**α-Synuclein Protofibrils Inhibit 26 S Proteasome-mediated Protein Degradation**

UNDERSTANDING THE CYTOTOXICITY OF PROTEIN PROTOFIBRILS IN NEURODEGENERATIVE DISEASE PATHOGENESIS

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The impaired ubiquitin-proteasome activity is believed to be one of the leading factors that contribute to Parkinson disease pathogenesis partially by causing α-synuclein aggregation. However, the relationship between α-synuclein aggregation and the impaired proteasome activity is yet unclear. In this study, we examined the effects of three soluble α-synuclein species (monomer, dimer, and protofibrils) on the degradation activity of the 26 S proteasome by reconstitution of proteasomal degradation using highly purified 26 S proteasomes and model substrates. We found that none of the three soluble α-synuclein species impaired the three distinct peptidase activities of the 26 S proteasome when using fluorogenic peptides as substrates. In striking contrast, α-synuclein protofibrils, but not monomer and dimer, markedly inhibited the ubiquitin-independent proteasomal degradation of unstructured proteins and ubiquitin-dependent degradation of folded proteins when present at 5-fold molar excess to the 26 S proteasome. Together these results indicate that α-synuclein protofibrils have a pronounced inhibitory effect on 26 S proteasome-mediated protein degradation. Because α-synuclein is a substrate of the proteasome, impaired proteasomal activity could further cause α-synuclein accumulation/aggregation, thus creating a vicious cycle and leading to Parkinson disease pathogenesis. Furthermore we found that α-synuclein protofibrils bound both the 26 S proteasome and substrates of the 26 S proteasome. Accordingly we propose that the inhibitory effect of α-synuclein protofibrils on 26 S proteasomal degradation might result from impairing substrate translocation by binding the proteasome or sequestrating proteasomal substrates by binding the substrates.

Parkinson disease (PD) is a common age-associated neurodegenerative disorder affecting ~2% of the population aged 65 years or older (1). The pathological hallmarks of PD are the loss of dopaminergic neurons in substantia nigra pars compacta and the presence of cytoplasmic inclusions called Lewy bodies. Lewy bodies contain dozens of proteins and lipids with α-synuclein (αSyn), which is deposited as fibrillar forms, as the predominant protein (2, 3). αSyn is a 140-amino acid protein found enriched in the presynaptic terminus. Structurally αSyn adopts a random coil conformation in solution, whereas the N-terminal amphipathic domain turns into an α-helical structure when associated on anionic membrane surfaces (4). The possible physiological functions of αSyn include regulating synaptic plasticity, dopamine neurotransmission, endoplasmic reticulum/Golgi trafficking, and acting as a molecular chaperone (5). An abundance of evidence has implicated αSyn in PD pathogenesis. Genetically three point mutations in the αSyn gene (A30P, E46K, and A53T) are associated with rare autosomal dominant forms of PD (6–8). In addition, duplication and triplication of the αSyn gene can cause early onset familial PD (9–12), indicating that simply increasing αSyn concentration is enough for disease pathogenesis. Biochemically αSyn aggregation is a nucleation-dependent process, which is exacerbated by some disease-causing mutants and C-terminal truncations (4). Importantly, animals overexpressing αSyn, especially disease-associated mutants, develop movement disorder accompanying αSyn aggregation, recapitulating some features of PD and several other neurodegenerative diseases related to αSyn aggregation including dementia with Lewy bodies and multiple system atrophy (13).

The impaired mitochondrial function and ubiquitin-proteasome activity are the two leading factors contributing to PD pathogenesis at least in part through accelerating αSyn aggregation (14). The ubiquitin-proteasome pathway is responsible for the degradation of the majority of intracellular proteins, and it plays essential roles in maintaining almost every aspect of cellular activities including gene transcription, protein translation, cell development, signal transduction, and protein quality control (15). The 26 S proteasome is a 2.5-MDa complex consisting of the 20 S proteasome and the 19 S regulatory complex (called PA700 in mammals). The eukaryotic 20 S proteasome has a heptameric, four-ring stacked structure arranged as salt; Amc, 7-amino-4-methylcoumarin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Ub, ubiquitin; PDDA, poly(diallyldimethylammonium) chloride; Z, benzyloxycarbonyl; Suc, succinyl. 

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α1−β1−γ/β1−γα1−γ. Three of the β subunits have distinct peptidase activities: β1 has a caspase-like activity, β2 has a trypsin-like activity, and β5 has a chymotrypsin-like activity. The six catalytic sites are housed inside the β chamber, which is sequenced from intracellular proteins by α chambers with sealed entrances (16). Interestingly the sealed entrance can be opened upon association with proteasomal regulatory complexes PA28, PA200, and PA700 (17). Only PA700, when associated on either or both ends of the 20 S proteasome that forms the 26 S proteasomes, has the ability to mediate the degradation of polyubiquitinated proteins. PA700 has 20 subunits, which possess activities to bind a polyubiquitin chain, bind denatured proteins, unfold protein substrates, catalyze substrate deubiquitination, and hydrolyze ATP (18, 19). Coordinated actions of these activities are necessary to mediate the degradation of polyubiquitinated proteins (20). Although both the 20 and 26 S proteasome can degrade unstructured proteins without a polyubiquitin chain modification (21, 22), the physiological role of the 20 S proteasome is still under debate.

