With the discovery of missense mutations (A53T and A30P) in a-synuclein (α-Syn) in several families with early onset familial Parkinson’s disease, α-Syn aggregation and fibril formation have been thought to play a role in the pathogenesis of α-synucleinopathies, such as Parkinson’s disease, dementia with Lewy bodies, and multiple system atrophy. As previous reports have suggested that α-Syn plays a role in lipid transport and synaptic membrane biogenesis, we investigated whether α-Syn binds to a specific lipid ligand using thin layer chromatography overlay and examined the changes in its secondary structure using circular dichroism spectroscopy. α-Syn was found to bind to acidic phospholipid vesicles and this binding was significantly augmented by the presence of phosphatidylethanolamine, a neutral phospholipid. We further examined the interaction of α-Syn with lipids by in situ atomic force microscopy. The association of soluble wild-type α-Syn with planar lipid bilayers resulted in extensive bilayer disruption and the formation of amorphous aggregates and small fibrils. The A53T mutant α-Syn disrupted the lipid bilayers in a similar fashion but at a slower rate. These results suggest that α-Syn membrane interactions are physiologically important and the lipid composition of the cellular membranes may affect these interactions in vivo.

α-Synuclein (α-Syn) belongs to a family of proteins including α-, β-, and γ-synucleins that are encoded in three different genes (1–7). α-Syn is expressed at high levels in the brain and enriched in neural synaptic terminals but its physiological function remains largely unknown. It has been suggested that α-Syn plays a role in neuronal plasticity because its expression in songbirds increased during learning (6). A role in neuronal development and in synaptogenesis has been proposed since α-Syn appears early in murine brain development and is redistributed from the cytosol to the nerve terminals where synaptosomes are located (8).

The association of α-Syn with neurodegeneration was identified by initial alanine to threonine mutation at codon 53 (A53T) which was linked to an autosomal dominant, early-onset familial Parkinson’s disease (PD) (9, 10). An additional missense mutation (A30P) was found in a second PD kindred (11) suggesting that the protein is of etiological significance. In addition, the integral α-Syn fragment, called non-αβ component, was found to be one of the major components of the insoluble fibrillar core of the Alzheimer’s disease senile plaques (4, 12). More recently, α-Syn has been shown to be one of the principal components of Lewy bodies that are intracellular inclusions with fibrillar morphology and are the neuropathological characteristics of PD and dementia with Lewy bodies (13–19). Immunocytochemistry has revealed that α-Syn-positive inclusions are present in astrocytic and oligodendroglial cells of PD-afflicted individuals (20, 21). α-Syn is also a major component of the glial and neuronal inclusions of multiple system atrophy (22–25). Therefore, α-Syn and its abnormal protein aggregation have been thought to play a role in the pathogenesis of these neurodegenerative diseases known as α-synucleinopathies (26–28).

In order to understand their etiology and pathogenesis, it is crucial to identify the normal function of α-Syn. The function of a protein is closely correlated with its three-dimensional structure, especially for proteins important in the pathogenesis of neurodegenerative diseases (29). From a protein folding viewpoint, α-Syn is a dynamic molecule whose secondary structure depends on its environment. For example, it has an unfolded random coil structure in aqueous solution (30), forms α-helical structure upon binding to acidic phospholipid vesicles (31), and forms insoluble fibrils with a high β-sheet content that resemble the filaments found in Lewy bodies (32).

