Membrane-bound α-Synuclein Has a High Aggregation Propensity and the Ability to Seed the Aggregation of the Cytosolic Form*

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α-Synuclein exists as at least two structural isoforms: a helix-rich, membrane-bound form and a disordered, cytosolic form. Here, we investigated the role of membrane-bound α-synuclein in the aggregation process. In a cell-free system consisting of isolated brain fractions, spontaneous and progressive aggregation of α-synuclein was observed in membranes starting at day 1, whereas no aggregation was observed in the cytosolic fraction in a 3-day period. The addition of antioxidants reduced the aggregation in the membrane fraction, implicating the role of oxidative modifications. When excess cytosolic α-synuclein was added to brain membranes, the rate of aggregation was increased, while the lag time was unaffected. Incorporation of cytosolic α-synuclein into membrane-associated aggregates was demonstrated by fractionation and co-immunoprecipitation experiments. In our recent study, we showed that mitochondrial inhibitors such as rotenone, induced α-synuclein aggregation in cells. In the present study using rotenone-treated cells, the earliest appearance of α-synuclein oligomeric species was observed in membranous compartments. Furthermore, α-synuclein-positive inclusions were co-stained with DiI, a membrane-partitioning fluorescent dye, confirming the presence of lipid components in α-synuclein aggregates. These results suggest that membrane-bound α-synuclein can generate nuclei that seed the aggregation of the more abundant cytosolic form.

α-Synuclein is a 140-amino acid protein that is highly expressed in the brain (1). Although the precise function of this protein remains unknown, it has been proposed that α-synuclein is involved in synaptic transmission, based on the phenotype of the knock-out mice and its enrichment in presynaptic terminals (2, 3). Induction of α-synuclein expression in canary brains during the song-learning period suggests that it may function in neuronal plasticity (4). The link between α-synuclein and neurodegenerative diseases was first suggested by the identification of autosomal dominant mutations in the α-synuclein gene in early onset familial Parkinson’s disease cases (5–7). Subsequently, α-synuclein was identified in its fibrillar form as one of the major components of Lewy bodies (LBs) in Parkinson’s disease and dementia with Lewy bodies (8–14), suggesting a possible etiologic significance of the protein in these neurologic conditions. α-Synuclein immunoreactivity was also found in the glial and neuronal cytoplasmic inclusions of multiple system atrophy (15–20). The pathogenic role of α-synuclein was further confirmed from the study, showing that the transgenic overexpression of this protein in fly brains resulted in a loss of dopaminergic neurons and the formation of LB-like inclusion bodies (21). Further studies with transgenic mice showed that overexpression of α-synuclein resulted in degenerative phenotypes, although some mice did not produce any apparent phenotype (22–25).

Since the discovery of fibrillar α-synuclein in LBs, the fibrilization of recombinant α-synuclein has been studied in vitro. Purified recombinant α-synuclein spontaneously forms typical amyloid fibrils in a nucleation-dependent manner (26–29), which appears to involve prefibrillar oligomeric intermediates (30, 31). Consistent with the proposed role of the aggregated form of α-synuclein in the pathogenesis of Parkinson’s disease, the disease-linked mutant forms tend to aggregate more rapidly than the wild type (30–32). One of the central issues regarding the mechanism of the α-synuclein aggregation is how the “natively unfolded” protein is transformed to highly ordered fibrils that are rich in cross-β-sheet structure. A recent study showed evidence for a monomeric intermediate with a partially folded conformation in the fibrillization process (33), suggesting that the gain of partial structure at the initial stage constitutes a rate-limiting step.

In vitro studies showed that α-synuclein binds to artificial liposomes of phospholipids with acidic head groups (34, 35). This binding seems to be mediated by seven imperfect repeats within the N-terminal half of the protein, which was proposed to form an amphipathic α-helix based on secondary structure predictions (36). Indeed, circular dichroism and nuclear magnetic resonance analyses demonstrated the structural transition from a random coil to an α-helix-rich conformation upon binding of α-synuclein to acidic phospholipid vesicles (34, 35, 37). Furthermore, mutagenesis of individual exons of α-synuclein showed a correlation between the lipid-induced α-helical content and the degree of binding to vesicles (38). Microscopic and fractionation studies showed that in cultured cells and brain tissue, a portion of α-synuclein is, indeed, associated with the membranous compartments (4, 39, 40). These experiments suggested the presence of two structurally distinct populations of α-synuclein in cells: an α-helix-rich, membrane-bound form and a disordered, free cytosolic form. Here, using both cell-free and intact cell systems, we provide evidence that the membrane-bound form has a higher tendency to aggregate than the cytosolic form and that the aggregates formed in the membrane act as “seeds” to accelerate the aggregation of the free cytosolic α-synuclein.