Several lines of evidence indicate a direct link of the impaired proteasome function to PD pathogenesis. Genetic mutations in PARK genes (PARK 2 encoding Parkin, an E3 ubiquitin-protein ligase, and PARK 5 encoding ubiquitin C-terminal hydrolase L1) encoding two components of the ubiquitin-proteasome pathway are associated with familial PD (23, 24). Histologically, immunohistochemical staining has revealed that Lewy bodies contain a great abundance of ubiquitin, ubiquitinated proteins, and proteasomal subunits (25, 26). Biochemical studies have shown the loss of proteasomal subunits and the impaired proteasomal activities in the substantia nigra pars compacta in sporadic PD (27). Furthermore, studies on animal models corroborate the involvement of proteasome dysfunction in the neurodegenerative process where administration of proteasome inhibitors induces dopaminergic neuronal death and the formation of αSyn/ubiquitin-containing inclusions in the surviving neurons (28–30) presumably because αSyn itself is a substrate of the proteasome (31, 32). Interestingly, overexpressing αSyn inhibits proteasomal activities in several mammalian cell lines (33–35), yeast (36), and a mouse model (37), suggesting that the elevation of αSyn concentration impairs proteasomal activity. Consistently, two recent reports showed that αSyn and its insoluble aggregated forms directly inhibit proteasome activity. Lindersson et al. (38) reported that αSyn filaments and oligomers specifically inhibit the chymotrypsin-like activity of purified 20 S proteasome using a fluorogenic peptide as the substrate. Snyder et al. (34) found that insoluble aggregated αSyn inhibits both ubiquitin-dependent and ubiquitin-independent protein degradation using HEK293 cell lysates or rabbit reticulocyte lysates as sources of the proteasome. Also they found that the chymotrypsin-like activity of purified 20 S proteasome is inhibited by both αSyn monomer and insoluble αSyn aggregates (34).

An emerging concept in the neurodegenerative disease field is that the potential cytotoxic species are soluble oligomeric intermediates, such as protofibrils, but not the insoluble fibrils deposited in diseased brains. Much evidence supports this concept from αSyn aggregation related to PD pathogenesis. In vitro studies showed that both earlier onset familial PD-causing mutations, A30P and A53T, promote αSyn protofibril formation relative to wild-type protein, whereas the A30P mutation retards fibril formation (39), suggesting that the pathogenic form is αSyn protofibrils or other soluble oligomers. Consistently, αSyn protofibrils, not fibrils, promote cell death when introduced in cell culture media (40, 41). Moreover, transgenic mice expressing human αSyn develop nonfibrillar intraneuronal inclusions, lose dopaminergic terminals, and show motor impairments (42). Introducing human αSyn gene into the substantia nigra of rats causes the formation of nonfibrillar inclusions and selective loss of dopaminergic neurons (43), supporting the idea that fibril formation is not necessary for neurodegeneration. Pathologically, recent studies demonstrate that the formation of soluble oligomers accompanies Alzheimer disease and PD pathology (40, 44), indicating that the well characterized in vitro nucleation-dependent protein aggregation process might occur in vivo as well. Whether the potential cytotoxic αSyn protofibrils could directly impair proteasome function, especially the physiologically important 26 S proteasome, is unknown. Here using fluorogenic peptides, unstructured proteins, and a polyubiquitinated protein as substrates, we examined the effects of three soluble αSyn species (monomer, dimer, and protofibrils) on the protein degradation activity of highly purified 26 S proteasomes.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Epoxomicin was purchased from BIOMOL International. Fluorogenic peptides were from Bachem. MG132 was from Sigma. Antibodies for the following proteins were purchased: anti-αSyn monoclonal antibody (BD Transduction Laboratories), anti-polyhistidine (His-1) and anti-β-actin (AC-15) (Sigma), anti-UbcH10 polyclonal antibody (Boston Biochem), anti-p21cip1 (c-19), anti-IkBa (C-21) (sc-371), and anti-Ub (P4D1) (Santa Cruz Biotechnology). Anti-p31 polyclonal antibody was a gift from Dr. G. N. DeMartino (University of Texas Southwestern Medical Center).

**Recombinant Protein Expression and Purification—**αSyn, His6-p21cip1, His6-UBCH10, Ub177, UbK48C, His6-E1, and GST-E25K were purified as described previously (20, 22, 41, 45). His6-securin was expressed in *Escherichia* coli and purified using nickel-nitritotriacetic acid (Qiagen) resins following the manufacturer’s instructions. Purified securin was kept in 20 mM Tris, pH 7.2, 200 mM NaCl, and 2 mM β-mercaptoethanol. PolyUb chains were synthesized according to a protocol established by Pickart and Raasi (46).

**Proteasome Purification—**PA700 and 26 S proteasomes were purified from bovine red blood cells as described previously (20, 47). 4% native PAGE for assaying the in-gel peptidase activity of the 26 S proteasome was performed according to a previous report (20).