The human α-Syn primary sequence (total 140 residues) is well conserved among vertebrates, with rodent and zebra finch α-Syn being 95 and 87% identical to the human sequence (1, 4, 6, 33). The amino-terminal sequence (residues 7–87) contains a series of amphipathic α-helical domains that may facilitate either membrane-protein or protein-protein interactions (Fig. 1). This region is composed of seven imperfect repeats of 11 amino acids, which contain a 6-residue core with the consensus sequence KTKEGV (Fig. 1B; Ref. 33). The central region of α-Syn (residues 61–95) is very hydrophobic, and this internal fragment was initially isolated from Alzheimer’s disease senile

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primary structure of human α-synuclein. A, α-synuclein contains three major regions including the NH2-terminal amphipathic α-helical domains, the non-Αβ component (NAC) of the insoluble fibrillar core of the Alzheimer’s disease senile plaques, and the acidic COOH-terminal region. B, the amino acid sequence of the amphipathic α-helical domains (residues 7–57) is aligned to show the 11-residue repeats containing the amino acids identical to the consensus sequence (underlined). Two missense mutations (A30P and A53T) associated with familial Parkinson’s disease are indicated by arrows.

Fig. 1. Primary structure of human α-synuclein. A, α-synuclein contains three major regions including the NH2-terminal amphipathic α-helical domains, the non-Αβ component (NAC) of the insoluble fibrillar core of the Alzheimer’s disease senile plaques, and the acidic COOH-terminal region. B, the amino acid sequence of the amphipathic α-helical domains (residues 7–57) is aligned to show the 11-residue repeats containing the amino acids identical to the consensus sequence (underlined). Two missense mutations (A30P and A53T) associated with familial Parkinson’s disease are indicated by arrows.

plagues (4). The carboxyl-terminal region is rich in glutamate residues and thus quite acidic. These primary and secondary structural features of α-Syn support a role for its interaction with lipid membranes.

There are a few lines of evidence suggesting that α-Syn may play a role in lipid transport and synaptic membrane biogenesis. First of all, its amphipathic α-helical domains of 11-residue repeats are reminiscent of those in the class Αβ apolipoproteins, which carry lipid molecules by reversibly binding to them (34). We and others have found that α-Syn undergoes a major structural transition from random coil to α-helical structure upon binding to lipid vesicles (31), supporting the possible role of α-Syn in lipid binding and transport. Interestingly, one of the PD-linked point mutations (A30P) abolished the ability of α-Syn to bind to the lipid vesicles (35), emphasizing the possibility that lipid binding is a normal function of α-Syn. Second, α- and β-synucleins were shown to be highly specific inhibitors of phospholipase D2, which hydrolyzes phosphatidylcholine to produce phosphatic acid and diacylglycerol (36). This suggests that the level and modification of α-Syn may affect phospholipase D2 activity, thereby modulating the cleavage of membrane lipids and membrane biogenesis (37). Third, α-Syn has been reported to co-localize with synaptic vesicles, suggesting its association with lipids (1, 38–40). Finally, it was recently reported that α-Syn regulates the size of the presynaptic vesicular pools (41). In brief, when the expression of α-Syn was suppressed with antisense oligonucleotide in primary hippocampal neuronal cultures, the number of synaptic vesicles was reduced significantly. This also suggests a role of α-Syn in synaptic membrane biogenesis and/or vesicle fusion and budding.

Given the evidence for α-Syn involvement in lipid transport and turnover in synaptic terminals, we investigated the possibility of a specific lipid ligand for α-Syn using thin layer chromatography (TLC) overlay and subsequent effect on secondary structure with circular dichroism (CD) spectroscopy. α-Syn was found to bind to acidic phospholipid vesicles and this binding was significantly augmented by the presence of phosphatidylethanolamine (PE), a neutral phospholipid. The interaction of α-Syn with lipids was further examined using in situ tapping mode atomic force microscopy (AFM). The association of soluble wild-type α-Syn with planar lipid bilayers resulted in extensive bilayer disruption with the concomitant formation of amorphous aggregates and small fibrils. Finally, we compared the wild-type and mutant (A53T) α-Syn with regard to their comparative ability to interact with membranes. These in vitro studies are aimed at elucidating the physiological role of α-Syn in the normal brain as well as the pathological role of the mutant α-Syn in PD and related neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl phosphatidylserine (POPS), brain L-α-phosphatidylserine (PS), brain L-α-phosphatidylethanolamine (PE), lyso brain plasmalogen phosphatidylethanolamine (lyso-PE), liver L-α-phosphatidyl ethanolositol (P1), brain sphingomyelin (sphingomyelin), 1-palmitoyl-2-oleoyl phosphatidic acid (POPA), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Poly(isobutyl methacrylate) was obtained from Aldrich. Monoclonal mouse anti-α-synuclein antibody (Synuclein-1) was from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated goat anti-mouse IgG was from Bio-Rad. Foil-backed polygram silica gel thin layer chromatography plates were from Macherey-Nagel (Germany).