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‡ The abbreviations used are: LB, Lewy body; PBS, phosphate-buffered saline; Aβ, amyloid β protein; GME, L-γ-glutamyl-L-cysteinyl ethyl glycinate.
**Materials and Methods**

**Rat Brain Fractionation**—The fractionation procedure was based on Huttner et al. (41). Frozen rat brains (~5 g) were chopped to smaller pieces before the addition of 50 ml of ice-cold isolation buffer (320 mM sucrose, 4 mM Heps, pH 7.4, 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor mixture (Sigma) and were homogenized with a Disruptor. The homogenized sample was centrifuged at 11,500 × g for 30 min. The pellet (P1) was discarded, and the supernatant (S1) was centrifuged at 11,500 × g for 15 min. The supernatant (S2) was saved until later in the procedure. The pellet (P2; crude synaptic vesicle fraction) was washed once with isolation buffer and centrifuged again. The pellet was resuspended with 10 ml of isolation buffer, and then 90 ml of ice-cold water was added for hypotonic lysis. It was then homogenized with the Disruptor to break synaptosomes to release synaptic vesicles. To the homogenized sample, 1 ml of 1 M Hepes, pH 7.4, was added and incubated on ice for 30 min before the centrifugation at 26,500 × g for 20 min. The pellet (LP1) was resuspended in 12.5 ml of isolation buffer. The supernatant (LS1) was centrifuged again at 200,000 × g for 1 h to obtain pellet (LP2; crude synaptic vesicles) and supernatant (LS2). The S2 fraction was centrifuged at 200,000 × g for 40 min to obtain pellets (P3; microsomal vesicles) and supernatant (S3; cytosolic fraction).

**Salt Wash Experiment**—P3 and LP1 fractions (50 μg each) from above were incubated at room temperature for 4 h in isolation buffer (320 mM sucrose, 4 mM HEPES, pH 7.4, protease inhibitor mixture) with an increasing amount of NaCl, ranging from 0 to 800 mM. The tubes were incubated at 300 μl, 50 μl, 10 μl, 5 μl, and 2.5 μl for LP3 and P3 fractions for 1 h or 26,500 × g for (LP1) for 20 min. The pellets were resuspended in 1 x Laemmli sample buffer. The final volume of the resuspended pellet material is the same as that of supernatant/1 x Laemmli sample buffer.

**Aggregation of α-Synuclein in Membrane Fractions**—P3, LP1, and LP2 fractions (200 μg) were incubated in isolation buffer (in a total volume of 550 μl) at room temperature for up to 2 h, and 100-μl aliquots were removed at each time point. P3 and LP2 (fractions from above) were centrifuged at 200,000 × g for 30 min, and the LP1 fraction was centrifuged at 26,500 × g for 20 min. The pellet and the supernatant were saved for Western blot analysis (see below). For comparison of α-synuclein aggregation in membrane and cytosolic fractions, P3, and S3 were incubated in isolation buffer at room temperature for up to 72 h. Membranes were solubilized by adding 10% Triton X-100 to make a final 1% concentration and incubated on ice for 20 min before the addition of 3 μl of a polyclonal anti-α-synuclein antibody, 66-8 (gift from Eliezer Masliah, University of California, San Diego, which recognizes an identical sequence to the rat α-synuclein but not the human α-synuclein sequence. After 5 h of incubation at 4 °C with rotation, protein G-Sepharose beads (30 μl; Sigma) were added and incubated for another 1 h at 4 °C. The beads were then washed four times with RIPA buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA) for 10 min each at 4 °C, and the proteins were eluted with 1 x Laemmli sample buffer.

