Characterization of Cytoplasmic α-Synuclein Aggregates

FIBRIL FORMATION IS TIGHTLY LINKED TO THE INCLUSION-FORMING PROCESS IN CELLS

He-Jin Lee and Seung-Jae Lee‡
From the The Parkinson’s Institute, Sunnyvale, California 94089

The α-synuclein fibrillation process has been associated with the pathogenesis of several neurodegenerative diseases. Here, we have characterized the cytoplasmic α-synuclein aggregates using a fractionation procedure with which different aggregate species can be separated. Overexpression of α-synuclein in cells produce two distinct types of aggregates: large juxtanuclear inclusion bodies and small punctate aggregates scattered throughout the cytoplasm. Biochemical fractionation results in an inclusion-enriched fraction and two small aggregate fractions. Electron microscopy and thioflavin S reactivity of the fractions show that the juxtanuclear inclusion bodies are filled with amyloid-like α-synuclein fibrils, whereas both the small aggregate fractions contain non-fibrillar spherical aggregates with distinct size distributions. These aggregates appear sequentially, with the smallest population appearing the earliest and the fibrillar inclusions the latest. Based on the structural and kinetic properties, we suggest that the small spherical aggregates are the cellular equivalents of the protofibrils. The proteins that co-exist in the Lewy bodies, such as proteasome subunit, ubiquitin, and hsp70 chaperone, are present in the fibrillar inclusions but absent in the protofibrils, suggesting that these proteins may not be directly involved in the early aggregation stage. As predicted in the aggregate model, disruption of microtubules with nocodazole reduced the number of inclusions and increased the size of the protofibrils. Despite the increased size, the protofibrils remained non-fibrillar, suggesting that the deposition of the protofibrils in the juxtanuclear region is important in fibril formation. This study provides evidence that the cellular fibrillation also involves non-fibrillar intermediate species, and the microtubule-dependent inclusion-forming process is required for the protofibril-to-fibril conversion in cells.

A group of human neurodegenerative diseases, such as Parkinson’s disease (PD), dementia with Lewy bodies (LBs), and multiple system atrophy, are characterized by cytoplasmic inclusion bodies that are mainly composed of α-synuclein fibrils (1, 2). Although direct role of fibrils and the inclusion bodies in the disease pathogenesis is the subject of intense debate, an increasing body of evidence suggests that the processes of fibrillation and inclusion formation are closely related to the disease mechanism. First, two missense mutations (A53T and A30P) that are responsible for the familial PD have been identified in α-synuclein gene (3, 4), and the mutant proteins have greater propensity for the self-association and aggregation than the wild type protein (5–7). Indeed, both mutations accelerated the formation of the pre-fibrillar oligomers in vitro, whereas the fibril formation was slowed by one of the mutations (7, 8). Second, transgenic animal models that were generated by the overexpression of human α-synuclein developed neuronal cytoplasmic inclusion bodies along with neuronal cell loss and behavioral defects (9–13). Some of these animals produce fibrillar inclusions (9, 12), but others generate only the granular aggregates (10). In a rat Parkinson’s model, established by a systemic administration of rotenone, nigrostriatal degeneration and the motor symptoms were also accompanied by α-synuclein-positive inclusion bodies (14). Therefore, the cellular mechanism of α-synuclein aggregation is likely to be linked to at least some aspects of the disease process. Although the process of α-synuclein fibril formation has been implicated in the pathogenesis of PD by genetic and biochemical evidence, the exact mechanism by which the processes of α-synuclein fibrillation and inclusion body formation contribute to neurodegeneration is currently unknown.