Expression and Purification of Recombinant α-Synuclein—Human α-Syn cDNAs, both wild-type and a mutant (A53T), were subcloned into the plasmid pET-28a (Novagen), using NcoI and HindIII restriction sites. α-Syn was overexpressed in Escherichia coli BL21 (DE3) via an isopropyl-1-thio-β-galactopyranoside-inducible T7 promoter. The bacterial pellet was resuspended in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride. The bacterial suspension was then sonicated for 30 s several times, boiled for 15 min, and ultracentrifuged at 150,000 × g for 30 min. The supernatant containing the heat-stable α-Syn was dialyzed against 50 mM Tris, pH 8.3, loaded onto a Q-Sepharose column (Amersham Pharmacia Biotech), and eluted with a 0–500 mM NaCl step gradient. The eluents were desalted and concentrated on a Centricon-10 (Millipore) in 5 mM phosphate buffer, pH 7.0, 0.4–4 ml of chloroform:methanol (2:1) and kept at 20 °C until use.

Binding of α-Synuclein to Synthetic Phospholipid Vesicles—Multimellar vesicles (MLVs) of various phospholipids were prepared by suspending the dry phospholipid film in PBS, pH 7.3, and vortexing for 10 min. Small unilamellar vesicles (SUVs) were subsequently prepared by sonicating MLVs using a probe sonicator for 10 s several times with a 20-s interval. Aliquots of each purified step were analyzed by SDS-polyacrylamide gel electrophoresis to confirm purity. Protein concentration was determined by Lowry assay.

Circular Dichroism Spectroscopy—CD spectra were recorded on a Jasco J-715 spectropolarimeter at room temperature. Incubated samples were placed in a 1-mm path length quartz cell to minimize the absorbance due to buffer components. Spectra were obtained from 195 to 250 nm, with a 0.5-nm step, 1-nm band width, and a speed of 20 nm/min. Three scans per sample were averaged. All spectra were corrected by subtracting background spectrum of the buffer or the lipid vesicles.

Extraction of Brain Lipids—Cerebellum (0.5–1 g) or temporal cortex from human brain (non-diseased, 92 yr-old male) was homogenized with a Polytron in 1.8 ml of PBS. Chloroform and methanol (2:1) were added to the brain homogenate to give a Folch partition. Chloroform:methanol:water (PBS) = 2:1:0.6. The mixture was vortexed vigorously and allowed to separate in a separatory funnel. After 3 h when both phases were clear, the lower phase was recovered and filtered. The upper phase was re-extracted with theoretical lower phase (chloroform: methanol:water = 86:14:1). The filtered lower phases were dried under a stream of nitrogen gas. The resulting lipid films were dissolved in 0.4–4 ml of chloroform:methanol (2:1) and kept at −20 °C until use. The final upper phase was lyophilized and dissolved in 200 μl of chloroform:methanol (2:1).