**Western Blot**—Western blotting was performed according to Lee et al. (42). Primary antibodies against α-synuclein used in this study were Syn-1 (BD Transduction Laboratories, San Diego, CA) and LB509 (StressGen Biotechnologies Corp., Victoria, Canada). Anti-γ-adaptin antibodies were purchased from BD Transduction Laboratories (San Diego, CA). Anti-calnexin antibody was purchased from StressGen Biotechnologies Corp. (Victoria, Canada).

**Electron Microscopy**—The vesicles were collected by centrifugation at 200,000 × g for 30 min. The membrane pellets were prefixed with Karnovsky’s fixative (4% paraformaldehyde, 2% glutaraldehyde, 0.1 M cacodylic acid, pH 7.4) at 4 °C for 12 h, suspended in cacodylate buffer (pH 7.4), and then postfixed with 1% osmium tetroxide for 2 h (43). Following dehydration, pellets were embedded in Epoxy, thin sectioned, stained with uranyl acetate and Reynolds lead citrate, and examined with a JEM-100CX-II electron microscope (Joel Ltd., Tokyo, Japan).

**Expression of Human α-Synuclein in COS-7 Cells and Preparation of Cytoplasmic Membrane Compartment**—Transformed African monkey kidney cell line COS-7 was maintained in Dulbecco’s modified Eagle’s medium (HyClone Laboratories Inc., Logan, UT) with 10% fetal bovine serum (HyClone Laboratories Inc., West Grove, PA) in blocking solution for another 30 min. After extensive washing for 1–2 h in PBS, Cy2-conjugated goat secondary antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) in blocking solution was added for 30 min. For CM-DI (Molecular Probes, Inc., Eugene, OR) staining, the cells were incubated with 0.1 μg/ml CM-DIl at room temperature for 1 h. The final concentration of CM-DIl was achieved by diluting the stock solution (1 mg/ml in Me2SO) in PBS at 1:10,000. Then the cells were briefly washed with 10% glycerol and washed overnight in PBS at 4 °C. The nuclei were stained with Hoechst 33258 (2 μg/ml; Molecular Probes) in PBS for 10 min and washed three times with PBS for 30 min before mounting with ProLong anti-fade reagent (Molecular Probes). Images were analyzed using the DeltaVision deconvolution microscope (Applied Precision Inc., Issaquah, WA) in the Cell Science Imaging Facility at Stanford University.

**RESULTS**

**Stable Association of Rat Brain α-Synuclein with Various Membrane Compartment**—To characterize membrane-associated α-synuclein, rat brain homogenate was fractionated to several subcellular compartments. Consistent with previous reports (4, 40), α-synuclein was broadly distributed to all of the fractions, and a small proportion was co-fractionated with membranes (Fig. 1). Based on a densitometric analysis, about 15% of α-synuclein in the S1 fraction was present in membrane fractions (P3, LP1, and LP2).

Studies showed that α-synuclein preferentially binds to the phospholipid liposomes that contain acidic head groups (34, 35). To test the involvement of electrostatic force in the membrane interaction, P3 and LP1 membrane fractions were incubated in different salt concentrations, and the membranes were subsequently isolated by centrifugation. α-Synuclein remained bound to the membranes throughout the NaCl strength range with no α-synuclein signal detected in the supernatant (Fig. 2). Perrin et al. (38) recently suggested that the interaction between hydrophobic residues of amphipathic α-helix and the membrane interior is important for the association of α-synuclein to the membrane. Our salt wash experiment agrees with this hydrophobic nature of the interaction. How-

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α-synuclein. All three fractions showed progressive accumulation of high molecular weight α-synuclein aggregates, which is accompanied by the reciprocal loss of monomers (Fig. 3). Our recent data showed that purified recombinant α-synuclein or human α-synuclein in a COS-7 cytosolic preparation did not form aggregates unless structural changes were induced in the protein by folding stresses, such as heat treatment. This observation prompted us to compare the aggregation tendency between the membrane-bound and cytosolic forms of α-synuclein from rat brain. P3 (microsomal vesicles) and S3 (cytosol) fractions were incubated at room temperature for up to 3 days, and the aggregation rate was compared. While the membrane-bound form produced high molecular weight aggregates, little aggregation was observed with cytosolic α-synuclein even after 3 days (Fig. 4A). Aggregation of α-synuclein in the membrane is somewhat selective, because other peripheral membrane proteins such as γ-adaptin and Rab4 did not form aggregates in the same period of incubation (Fig. 4B). However, the aggregation is not exclusive to α-synuclein, because calnexin, a molecular chaperone with a transmembrane domain, underwent an extensive aggregation as well (Fig. 4B).