In dilute solution, α-synuclein does not have stable structure (15) except for some residual helical structure in the N terminus of the protein (16). Fibrillation of α-synuclein is a nucleation-dependent process (17) and is initiated by acquiring a partially folded conformation (18), which is subsequently stabilized by self-association (19). Prior to the formation of the fibril, the end product of the process, several non-fibrillar oligomeric aggregates, or protofibrils, were identified (20). Earliest and most common protofibrillar species are in a spherical shape with average height of 4.2 nm in case of wild type protein (21). The spherical protofibrils are thought to undergo head-to-tail associations to form elongated chain-like (22), and ring-like protofibrillar species (21). In their search for a potential pathogenic mechanism for α-synuclein protofibrils, Lansbury and colleagues (23, 24) demonstrated that only protofibrillar α-synuclein bind tightly and permeabilize synthetic vesicles in a size-selective manner, suggesting membrane disruption via a pore-like mechanism. This hypothesis is supported by the findings that the two pathogenic mutations (A53T and A30P) promote the formation of annular pore-like protofibrils (25) and result in an increased permeabilization activity relative to the wild type protein (24). Although some of the basic processes of α-synuclein aggregation, including the protofibrils with different morphologies, have been characterized in vitro, little is known about the fibrillation process or the intermediate protofibrillar species in cells.
To understand the mechanism of α-synuclein aggregation in cells, the following questions need to be addressed: (i) What is the end product of an α-synuclein aggregation process in cells? (i.e. is it the fibril or other aggregate form?) (ii) What are the intermediates that precede α-synuclein fibrillation in cells? (iii) Is the fibrillation process linked to the inclusion formation process, and if so, how? Here, using a combination of biochemical fractionation, immunofluorescence labeling, and electron microscopy (EM), we show that cells produce inclusion bodies that are filled with α-synuclein fibrils. Before the appearance of the fibrillar inclusions, small non-fibrillar aggregates are formed, and the basic properties of these aggregates suggest that they are the cellular equivalent of the protofibrils. Our study also provides evidence that the inclusion-forming process, which is characterized by the transport of α-synuclein protofibrils and other cellular components to the pericentriolar region, is required for the conversion of protofibrils to fibrils.

MATERIALS AND METHODS

Expression of Human α-Synuclein and Induction of Aggregation—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.) in a 37 °C/5% CO2 humidified incubator. To express α-synuclein, COS-7 cells were split on 100-mm tissue culture dishes 1 day prior to infection to obtain ~90% confluence on the day of infection. For infection, recombinant adenoviral vector (26) was added to each dish at a multiplicity of infection (m.o.i.) of 75. After 90 min of incubation at 37 °C, 9 ml of fresh medium was added and the cells were maintained at 37 °C. The cells were then split the next day to ~40% confluence and maintained for another 48 h or as indicated. Note that, in our culture condition, the time course of aggregation varies depending on the size of the culture dish used. For example, cells cultured in a 100-mm dish show more rapid aggregation than the cells in smaller dishes.

Fractionation of α-Synuclein Aggregates from COS-7 Cells—COS-7 cells expressing α-synuclein were washed twice with cold phosphate-buffered saline (PBS) before addition of buffer T (20 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl2, 0.25 mM sucrose, 1% Triton X-100, protease inhibitor mixture (Sigma)) to each dish. After a 5-min incubation at room temperature, the supernatant containing Triton-soluble proteins was carefully removed from plates. After gentle washing of dishes with PBS, the Triton-insoluble materials were scraped in buffer N (0.1 M Na2CO3, pH 11.5, protease inhibitor mixture), resuspended by repeated pipetting, and incubated on ice for 5 min. The extract was then centrifuged at 80 × g for 10 min. The pellet containing big inclusions was resuspended in 1× Laemmli sample buffer (SB) for Western blot analysis, in PBS for immunofluorescence staining, or in 0.1 M phosphate buffer for EM. The supernatant containing small aggregates was then overlaid onto a discontinuous density gradient of 2.5%, 25%, and 35% iodixanol (3:3:1, v/v) in buffer T and centrifuged at 50,000 × g for 30 min. The fractions were obtained from the top of the gradient, diluted with 2 volumes of PBS, and centrifuged at 16,000 × g for 10 min. The pellets from fractions were then resuspended in 1× SB for Western blot analysis, in PBS for immunofluorescence staining, or in 0.1 M phosphate buffer for EM. Western Blotting—Western blot analysis was performed according to the procedure described in Lee et al. (27). LB509 monoclonal anti-α-synuclein antibody was purchased from Zymed Laboratories (South San Francisco, CA).