Thin Layer Chromatography (TLC)—Chloroform, methanol, and 0.25% RCI were combined at a ratio of 5:4:1 and used as a standard TLC.
running solvent. Human cerebellar lipid extracts along with phospholipid standards (2–15 μg per lane) were applied to silica gel TLC plates and run until the solvent front was ~1 cm below the top of the plate. After being air-dried, the TLC plate was cut in half, turning to twin plates, for the detection of total lipids and the other for the overlay with α-Syn. For total lipids, the TLC plate was sprayed with a mixture of 10% CuSO₄, H₂O and 8% H₃PO₄ and heated for 5 min at 170 °C (43). To detect PE and PS, which contain amide groups, the TLC plate was alternately sprayed with 0.5% ninhydrin (Sigma) in a mixture of 20 ml of glacial acetic acid and 80 ml of ethanol and heated for 5 min at 110 °C.

**Lipid Overlay with α-Synuclein**—TLC plates were treated with poly(isobutyl methacrylate) to prevent silica loss and to orient the lipid head groups suitable for ligand binding (44). To block nonspecific binding, plates were incubated in 0.6% gelatin with shaking for 1 h at 37 °C. After a brief rinse with Tris-buffered saline (TBS), pH 7.6, the TLC plate was incubated with 2 μg of α-Syn in 7 ml of TBS overnight at room temperature. After rinsing with TBS, the TLC plate was incubated with monoclonal anti-α-synuclein antibody. After extensive washing, synuclein immunoreactivity was detected by peroxidase-conjugated goat anti-mouse antibody and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Atomic Force Microscopy**—Solution tapping mode atomic force microscopy images were acquired on a Digital Instruments NanoScope IIIA MultiMode scanning probe microscope (Digital Instruments, Santa Barbara, CA) using 120-μm oxide-sharpened silicon nitride V-shaped cantilevers installed in combination contact/tapping mode liquid flow cell. The AFM images were acquired using the E scanning head, which has a maximum lateral scan area of 14.6 μm × 14.6 μm. All imaging was performed at tip scan rates between 1.25 to 2 Hz, using cantilever drive frequencies of ~9.9 kHz. All images were captured as 512 × 512 pixel images and were low-pass filtered. Feature size and volumes were calculated using the Digital Instruments NanoScope software (version 4.2.1) and shareware image analysis program, NIH-Image (version 1.62). In situ tapping mode atomic force microscopy experiments were performed by sequential addition of the solutions to the AFM fluid cell. Planar bilayers were formed by directly injecting ~500 μl of the ~1 mg/ml POPC/POPS suspension into the AFM fluid cell, previously sealed against a piece of freshly cleaved mica and allowing the vesicles to fuse in situ. The cell was flushed with neat buffer and reference tapping mode atomic force microscopy height and phase images were acquired in PBS, pH 7.4, to confirm formation of stable lipid bilayers. Approximately 500 μl of α-Syn in PBS at 0.1 mg/ml were injected directly into the AFM cell and imaging initiated 30 min after the protein was introduced.

**RESULTS**

**Binding of α-Syn to PS and PE Examined by TLC Overlay**—α-Syn has been reported to bind to rat brain vesicles (35) and synthetic phospholipid membranes (31). This prompted us to apply TLC overlay to determine whether α-Syn exhibits any specificity for a particular lipid extracted from human brain. Total extracts obtained from either cerebellum or temporal cortex were separated on a TLC plate and incubated with purified α-Syn. Approximately 9–11 individual lipid bands were observed (Fig. 2A, lane 1) and only two showed any significant interaction with α-Syn (Fig. 2B, lane 1). In order to identify these α-Syn-binding lipids, commercially available lipids were run on TLC plates as references and overlaid with α-Syn (Fig. 2, A and B, lanes 2–6). α-Syn was found to bind to phosphatidylethanolamine (E. coli and brain PE), lyso-PE (brain lyso-PE), phosphatidylinositol (liver PI) and showed a detectable but much weaker interaction with phosphatidylserine (POPS and brain PS) (Fig. 2B). No interaction was observed for phosphatidylycholine (POPC), phosphatidic acid (POPA), sphingomyelin (brain sphingomyelin), or cholesterol (Fig. 2B). Nonspecific immunoreactivity was not observed with similar TLC-separated lipids exposed to the anti-synuclein antibody alone (i.e. without prior incubation with recombinant α-Syn) or with the secondary antibody (data not shown). It was surprising that α-Syn did not bind to phosphatidic acid (PA) on TLC plate (Fig. 2, lane 6) since α-Syn was reported to bind to the SUVs containing acidic phospholipids, such as PS, PI, and PA (31). This discrepancy can be attributed to the physical properties of the TLC overlay method. These findings suggest the possibility of some specificity of α-Syn for particular lipids which may be relevant to its function. However, the overlay technique may not identify all α-Syn binding lipids (e.g. phosphatidic acid) due to these technical constraints. Significant binding of α-Syn to brain PE was observed which may reflect a similar in vivo interaction.