**Antioxidants Inhibit the Aggregation of Membrane-bound α-Synuclein**—Lipids with unsaturated fatty acyl chains are vulnerable to oxidation, and α-synuclein aggregates are known to be stabilized, if not promoted, by oxidative modifications (47). To determine the role of oxidation, we examined the effect of antioxidants on the aggregation of membrane-bound α-synuclein. The addition of either glutathione monoethyl ester (GME; a membrane-permeable derivative of glutathione) or Trolox (water-soluble derivative of vitamin E) to the incubation drastically reduced the aggregation of α-synuclein (Fig. 5). This finding suggests that oxidative modifications in membrane lipids and/or α-synuclein promote the aggregation.

To investigate possible morphological changes of the vesicles during the incubation, the vesicles in the P3 fraction were examined by electron microscopy. As shown in Fig. 6, no apparent morphological change was observed during the incubation or in the presence of GME. The vesicles were heterogeneous in size, ranging from 30 to 270 nm in diameter with a few multilamellar structures, but no significant change in vesicle size was found after incubation (Fig. 6, graph). This result suggests that the aggregation of α-synuclein in the membrane is not a secondary effect of vesicular alterations, such as membrane fusion or fragmentation.

### Antioxidants Inhibit the Aggregation of Membrane-bound α-Synuclein

**Aggregates Formed in the Membrane Can Seed the Aggregation of Cytosolic α-Synuclein**—Since the majority of α-synuclein in the cells is in a free soluble state, cytosolic α-synuclein might play a critical role in the overall aggregation process, even if the aggregation is initiated by membrane-bound forms. To address the dynamic relationship between the membrane-bound and cytosolic forms in the aggregation process, the rat brain P3 fraction was mixed with a cytosolic preparation from COS-7 cells that expressed human α-synuclein, and the aggregation rate was compared with a mixture of P3 and control cytosol from empty vector-treated cells. To mimic the in vivo ratio, excess cytosolic α-synuclein (>6-fold, estimated based on the intensity of Western blot) was added to the membrane fraction. The lag time did not seem to differ significantly between the incubation with excess cytosolic α-synuclein and the incubation with control cytosol without α-synuclein, since they formed a similar amount of aggregates at day 1 (Fig. 7). This finding suggests that at the early stages, the membrane-bound...
form of α-synuclein is selectively aggregated (also see below). On the other hand, the growth rate of the aggregates is significantly increased in the presence of cytosolic α-synuclein compared with the one with the control cytosol that lacks α-synuclein. The increase is not due to the self-assembly of cytosolic α-synuclein, because the cytosolic form alone did not form aggregates (Fig. 7). Thus, the aggregation of membrane-bound α-synuclein provides the seeds that are responsible for an accelerated aggregation of the less aggregation-prone but abundant cytosolic form.

The seeding of cytosolic α-synuclein by the membrane-bound form was further confirmed by flotation experiments. A mixture of rat brain microsomal vesicles and the cytosolic preparation from COS-7 cells that express human α-synuclein was fractionated, and the membranes and membrane-bound proteins were isolated at different time points (Fig. 8). Flotation of the membrane was employed, instead of sedimentation, to rule out the possible incidental co-sedimentation of aggregates with membranes. Cytosolic α-synuclein was distinguished from the rat brain membrane-bound form using the LB509 antibody that selectively recognizes human α-synuclein. Analyses of the proteins in buoyant and nonbuoyant fractions showed that cytosolic α-synuclein that was converted to the high molecular weight aggregates were found only in the buoyant membrane.

**Fig. 3.** Stable association and spontaneous aggregation of α-synuclein in the membrane. Fractions P3 (A), LP1 (B), and LP2 (C) were incubated at room temperature for up to 72 h, and the dissociation rate was analyzed by measuring α-synuclein in the supernatant at different time points after centrifugation. No α-synuclein was detected in the supernatants, indicating extremely slow dissociation. The arrows indicate high molecular weight α-synuclein, and the arrowheads indicate monomer (M).