Immunofluorescence Staining of Cells and Aggregate Fractions—The procedure for cell staining has been described elsewhere (26). Briefly, cells grown on poly-l-lysine-coated coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Coverslips were then blocked in blocking solution (PBS, 5% bovine serum albumin). Primary antibodies in blocking solutions were added at dilutions described below and incubated for 30 min: LB509 monoclonal α-synuclein antibody (1:1000, Zymed Laboratories Inc.), 7071 polyclonal α-synuclein antibody (1:1000, provided by Peter Lansbury at Harvard Medical School, Boston, MA), anti-ubiquitin antibody (1:1000, Chemicon), anti-Hsp70/Hsc70 antibody (1:200, StressGen), anti-β-amyloid protein (1:200, provided by Richard Scholze at Calbiochem-Novabiochem Corp., San Diego, CA). Coverslips were extensively washed for 1–1.5 h with PBS before addition of fluorescent dye (Cy3, Cy2, or Rhodamine Red X-conjugated goat anti-mouse or anti-rabbit antibodies (1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in blocking solution for 30 min. Stainings with Thioflavin S (Sigma) and Hoechst 33258 (Molecular Probes, Inc., Eugene, OR) were performed according to Lee et al. (26). For staining of obtained fractions, the samples in PBS were placed on coverslips and let partially dry. After fixing the coverslips in 4% paraformaldehyde in PBS for 30 min, the staining was done in the same manner as described above.

Electron Microscopy—COS-7 cells expressing α-synuclein and α-synuclein fractions in PBS were prepared for section as described in Bouley et al. (28). For the immunolabeling, the sections were incubated in 1% gelatin in PBS and then in 0.03 M glycine in PBS. After blocking in 2% bovine serum albumin in PBS, the sections were incubated with LB509 antibody (1:250) in 2% bovine serum albumin in PBS, followed by 10-nm gold-conjugated goat anti-mouse IgG antibody. The sections were stained with 1% uranyl acetate and lead citrate for 5 min each. For negative staining of fibrils from the inclusion bodies, the pellet from an 80 × g spin was rinsed and then resuspended in PBS and incubated in 1% SDS for 20 min at room temperature. After the incubation, the sample was centrifuged at 80 × g to remove undissolved inclusion bodies, and the supernatant was centrifuged at 100,000 × g for 30 min. The pellet was then resuspended in 50 mM Tris, pH 7.4, and then placed on a carbon-coated grid. The grid was incubated with LB509 antibody (1:250), followed by goat anti-mouse IgG antibody conjugated with a 10-nm gold particle, and after two rinses with distilled water, it was stained with 1% uranyl acetate. The images were obtained using a Philips CM 12 electron microscope.

RESULTS

To characterize the cytoplasmic α-synuclein aggregates, we established a mammalian cell culture model using COS-7 cells and recombinant adenoviral vector. In this model, expression of monomer is saturated at an m.o.i. of about 25, and higher expression over the saturation point induces the spontaneous aggregation, which shows an exponential increase with respect to the increasing m.o.i (40). Alternatively, inhibitors of mitochondrial respiratory chain, such as rotenone, greatly enhance α-synuclein aggregation at the expression level slightly above the saturation point (m.o.i. of 35), in which the spontaneous aggregation is minimal (26). In this study, the spontaneous α-synuclein aggregation was induced in COS-7 cells at m.o.i. 75, and the resulting cytoplasmic aggregates were characterized using biochemical fractionation combined with imaging analyses.

Similar to what was observed in the rotenone-induced α-synuclein aggregation (26), spontaneous aggregation at m.o.i. 75 resulted in two types of aggregates that are distinct in their sizes and cytoplasmic distributions. One type is represented by small punctate aggregates that are dispersed throughout the cytoplasm, and the other is large juxtanuclear inclusion bodies (Fig. 1). The juxtanuclear inclusion bodies bind to thioflavin S (Fig. 1B), a fluorescent dye specific for the highly ordered cross β-sheet structure, thus an indicative of the amyloid-like fibrillar structures (see below for the ultrastructural analysis). In contrast, the small punctate aggregates are thioflavin S-negative (Fig. 1A), indicating a non-fibrillar conformation. The difference in the dye binding properties suggests that these two types of aggregates have different structural properties.