When these reference lipids were compared with the brain lipid extracts in parallel on a TLC plate, the location of purified brain PE (Fig. 2, lane 2, upper band) coincided with the upper band of the α-Syn binding lipid bands from the brain lipid extracts (Fig. 2B, lane 1, upper band). The lower lipid band present in the brain lipid extracts ran similarly to purified PS, PI, and lyso-PE. In order to confirm that the α-Syn-positive bands are PE and PS, the lipid samples on the TLC plate were examined using the ninhydrin reaction. This reaction is specific for amide and amine groups that are only found in PE and PS. Both overlay positive bands and reference PS and PE were stained with ninhydrin, confirming that the overlay positive bands are PE and PS, respectively (data not shown).

**Binding of α-Syn to Phospholipid Vesicles**—In addition to the solid phase assay, we screened a variety of phospholipids for α-Syn binding, using phospholipid liposomes. It has been reported that α-Syn binds to SUVs containing acidic phospholipids, which results in an increased α-helical conformation in α-Syn (31). We report herein the following novel findings regarding the association of α-Syn with lipids. (i) The binding of α-Syn with acidic phospholipids is dose-dependent; (ii) the extent of lipid binding depends rather on the availability of the phospholipids than on the size of lipid vesicles (SUVs versus MLVs); (iii) α-Syn binding to phospholipid vesicles increases drastically when an acidic phospholipid is mixed with PE instead of PC; and (iv) α-Syn binding to acidic phospholipid (with PC or PE) vesicles is inversely proportional to the ionic strength.

In the first instance, α-Syn was incubated with POPC or POPC:POPS (1:1, w/w) SUVs. CD spectroscopy was used to monitor changes in α-Syn secondary structure upon binding to the lipid vesicles. As shown in Fig. 3A, the spectrum of α-Syn in PBS, pH 7.3, showed a minimum ellipticity at 200 nm, indicating a random coiled secondary structure. In the presence of POPC SUVs, the CD spectrum revealed a subtle shift toward α-helical conformation. In the presence of POPC:POPS SUVs, the spectrum clearly showed a typical α-helical secondary structure with the characteristic minima at 208 and 222 nm. The intensity of the α-helicity increased as the concentration of
Fig. 3. Changes in the secondary structure of \(\alpha\)-synuclein examined by CD spectroscopy. A, \(\alpha\)-synuclein at 0.2 mg/ml was examined in PBS (——) or in the presence of POPC SUVs (---) or POPC/POPS (1:1 wt/wt) SUVs (••••) at 2 mg/ml. \(\alpha\)-synuclein at 0.2 mg/ml was examined with increasing weight ratios of lipid to protein; \(\alpha\)-synuclein in PBS (——) or in the presence of POPC/POPS SUVs at 0.5 mg/ml (1:2.5, protein/lipid)(•••), 1 mg/ml (1:5)(••••), 2 mg/ml (1:10) (- - - -), and 4 mg/ml (1:20) (••••).  