**Fig. 4.** Aggregation of α-synuclein is more favorable in the membrane than the cytosolic fraction. A, the rates of α-synuclein aggregation in the P3 and S3 fractions were compared at room temperature by Western blotting using the Syn-1 antibody. Input material was adjusted so as to have an equal amount of α-synuclein in each incubation. B, P3 fraction was incubated at room temperature, and the aliquots removed at given time points were subjected to Western blotting using Syn-1, anti-Rab4, anti-γ-adaptin, or anti-calnexin antibody. The arrowhead and vertical line on the right side of each gel indicate monomeric protein and stacking gel portion, respectively.

**Fig. 5.** Effects of antioxidants on the aggregation of membrane-bound α-synuclein. P3 fraction was incubated alone or in the presence of GME or Trolox for up to 3 days. Aliquots removed at given time points were subjected to Western blotting using Syn-1 antibody. At the start of the incubation, the concentrations of the antioxidants were both 1 mM, and an additional 1 mM was added everyday. The stacking gel portion is indicated on the right side of the gel.
fraction (Fig. 8B). Cytosolic α-synuclein alone did not form any aggregates; nor did it float to the buoyant fraction. The conversion of cytosolic α-synuclein to the membrane-bound high molecular weight species was preceded by the aggregation of microsomal α-synuclein. These results suggest that the nucleation process occurs preferentially among the membrane-bound α-synuclein and that the fast growth of aggregates can be achieved by recruiting the cytosolic α-synuclein to the membrane-bound seeds. Co-aggregation of the membrane-bound form and cytosolic form was further confirmed by a co-immu-
noprecipitation experiment (Fig. 8C). In this experiment, α-synuclein aggregates can be induced in cultured cells by inhibitors of the mitochondrial electron transport chain, such as rotenone and oligomycin. To determine the subcellular distribution of aggregates in the cells, especially in the early stage of aggregation, COS-7 cells that overexpress α-synuclein were treated with rotenone, and the cell homogenates were fractionated to three parts: 1,000 × g pellet (P1), membrane fractions (M), and cytosol (C) (Fig. 9A). Since the high molecular weight aggregates became apparent after 2 days of rotenone treatment, we chose to examine the time course up to 2 days with special attention to the period between 24 and 48 h. Small oligomeric species of about 90 kDa appeared only in the M fraction and increased through 36 h (Fig. 9B). The identity of this 90-kDa protein as an α-synuclein oligomer was confirmed by a series of polyclonal and monoclonal antibodies against α-synuclein (data not shown). As the level of the 90-kDa protein decreased in the M fraction by 48 h, higher molecular weight aggregates increased in the P1 fraction, suggesting a precursor-product relationship. During this period, no aggregation was detected in the cytosolic fraction. These results suggest that in rotenone-induced α-synuclein aggregation, the earliest process of nucleation may occur in the membranous environment.

This interpretation was further supported by the presence of lipid components in α-synuclein aggregates. We used Dil, a fluorescent compound that partitions into the lipid phase, to detect lipid components in the α-synuclein-positive aggregates in rotenone-treated cells (Fig. 10). Unfortunately, attempts to stain the small aggregates with Dil were not successful because of a high overall background cell staining at higher Dil concentrations. At lower Dil concentration, as shown in Fig. 10, only large inclusions were stained, indicating dense accumulation of lipids in the inclusions. The cells infected with the control adenovirus (adeno/vec) or the cells infected with adenov/α-syn, but without the rotenone treatment, did not produce any Dil-positive inclusions (data not shown).