Previous immunohistochemical studies of human LBs showed that they are also stained with ubiquitin (29), proteasomes (30), and molecular chaperones, such as hsp70 (11). To determine whether the inclusion bodies in our cell system contain these proteins, we co-stained the cells for α-synuclein with ubiquitin, α-subunit of 20 S proteasome, or hsp70. Like human LBs, large juxtanuclear inclusion bodies were positive for all these proteins (Fig. 1B). On the other hand, most of the peripheral punctate aggregates were not stained with ubiquitin, the proteasome subunit, or hsp70 (Fig. 1A). Interestingly, the cells with small punctate aggregates display brighter staining in the perinuclear region for ubiquitin, hsp70, and 20 S α-subunit proteins than the cells without aggregates (data not provided).
shown), where they show some degree of co-localization with α-synuclein punctate aggregates (Fig. 1A). Mitochondria and lysosomes are also elevated and accumulated in the perinuclear region along with the small punctate α-synuclein aggregates (40). The accumulation of these proteins and organelles, which are required for the degradation of macromolecules in the perinuclear region, at what appears to be an early stage of inclusion formation, supports the hypothesis that the inclusion-forming process represents how the cells deliberately sequester and degrade abnormal protein aggregates at the specialized subcellular location.

To further characterize different types of α-synuclein aggregates, we have established a procedure by which these aggregates are separated into different fractions (Fig. 2A). Cells that produce α-synuclein aggregates were extracted with 1% Triton X-100 in the culture dish, and the Triton-soluble proteins were gently removed. Under this condition, the soluble monomeric α-synuclein was completely removed, and the aggregates remained in the culture dish, probably due to their association with unknown structures. The remaining Triton-insoluble portion was then scraped in basic pH buffer, in which the aggregates were dissociated from the cellular structures, and centrifuged at 80 g. After the centrifugation at 80 g for 10 min, the pellet fraction was found to contain exclusively the large inclusion bodies, whereas the supernatant contained predominantly small spherical aggregates (data not shown). In an attempt to further separate the different α-synuclein aggregates, the supernatant was then subjected to a density gradient centrifugation as described in Fig. 2A. Western blot analysis of the fractions (fr) identified three distinct fractions containing SDS-resistant α-synuclein aggregates (fr 2, fr 4, and 80 g pellet) (Fig. 2B). Although fr 7 also contains α-synuclein aggregates in some cases, immunofluorescence and immuno-EM studies suggested that this fraction consists of clumps of heterogeneous α-synuclein aggregates, which appear to be a mixture of the aggregates found in fr 2 and fr 4 (data not shown).

Dual fluorescence labeling shows that fr 2 and 4 contain small punctate aggregates that are thioflavin S-negative, whereas the 80 g pellet contains large inclusion bodies that are thioflavin S-positive (Fig. 3A), confirming the in situ immunofluorescence results (Fig. 1). Also consistent with the in situ immunofluorescence staining, the small aggregates do not contain ubiquitin, hsp70, or 20 S proteasome α-subunit, whereas the large inclusion bodies in the 80 g pellet were positive for these proteins (Fig. 3, B–D). The inclusion bodies were found exclusively in the 80 g pellet. These results confirm that the cells produce different types of α-synuclein aggregates with distinct conformational and compositional properties and that the separation of these aggregates can be achieved using our procedure.

To further analyze the ultrastructural features of α-synuclein aggregates, the fractions were examined by immuno-EM. The aggregates in both fr 2 and fr 4 appear to be non-fibrillar spheres (Fig. 4, A and B). To compare the size distributions of aggregates in fr 2 and fr 4, we measured the diameter of each aggregate labeled with α-synuclein antibody (Fig. 4C). Those aggregates that are irregular in shape were measured across the longest and shortest axes, and the median value was calculated. Random measurement of the diameter of these spheres shows the different size distributions in these fractions, and the calculated mean diameters for fr 2 and fr 4 were 24 and 34 nm, respectively.