Fig. 4. CD spectra of \(\alpha\)-synuclein bound to the preformed MLVs versus \(\alpha\)-synuclein incorporated into MLVs. \(\alpha\)-Synuclein at 0.2 mg/ml was examined in PBS (——) or in the presence of POPC/POPS MLVs (- ••• •••) at 2 mg/ml. Alternatively, \(\alpha\)-synuclein was incorporated into POPC/POPS MLVs by making MLVs (2 mg/ml) in PBS containing \(\alpha\)-synuclein (0.2 mg/ml) (••••). A, wild-type \(\alpha\)-synuclein. B, A53T mutant \(\alpha\)-synuclein.

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POPC/POPS was increased from 0.5 mg/ml (lipid:protein = 2.5:1, w/w) to 2 mg/ml (lipid:protein = 10:1, w/w) (Fig. 3B). The spectrum did not change significantly when the POPC/POPS concentration was increased from 2 to 4 mg/ml, indicating that the binding of \(\alpha\)-Syn to POPC/POPS SUVs was saturated at a lipid concentration of 2 mg/ml.

Second, we examined the interaction of \(\alpha\)-Syn with SUVs in comparison to MLVs since the curvature of SUVs has been suggested to affect protein-lipid interactions (31). When preformed POPC/POPS MLVs at 2 mg/ml were added to \(\alpha\)-Syn in PBS, the CD spectrum did not change significantly from its random coiled structure (Fig. 4). This seemed to be due to the fact that only the outermost lipid bilayer is available for \(\alpha\)-Syn binding and the inner layers are inaccessible to \(\alpha\)-Syn, resulting in an actual concentration of the available lipids much less than 2 mg/ml. In contrast, when POPC/POPS MLVs (2 mg/ml) were made in PBS containing \(\alpha\)-Syn, the CD spectrum showed a characteristic \(\alpha\)-helical structure (Fig. 4), similar to the one obtained by adding POPC/POPS SUVs at 2 mg/ml to \(\alpha\)-Syn in PBS. Therefore, the extent of lipid binding was dependent on the availability of lipids rather than on the size of the vesicles (SUVs versus MLVs). The A53T mutant \(\alpha\)-Syn was also examined by CD in all the experiments in order to compare the secondary structure of the mutant with the wild-type \(\alpha\)-Syn. CD spectra of A53T mutant (Fig. 4B) were similar to the wild-type (Fig. 4A) in all conditions, suggesting that the average secondary structure of A53T \(\alpha\)-Syn upon lipid binding is similar to the wild-type.

Third, we examined if \(\alpha\)-Syn binds to PE-containing vesicles because \(\alpha\)-Syn was found to bind to PE on TLC plate. PE itself cannot form stable vesicles due to its tendency to form a hexagonal phase (45). Therefore, SUVs of an equimolar mixture of PC and PE as well as 100% PE dispersed in buffer were examined for \(\alpha\)-Syn binding. \(\alpha\)-Syn did not undergo a conformational change in the presence of PC/PE SUVs or PE suspension (data not shown). Although PE suspension and PC/PE SUVs did not cause an increase in the \(\alpha\)-helicity of \(\alpha\)-Syn, PE in the presence of all acidic phospholipids caused an appreciable increase in its \(\alpha\)-helicity (Fig. 5). Surprisingly, PA and PI did not induce a significant conformational change in \(\alpha\)-Syn when presented with PC (Fig. 5). In contrast, when PA and PI were combined with PE (1:1, w/w), the \(\alpha\)-helicity of \(\alpha\)-Syn increased significantly (Fig. 5), indicating its increased interaction with PE/MA (and PE/PI) vesicles. Therefore, PE seems to help the lipid vesicles to bind to \(\alpha\)-Syn possibly by providing a monolayer negative curvature strain to the vesicles, which can be relieved by \(\alpha\)-Syn binding. Alternatively, since PE has a smaller hydration shell than PC, it might allow closer protein-membrane contact, as proposed for protein kinase C (46).