DISCUSSION

α-Synuclein exists as two different structural conformers in vivo: a membrane-bound form with a stable α-helical structure and a free cytosolic form that is essentially disordered (34, 35, 37). Since the aggregation of α-synuclein is accompanied by or even preceded by structural changes (33), the microenviron-mental and structural differences of these two forms raised an interesting question as to how they differ in aggregation propensity. In this study, using various subcellular fractions isolated from rat brain homogenate, we show that the membrane-bound form has a higher tendency to form aggregates than the cytosolic form. Oxidative modifications in lipids and/or the protein itself seem to play a role in the aggregation in the membrane. Moreover, the aggregation of cytosolic α-synuclein can be “seeded” by preformed membrane aggregates. The addition of excess cytoplasmic α-synuclein did not affect the lag time but only increased the rate of aggregate growth. Therefore, we propose that although it represents only a small fraction of total cellular α-synuclein, the membrane-bound form may have the ability to give rise to nuclei that seed the aggregation of the far more abundant cytosolic α-synuclein. Recently, Perrin et al. (48) reported that the interaction of α-synuclein with long chain unsaturated fatty acids rapidly promoted α-synuclein aggregation. Sharon et al. (49) also sug-gested that brain α-synuclein occurs in lipid-containing high molecular weight aggregates. These findings, along with ours in the present study, point to the importance of α-synuclein/ lipid interaction in multimerization of this protein. Although the importance of membrane-bound α-synuclein in the early stage of inclusion formation is not fully understood in vivo, our data suggest the role of the membrane-bound form in the aggregation in a cell-based system. During the rotenone-induced α-synuclein aggregation in intact cells, the earliest oligomeric species appeared in the membranous compartments, not in the cytosol. The α-synuclein-immunopositive inclusions in these cells showed a strong Dil staining, which suggests an
abundance of lipid components. It is also noteworthy that in transgenic mice that overexpress human α-synuclein in neurons, small aggregates were found on the surface of the rough endoplasmic reticulum (22) and that in the human brain LBs have lipid components (50).

The membrane-bound form of α-synuclein represents only a small fraction of total brain α-synuclein. However, our study showed that this membrane-bound form might be a nucleating species because of its higher propensity for aggregation and its seeding ability. An interesting analogy can be found in the aggregation of amyloid β protein (Aβ). Aβ40 is the major species in plasma and cerebrospinal fluid, while Aβ42 represents only about 10% of total secreted Aβ (51). However, it is widely believed that Aβ42 is responsible for the nucleation of amyloid formation, because it has a higher tendency to aggregate, and Aβ42 aggregates can seed Aβ40 in vitro (52). This idea was supported by the finding that diffuse plaques, which are believed to be the precursor for senile plaques, are composed of Aβ42 in Down’s syndrome (53), whereas senile plaques themselves contain both Aβ40 and Aβ42 (54). These findings provide the basis for a model in which highly amyloidogenic Aβ42 initiates the aggregation process by forming nuclei, which seed the aggregation of the less aggregation-prone but far more abundant Aβ40. This model predicts that the selective increase in Aβ42 would accelerate the overall rate of amyloid formation, resulting in an earlier onset of the disease. In fact, mutations in amyloid precursor protein and presenilins that are linked to early onset familial Alzheimer’s disease specifically increased Aβ42 (55). Therefore, since the selective increase of Aβ42 may be a critical early event in Alzheimer’s disease pathogenesis, the increase in the membrane-bound population of α-synuclein could trigger the aggregation and neurodegeneration processes in α-synucleinopathies.

Alternatively, altering the lipid composition or chemically modifying membrane lipids could lead to changes in membrane structure that promote aggregation. The increase in oxidative damage of membrane lipids is a common phenomenon in aged brains (56, 57). Koppaka et al. (58) recently showed that oxidized phospholipid membranes promote the β-sheet conformation in Aβ42. It is also noteworthy that inclusion bodies and small granular particles that are α-synuclein-positive are exclusively found within lipofuscin or neuromelanin deposits in the lower brain stem nuclei of Parkinson’s disease patients (59). Lipofuscin is an age-linked neuronal deposit composed of oxidized lipids and proteins, hence an indicator for oxidative stress (60). We have shown in the present study that antioxidants can inhibit the aggregation of membrane-bound α-synuclein. This suggests that oxidative damage to membranes might promote the aggregation of α-synuclein. Oxidation of membrane lipids may promote α-synuclein aggregation by adopting an environment that favors the self-assembly of α-synuclein or by leading to oxidative modifications of α-synuclein that cause structural changes in the protein and make the protein more susceptible to aggregation (47, 61–63).

