in co-transfection experiments, α-synuclein inhibited pervanadate-stimulated PLD activity. This suggests that α-synuclein can inhibit both forms of PLD. The ability of α-synuclein to inhibit PLD1 in cells might reflect differences between in vitro and in vivo environments. Recently, α-synuclein was reported to inhibit PKC activity in HEK 293 cells (15), which could contribute to the inhibition of PLD activity. Inhibition of PKC by α-synuclein could contribute to the decrease in pervanadate-stimulated PLD activation in cells overexpressing α-synuclein. However, it is possible that this inhibition might also result from a direct interaction between

**FIG. 10.** Formation of eosinophilic cytoplasmic inclusion bodies. **A**, HEK 293 cells co-transfected with constructs encoding NAC (amino acids 61–95 of α-synuclein) and full-length synphilin-1 develop cytoplasmic eosinophilic inclusions when stained with H & E. The inclusions were hardly detected in cells co-transfected with constructs encoding NAC plus PLD1 or NAC plus PLD2. **B**, quantification of eosinophilic inclusion formation in HEK 293 cells transfected with various constructs. Approximately 6% of cells co-transfected with constructs encoding NAC and synphilin-1 co-stained eosinophilic inclusions, whereas co-transfection of constructs encoding NAC and PLD1 or PLD2 resulted in the formation of less than 1% of eosinophilic inclusion. Values are expressed as percentages of cells containing eosinophilic protein aggregates with pinkish color relative to total cells. Results are the means ± S.D. of three independent experiments.
PLD and a-synuclein. Here we demonstrated that a-synuclein is constitutively associated with PLD1 in cells, and a-synuclein forms a triple complex with PLD and PKCa in a ligand-independent manner. The association between a-synuclein and PKC is consistent with prior results observed by Ostrerova and colleagues (15). We also demonstrated that a-synuclein associates with PLD2 in co-transfected cells. We found that a-synuclein co-immunoprecipitated with endogenous PLD from rat brain tissue and is co-localized with PLD in neurons in the substantia nigra pars compacta of rat brain, indicating that these two proteins interact in vivo. The PLD1 binding site in a-synuclein resides in the amino acid residues 1–95 containing 6 or 7 conserved repeats with the consensus core sequence KTKEGV.

a-Synuclein has been shown to interact with phospholipids. This interaction is also facilitated mainly by a conserved NH$_2$-terminal 95 residues, which changes its structure from “unfolded” to a-helical upon binding to lipids (12). Because of a-synuclein's ability to interact with lipids and their association with synaptic vesicles, it has been suggested that synucleins might be involved in intracellular vesicular trafficking (43). Tau interacts with the acidic COOH-terminal region (residues 89–140) of a-synuclein through its microtubule-binding domain. This opens the possibility that a-synuclein might have a bridging function that might serve to bring different classes of ligands together. a-Synuclein binds to a region between amino acids 1 and 331 of PLD1. This region contains the NH$_2$-terminal pleckstrin homology and phox domains, which are known to be involved in protein-protein interaction as well as binding of phospholipids (44). The interaction sites of PLD2 with a-synuclein showed similar patterns as with PLD1 (data not shown). a-Synuclein did not affect the activity of PLC and other lipid-modifying enzymes and did not associate with PLC-β1 in cells overexpressing a-synuclein, suggesting that the effect of a-synuclein on PLD is specific.

Protein-tyrosine phosphorylation is thought to be important in regulating synaptic function and plasticity (45, 46). It was reported recently that a-synuclein can be tyrosine-phosphorylated by the Src family tyrosine kinase in a co-transfection experiment and in vitro using purified kinases (37). This tyrosine phosphorylation occurs primarily on tyrosine 125. It is difficult to speculate on the functional consequences of tyrosine phosphorylation of a-synuclein, because its normal function has not been elucidated definitively. The putative role of a-synuclein in regulating intracellular vesicular trafficking and signaling appears particularly interesting. Mice lacking a-synuclein show abnormal dopamine release (47). a-Synuclein exists in the cytoplasm in presynaptic neurons, but is also loosely associated with synaptic vesicles (48). Covalent modification, such as phosphorylation, is a likely candidate for regulation of α-synuclein at the synapse, and covalent modification could be important in modulating its function. Although the functional consequences of phosphorylation of the tyrosine 125 residue of a-synuclein remain to be elucidated, tyrosine phosphorylation could regulate the ability of α-synuclein to bind synaptic vesicles and thereby regulate protein-protein interactions. Here we demonstrate that the phosphorylation state of tyrosine 125 of α-synuclein modulates the activity of PLD. A mutation Tyr$^{125}$ to Phe in α-synuclein (Y125F) that mimics dephosphorylation increases the ability of α-synuclein to inhibit pervanadate-induced PLD activation. This regulatory axis could affect exocytosis, because PLD is thought to be an important component of the exocytotic machinery (49).

The discovery of abnormal protein aggregates or accumulation has been described in a number of neurodegenerative diseases. We tried to investigate that the interaction between α-synuclein and PLD isozymes has a role in inclusion body formation in PD. Recently, it was reported that when constructs encoding portions of α-synuclein and synphilin-1 are co-transfected in mammalian cells, the cells formed eosinophilic cytoplasmic inclusions resembling the Lewy bodies of PD (16). Although α-synuclein aggregates by itself in vitro, it may be that aggregation in vivo is facilitated by an associated protein such as synphilin-1. When constructs encoding synphilin-1 and full-length α-synuclein or the non-Aβ component AD amyloid (NAC) portion of α-synuclein were co-transfected, we observed that ~6% of cells had cytosolic phase-dense inclusions. Cytosolic inclusions were eosinophilic when stained with H & E. In contrast, when constructs encoding PLD isozyme (PLD1 or PLD2) were co-transfected along with α-synuclein or NAC, only ~1–2% of cells had eosinophilic inclusions, which is similar to that present in control cells. Thus, our data suggest that PLD isozymes may not modulate α-synuclein aggregation.

In summary, our study suggests that α-synuclein modulates the activity of PLD by protein-protein interactions, but this interaction might not be involved in regulation of the formation of cytoplasmic inclusion bodies in mammalian cells. However, we cannot rule out the possibility that PLD does modulate α-synuclein aggregation in the brain, because the environment of HEK 293 cells differs from that of the aged human brain. Although the function of both α-synuclein and PLD remains largely unknown, identification of binding partners and examination of how and where the complexes form in the cell provide important tools for understanding the physiology of PD.

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