Fourth, since \(\alpha\)-Syn binding to PC/PS vesicles was thought to be electrostatic, we investigated the influence of ionic strength on \(\alpha\)-Syn association with PC/PS and PE/PS vesicles (Ref. 31, Fig. 6). The \(\alpha\)-helicity of \(\alpha\)-Syn was greatest at 0 mM NaCl and decreased with increasing NaCl concentration, indicating that the electrostatic interaction between \(\alpha\)-Syn and PC/PS vesicles was inhibited by salt. PE/PS vesicles were more resistant to the salt-induced inhibition of \(\alpha\)-Syn binding to the vesicles than PC/PS. The extent of \(\alpha\)-Syn binding to PE/PS vesicles was significantly greater than its binding to PC/PS vesicles at 100–400 mM NaCl which includes a physiological salt concentration (Fig. 6). This result confirms the specific binding of \(\alpha\)-Syn to PE, as shown by TLC overlay.

Disruption of Acidic Phospholipid Bilayer by \(\alpha\)-Syn—Based on the results on the binding of \(\alpha\)-Syn to acidic phospholipid vesicles, the interaction of \(\alpha\)-Syn with planar supported bilayers of POPC/POPS (1:1) prepared by in situ vesicle fusion to
mica was examined by in situ AFM. The addition of α-Syn at 0.1 mg/ml in PBS buffer resulted in the gradual formation and expansion of bilayer holes over the course of several hours (Fig. 7). Close inspection of the bilayer and the defect sites revealed the presence of small aggregates and putative fibrils lying against the exposed mica surface (Fig. 7C, arrows). The aggregates with a width of 2–3 nm and a thickness of ~2 nm are likely aggregated α-Syn or possibly α-Syn-lipid complexes (Fig. 7B, inset). While intermittent contact AFM height imaging provides details on surface topography, phase imaging measures the relative phase shift between the applied and detected tip oscillation during imaging, providing information related to specific adhesive interactions between the scanning tip and the sample surface (47–50). Phase images collected simultaneously with the topographical images acquired by in situ tapping mode AFM revealed variations in phase image contrast between the intact lipid bilayers and these aggregates, which confirms that the aggregates are not simply lipid artifacts.

To investigate whether there is any difference in lipid binding associated with the A53T mutant α-Syn, we employed in situ AFM to examine the A53T α-Syn binding to supported planar bilayers of POPC/POPS. As in the case of the wild-type α-Syn, extended duration in situ AFM revealed the gradual expansion of bilayer defects (Fig. 8). The rate of bilayer disruption was qualitatively slower in the case of the A53T α-Syn mutant, which suggests that the mutant may be less effective in membrane binding and membrane disruption. We did not find any aggregated materials on the surface of the bilayers for both the wild-type and A53T mutant α-Syn. However, we observed that the morphology of the A53T mutant α-Syn aggregates was different from the wild-type. The wild-type α-Syn tended to form elongated aggregates with a width on the order of 2–3 nm and a thickness of ~2 nm (Fig. 7B, inset) whereas the A53T mutant α-Syn formed small ellipsoidal aggregates with a dimension of 4 × 3 × 1.5 nm (Fig. 8D, inset).

**DISCUSSION**

The primary sequence of α-Syn is highly conserved among vertebrates and its NH$_2$-terminal amphipathic α-helical domain is similar to the class A$_1$ apolipoproteins. Apolipoproteins bind and solubilize lipids to form the various plasma lipoprotein particles. Class A amphipathic helices have cationic residues near the hydrophobic-hydrophilic interface and anionic residues that oppose the hydrophobic face (51). However, the α-Syn sequence is much more conserved across species than the apolipoprotein sequence, suggesting that α-Syn is more than just a lipid carrier. It is possible that the functions of α-Syn, including lipid transport, require the protein to adopt a variety of structures, depending on the availability of binding partners. In order to do so, its sequence needs to be carefully balanced with respect to its conformational propensity for forming α-helix, β-sheet, and random structure as well as its amphiphilicity. This may explain the specific conservation in the α-Syn sequence. It is also possible that α-Syn binds to specific proteins *in vivo*, such as synphilin-1 (52), which can also explain the highly conserved sequences.