EM analysis confirmed that the large inclusion bodies were enriched in the 80 g pellet, and they were immunostained with α-synuclein antibody (Fig. 5A). However, in contrast to the small spherical aggregates in fr 2 and fr 4, the inclusions are filled with fibrillar structures. To further characterize the fibrils of these inclusion bodies, the 80 g pellet was disrupted in 1%
 SDS, and the released fibrils were collected by 100,000 × g centrifugation. The fibrils isolated from the inclusions were 8–13 nm in width and were clearly labeled with α-synuclein antibody (Fig. 5B). These fibrils resembled the ones isolated from human LBs (29) or the inclusions of the transgenic mice (12). Thicker structures, which appear to be fibril bundles, were also frequently observed (Fig. 5C). These ultrastructural morphologies, along with the thioflavin S binding property, demonstrate that the α-synuclein aggregation process in our cell model produces the inclusion bodies that are composed of α-synuclein fibrils, thus suggesting that this process of inclusion body formation in these cells may resemble the process of LB formation in human brain.

To gain insights into the dynamic relationship between different aggregate species, we investigated the time-dependent formation of the small spherical aggregates and the fibrillar inclusions in the 80 × g supernatant and pellet fractions, respectively. As shown in Fig. 6A, the small aggregates in the 80 × g supernatant became apparent as early as 48 h post-infection and increased with time. On the other hand, the fibrillar inclusions in the 80 × g pellet did not appear until 60 h (Fig. 6A). This result suggests that the small spherical aggregates are formed before the formation of fibrillar inclusions. We then investigated the time course for the two subspecies of spherical aggregates. Fractionation of the spherical aggregates at different time points shows that the fr 2 aggregates are the
earliest species formed, appearing at 48 h (Fig. 6B, top panel). The fr 4 aggregates were formed slightly later at around 54 h (Fig. 6B, second panel). Both species increased progressively at later time points, but the increase was more dramatic for the fr 4 aggregates (Fig. 6B). The fact that these two fractions show differences in both the time of appearance and the rate of increase suggests that they are distinct aggregate species and that the fr 2 aggregates may be the precursors for the fr 4 aggregates.

It has been demonstrated with a number of proteins that the juxtanuclear inclusion formation requires the microtubule-dependent transport of peripheral small aggregates to the pericentriolar region (31, 32). To test whether microtubule-dependent transport is important for the formation of α-synuclein inclusion body, we treated the cells with a microtubule-disrupting agent, nocodazole, and assessed the inclusion formation in two ways. First, the number of inclusion bodies was counted after the immunofluorescence staining (Fig. 7A). Second, the relative amounts of inclusion bodies and small non-fibrillar aggregates were measured after the fractionation (Fig. 7B). Both analyses clearly show the decrease of the inclusion bodies after nocodazole treatment, implicating the importance of microtubule-dependent transport system in the inclusion formation. Furthermore, the fractionation experiment shows that the reduction of inclusion bodies is accompanied by the increase of the small aggregates (Fig. 7B), confirming that nocodazole inhibits the transport of the small aggregates rather than the aggregation per se. Interestingly, when the microtubule is disrupted, bigger foci were frequently found in the periphery of the cell (Fig. 7C), which implies that the peripheral aggregates can grow bigger to a certain extent, if their transport to the pericentriolar region is blocked. This finding raised a question as to whether the large foci that are grown in the periphery of the cell can acquire the characteristics of the juxtanuclear inclusion bodies. To answer this question, we investigated the thioflavin S binding property and the immunoreactivities for the proteins that are found in the inclusion bodies. Unlike the juxtanuclear inclusion bodies, the large peripheral foci are thioflavin S-negative, indicative of the non-fibrillar nature of the aggregates and devoid of ubiquitin, hsp70, and 20 S proteasome α-subunit (Fig. 7C). These data suggest that fibrillation and acquisition of the auxiliary proteins are not spontaneous consequences of the aggregate growth, rather, they are closely linked to the microtubule-dependent inclusion-forming process.