There are many examples of amyloid formation characterized by α-helix to β-sheet transition (64), which often involves partially folded intermediates (65). Although structural information is not available at this point, it is reasonable to assume that the aggregation of membrane-bound α-synuclein is accompanied by a transition from α-helix to β-sheet, which probably involves structural intermediates. The temporal and spatial resolution of these events in cells would offer insight into the detailed mechanism of LB-like inclusion formation. Particularly interesting in this regard is that at least the initial stage of aggregation occurs in membranous compartments in rotenone-treated cells. Our previous study showed that at least the initial stage small aggregates are thioflavin S-negative, whereas mature perinuclear inclusions are thioflavin S-positive, suggesting that a highly ordered cross-β structure may be obtained in the later stages. Therefore, small oligomeric α-synuclein formed in membranous compartments may still have a partially unfolded helical conformation. A recent study using a helix-turn-helix peptide model showed that the self-oligomerization of partially folded, but still helix-rich, structural intermediates preceded the structural transition to β-sheets (66). Membrane-bound α-synuclein appears to be inserted into the membrane interior probably through the hydrophobic faces of the amphipathic helices (Fig. 2 and Ref. 38). One could speculate that a slight structural perturbation in the helical region may expose some of the hydrophobic residues that can remain clustered only in a helical context to the polar environment, resulting in a conformation that would favor the self-assembly through hydrophobic interaction.

A recent in vitro study showed that nonfibrillar oligomeric forms of α-synuclein had a higher affinity for liposomes than monomers and fibrils, suggesting that oligomeric α-synuclein may affect membrane structure or integrity (45). In fact, in the same study, it was demonstrated that oligomeric α-synuclein had the ability to permeabilize liposomes in vitro, whereas monomer and fibrils did not. Here, we show that the early stage of α-synuclein aggregation occurs in a membrane-bound state, resulting in membrane-associated oligomeric α-synuclein. These early stage aggregates do not appear to be fibrillar amyloids, because they are not stained with thioflavin S. Concluding the membrane permeabilization activity of prefibrillar oligomeric α-synuclein, aggregates of α-synuclein formed in membranes might in turn perturb membrane integrity. Investigations on the structure and integrity of subcellular membranous compartments in α-synuclein aggregate-containing cells should bring new insights as to how α-synuclein aggregates might affect the cell viability. In conclusion, our study offers the possibility of unraveling the elusive relationship between protein aggregation and cellular degeneration, since membrane-bound small aggregates of α-synuclein might not only initiate the inclusion formation but also reduce the cell viability by affecting the integrity of cellular membranes.

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REFERENCES
1. Ueda, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D. A., Kondo, J., Ihara, Y., and Saitoh, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 11252–11256
2. Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W. H., Castillo, P. E., Shinsky, N., Verduco, J. M., Armanini, M., Ryan, A., Hynes, M., Phillips, H., Sulzer, D., and Rosenthal, A. (2000) Neuron 25, 299–252
3. Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., de Silva, H. A., Kettel, A., and Saitoh, T. (1995) Neuron 14, 467–475
4. George, J. M., Jin, H., Woods, W. S., and Clayton, D. F. (1985) Neuron 13, 361–372
5. Polymereoupos, M. H., Lavedan, C., Leroy, I., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenfors, E. S., Chandrasekharappa, S., Athanassiou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Science 276, 2045–2047
6. Kruger, R., Kuhn, W., Muller, T., Waistalla, D., Grascher, M., Kosel, S., Przuntek, H., Epplen, J. T., Schols, L., and Ries, O. (1998) Nat. Genet. 18, 106–108
7. Papadimitriou, A., Veletz, V., Hadjigeorgiou, G. M., Patriziou, A., Hirano, M., and Anastasopoulos, I. (1999) Neurology 52, 651–654
8. Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) Nature 388, 839–840
9. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and Goedert, M. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 6469–6473
10. Baba, M., Nakajo, S., Tu, P. H., Tomita, T., Nakaya, K., Lee, V. M., Trojanowski, J. Q., and Iwatsubo, T. (1998) Am. J. Pathol. 152, 879–884
11. Takahashiy, K., Matsunoto, K., Takayama, K., Yoshimoto, M., and Takahashi, H. (1997) Neurosci. Lett. 239, 45–48
12. Arima, K., Ueda, K., Sunohara, N., Hirai, S., Izumiyama, Y., Tenozuka-Uehara, H., and Kawai, M. (1998) Brain Res. 808, 95–100
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