Replacing PC with PE in acidic lipid vesicles increased the binding of α-Syn to these vesicles drastically (Fig. 5). PE has been found to facilitate the interaction between membranes and a few cytoplasmic proteins, including protein kinase C (46). Although both PE and PC are neutral phospholipids that exhibit similar electrostatic properties, they differ in their head group orientation, lipid bilayer packing, and hydrogen-bonding capacity (45). One of the differences between PE and PC is the size of their head groups. The surface area of PC head group is 47–54 Å$^2$ which is compatible with the minimum cross-sectional area of 50 Å$^2$ required by two liquid-crystalline hydrocarbon chains (45). Conversely, the PE head group has a surface area of 35–42 Å$^2$ which is smaller than the minimum requirement for two hydrocarbon chains. When the zwitterionic head groups are tightly packed, PE forms a lipid monolayer with high negative curvature that eventually leads to the formation of a hexagonal phase (45). It has been reported that the membrane monolayer negative curvature strain is relieved by class A amphipathic helices of the apolipoproteins (51, 53). Since α-Syn also contains class A amphipathic helical domains,
α-Syn in the presence of PE/acidic lipid vesicles is thought to relieve the negative curvature strain and instantaneously stabilize the vesicles, resulting in its drastic binding to these vesicles, whereas α-Syn did not bind to PC/acidic lipid vesicles as much (Fig. 5).

The lipid composition of synaptic vesicles has been estimated to be 25–32% PC, 15–24% PE, 4–8% PS, 3–8% sphingomyelin, 2–3% PI, 0–1% PA, and 33–38% cholesterol (31, 54). The negative curvature strain caused by the relatively high PE content as well as the surface defect caused by the curved surface of the small synaptic vesicles would favor α-Syn binding to these vesicles, and thus stabilizing the membranes. The failure of α-Syn to bind to the membranes, possibly caused by its mutations, will break the equilibrium between membrane-bound and free α-Syn and may cause the aggregation of free α-Syn. This possibility is consistent with our AFM data discussed below.

Our AFM results suggest that the A53T mutant α-Syn may be less effective than the wild-type α-Syn in binding to or disrupting the membranes. This may be a consequence of the more rapid oligomerization kinetics exhibited by the mutant relative to the wild-type protein (55–58). The fibrillar form, which typically has a β-sheet structure, is not expected to bind to membranes as effectively as the monomeric form, which has been shown to form α-helical structure when bound to membranes. An alternative possibility is that the A53T mutant is structurally defective in binding to the membrane by having threonine at position 53 instead of alanine. Alanine is one of the strong α-helix formers whereas threonine is indifferent in α-helical propensity (59). Considering the correlation between the α-helix formation of α-Syn and membrane binding, the A53T point mutation may have a negative effect on α-helix formation, resulting in reduced membrane binding. However, the binding of the A53T mutant α-Syn to phospholipids as determined, for example, by CD spectroscopy was qualitatively identical to the wild-type protein. This may be due to the inability of the spectroscopy technique to quantitate small differences in lipid binding. In contrast, the obvious differences in the extent of membrane disruption of wild-type as compared with mutant α-Syn as indicated by AFM appear to be significant.

The reduced membrane binding of the mutant α-Syn in vivo will result in a higher cytosolic concentration of α-Syn, leading to fibril formation which is a concentration-dependent process (32, 60). This may explain the pathogenesis of the early-onset familial PD linked to the point mutations in α-Syn, at least in part, since fibril formation has been shown to be toxic to neurons (61). Therefore, we suggest that the normal function of α-Syn in synaptic terminals includes its proper interaction with lipids, and the mutation-caused defective interaction of α-Syn with lipids is responsible at least for a part of the disease process in the early-onset familial PD.

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