**DISCUSSION**

In our previous study, we have demonstrated that overexpression of α-synuclein or exposure of cells to mitochondrial inhibitors produces two distinct forms of α-synuclein aggregate; small punctate aggregates that are scattered throughout the cytoplasm and large juxtanuclear inclusion bodies. Here, we have characterized the structural nature of these aggregates and investigated the relationship between them in the context of inclusion forming process. Using biochemical fractionation and EM analysis, we have demonstrated small aggregates are non-fibrillar spheres, and the inclusion bodies are filled with α-synuclein fibrils. Time-dependent analysis shows that small non-fibrillar aggregates precede the formation of fibrillar inclusions. An anti-microtubule agent, nocodazole, causes a reduction in the number of fibrillar inclusions and the accumulation of non-fibrillar aggregates in the cytoplasm, suggesting that these non-fibrillar aggregates are the precursors of the fibrillar aggregates in the inclusion bodies. Prototibrils are described as non-fibrillar aggregates that precede the fibril formation and are often enriched with α-synuclein fibrils. Although the conformational characteristics of the cytoplasmic aggregates are not yet available, the ultrastructural and kinetic properties suggest that the small non-fibrillar aggregates are the cellular equivalents of the protofibrils.

In solution, fibrillation of α-synuclein, which involves a number of metastable intermediate species, including various protofibrils, is a continuous process, because all the monomers and assembly intermediates are freely diffusible and available for the molecular interactions. However, in cells, fibrillar α-synuclein aggregates are found exclusively in the juxtanuclear inclusions, and interfering with the transport of protofibrils to the pericentriolar region inhibits the fibril formation. These findings support the hypothesis that monomer to protofibril conversion and protofibril to fibril conversion are spatially separate processes; the former seems to be a diffusion-limited reaction that occurs throughout the cytoplasm, whereas the latter is restricted only in the pericentriolar region. In cells, the protofibrils seem to be associated with detergent-insoluble structures, which limits their chance to interact with one another. When microtubule-mediated transport is disrupted with nocodazole, peripheral protofibrils become larger than normal but do not turn into fibrils. Thus, it is only after the protofibrils are transported and deposited in the pericentriolar region that the interactions between the protofibrils are allowed to undergo the transformation into fibrils.

Protofibrillar intermediates (often in a spherical morphology) have been found in virtually all fibrillation characterized, thus becoming a universal mechanism of amyloid-like fibril formation (33-36). However, the mechanism of the protofibril-

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**Note:**

1. H.-J. Lee and S.-J. Lee, unpublished data.
to-fibril transition is not clearly understood. In vitro, most α-synuclein protofibrils are either in a spherical shape or in the morphologies (chain-like and annular pore-like structures) that appear to be produced by the association of the spherical protofibrils (20, 21). This observation implies that the protofibril spheres can self-associate to form higher-order structures. A similar mechanism has been proposed in the fibrillation of sup35 protein (N-terminal and mid-domain fragment) in which oligomer-oligomer interactions precede the conformational changes to amyloid-like fibrils (34). Our observation that the fibril formation occurs only when the protofibrils are concentrated and allowed to interact with one another also supports the possibility that the fibril formation in vivo could be driven by protofibril/protofibril interactions. However, our findings do not exclude the role of monomers in the cellular fibrillation process. Direct incorporation of monomers into the fibrils could also play a role in fibril growth in vivo. In vitro studies show that when the protofibrils are populated in solution, the transition from spherical aggregates to the amyloid-like fibrils is a spontaneous process. However, in cells this process may be
assisted by the proteins that are co-accumulated with α-synuclein aggregates in the pericentriolar region, such as molecular chaperones.

Microtubule-dependent deposition of peripheral aggregates into the pericentriolar region has been documented with several other proteins, and the resulting inclusion bodies are referred to as aggresomes (37, 38). Aggresome represents one of the end points of cellular responses to misfolded proteins, with other competing end points being degradation and refolding. Although many proteins share the same mechanism for the deposition in the pericentriolar region, the physical states of the final aggregated forms seem to be determined by the conformational properties of individual proteins. For example, aggregates of cystic fibrosis transmembrane conductance regulator (31) or GFP-250 (32) remain granular in shape, whereas α-synuclein aggregates are transformed into fibrils after deposition in the aggresomes. If the recent proposal is correct that protofibrils are the pathogenic species (20), accelerating the transformation of disease-associated protofibrils into fibrils would have evolutionary advantage. Developing noninvasive ways to interfere with the transport of protofibrils to the inclusion-forming site or the structural transition to fibrils will allow us to assess the role of these processes in α-synuclein-mediated cell death.

Inclusion bodies, either the ones that are produced in our cell system or LBs in human brain, contain protein components other than α-synuclein fibrils, such as proteasome subunits (30), ubiquitin (29), and molecular chaperones (11). Their presence in inclusion bodies raises the question of whether they co-aggregate with α-synuclein or even promote the aggregation process. Our study shows that α-synuclein protofibrils, both the ones in the cells or isolated from the cells, are not stained for these proteins, but they are co-deposited in the perikaryal region with α-synuclein protofibrils. In a previous study, we have shown that early stage α-synuclein oligomerization in crude cytosol preparation is highly self-selective (19). In agreement with our findings, Rajan et al. (39) have recently demonstrated that aggregation of misfolded proteins is the result of highly specific self-association, rather than nonspecific interactions between unrelated proteins. Therefore, it is unlikely that the proteins that co-exist in the inclusion bodies play a direct role in promoting α-synuclein aggregation. Rather, it seems more likely that the presence of protein degradation machineries and the molecular chaperones is an indication of an attempt by the cell to clear these protein deposits.

Fractionation of protofibrillar aggregates resulted in the separation of two subspecies with distinct size distribution. It is not entirely clear at the moment whether they represent two distinct intermediates in a linear process or one of them is an artificial outcome that are formed during the extraction procedure, such as fragmentation of larger aggregates to smaller pieces or large clumps of aggregates due to an incomplete resuspension. If they were the same species, it would be predicted that their kinetic behavior is identical. However, our

Fig. 7. Nocodazole treatment inhibits the progression of peripheral α-synuclein aggregates into juxtanuclear fibrillar inclusions. A, percentage of cells with inclusion bodies. COS-7 cells overexpressing α-synuclein were treated with MeSO or nocodazole (10 μg/ml) for 48 h before the immunofluorescence labeling for α-synuclein and counting. The numbers above each bar indicate the number of cells with inclusion bodies per the total number of cells counted. For each sample, ten random fields were selected for counting. B, reduction in inclusion bodies and concomitant increase of small non-fibrillar aggregates after the nocodazole treatment. COS-7 cells expressing α-synuclein were treated with MeSO or nocodazole for 24 h before extraction, and the aggregates were fractionated according to the procedure described in Fig. 2. The fractions were analyzed by Western blotting with LB509 antibody. The stacking gel portion is indicated as a line on the right side of the gel. Txsol, Triton X-100-soluble fraction; −, dimethyl sulfoxide (MeSO); +, nocodazole. C, enlarged peripheral α-synuclein aggregates were found in the cells that were treated with nocodazole. These enlarged peripheral foci did not stain with thioflavin S or the antibodies against ubiquitin, hsp70, or 20 S proteasome α-subunit.
time-course experiment shows that these two species have clearly distinctive kinetics: the smaller species appears first, followed by the larger one. Thus, it is likely that they are distinct entities and may be related in a linear process.

In conclusion, we have demonstrated that α-synuclein fibrillation is tightly associated with the microtubule-dependent inclusion-forming process, which seems to be necessary for the protofibril-to-fibril transition. The ability to separate different species and semi-quantitatively analyze them will allow the detailed characterization of the α-synuclein aggregation process in cells.

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