**Preparation and Separation of αSyn Monomer, Dimer, and Protofibrils—**αSyn protofibrils were prepared by a method established by Lansbury and co-workers (48). To increase protofibril production, purified αSyn was repeatedly lyophilized and redissolved two to four times. Soluble samples were loaded onto a Superdex 200 gel filtration column on a fast performance liquid chromatography system. The concentration of protofibrils was quantitated by measuring the density of Coomassie-
stained SDS-PAGE using bovine serum albumin as a standard and assuming an average molecular mass of 1,500 kDa. The concentrations of αSyn monomer and dimer were measured using $\varepsilon_{280} = 5,960 \text{ cm}^{-1} \text{ M}^{-1}$ and $\varepsilon_{280} = 11,920 \text{ cm}^{-1} \text{ M}^{-1}$, respectively.

Circular Dichroism (CD) Spectroscopy—Far-UV CD spectra were collected at 22 °C on a JASCO-810 spectropolarimeter using a cuvette with a 0.1-cm optical path length. The data were acquired at 0.2-nm intervals with an average of eight scans. Absorptions from individual buffer were subtracted as backgrounds.

Atomic Force Microscopy—Atomic force microscopy measurements were collected using a Nanoscope IIIa system (Digital Instruments Inc., Santa Barbara, CA) operating in the tapping mode. Tapping mode etched silicon tips with a cantilever length of 125 μm and a characteristic frequency of 300–330 kHz were used for image acquisition. Protein samples were prepared according to the following procedures: 1) dipping the silicon wafers into 10 mM poly(diallyldimethylammonium) chloride (PDDA) solution for 10 min, 2) rinsing the PDDA-treated silicon wafers with Milli-Q water for 2 min, 3) dipping the washed silicon wafers into protein samples for 10 min, and 4) rinsing the protein-bound silicon wafers with Milli-Q water for 2 min. The advantages of using PDDA treatment are as follows. 1) PDDA is uniformly adsorbed on the silicon wafer, and the thickness of the PDDA layer is less than 0.3 nm so it does not have an obvious effect on the atomic force microscopy measurement of binding proteins. 2) The positive-charged PDDA layer allows the negative-charged proteins to be stably adsorbed on the silicon matrix due to the electrostatic attraction.

Assay of Proteasomal Peptidase Activities and Deubiquitination Activity—For the proteasomal peptidase assay, Suc-Leu-Leu-Val-Tyr-Amc, Z-Leu-Leu-Arg-Amc, and Z-Leu-Leu-Glu-Amc fluorogenic peptides were used to monitor the chymotrypsin-, trypsin-, and caspase-like activities of the 26 S proteasome, respectively. Appropriate concentrations of αSyn monomer, dimer, or protofibrils as indicated in the figure legend were incubated with 10 mM 26 S proteasome in buffer A (20 mM Tris, pH 7.2, 50 mM NaCl, 5 mM MgCl2, 1 mM ATP, and 1 mM dithiothreitol) for 5 min. 10-μl mixtures were then added to 200 μl of 50 mM fluorogenic peptide substrates in 20 mM Tris, pH 7.2, and 2 mM β-mercaptoethanol. For the proteasomal deubiquitinase assay, 5 mM 26 S proteasome and 250 mM αSyn protofibrils in buffer A were preincubated for 5 min before adding 500 nM Ub-Amc. Fluorescence of released Amc was continuously monitored using a Synergy HT plate reader (BioTek) with an excitation/emission filter set at 360/40 and 460/40 nm, respectively. Activities were calculated by the slopes of the linear portions of the fluorescence curves. The results represented an average of three independent experiments.

Assaying Proteasomal Degradation of Non-ubiquitinated Proteins and Polyubiquitinated Protein in Vitro—Lys-48-linked tetraubiquitin-conjugated Ubch10 (Ub4-UbcH10) was synthesized according to a recent report (20). Purified 26 S proteasomes were preincubated at 37 °C for 30 min in buffer A with 1 × PA700 to promote the assembly of doubly capped 26 S proteasome. Degradation of αSyn monomer, dimer, and protofibrils; p21cip1; securin; or Ub4-UbcH10 was performed in buffer A except that 125 mM NaCl was present in the buffer for the degradation of p21cip1 and securin. Concentrations of the proteasome and substrates are indicated in the figure legends. To determine the effect of αSyn monomer, dimer, and protofibrils on the degradation of p21cip1, securin, and Ub4-UbcH10, 1-, 5-, 25-, or 125-fold molar excess amounts of αSyn species were preincubated with the 26 S proteasome for 5 min prior to the supplementation of each substrate. At each designated time point, samples were withdrawn, and reactions were stopped by adding 5 × SDS sample buffer. Degradation was evaluated by immunoblotting using appropriate antibodies.

Assaying αSyn Aggregation in Cultured Cells—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 250 ng/ml streptomycin in a 5% CO2, 95% air atmosphere. At 50–60% confluence, the cells were transfected with empty pCDNA 3.1(+) vector (Invitrogen) or the vector containing a myc-αSyn-HA insert using ExGen 500 transfection reagent (Fermentas). 48 h post-transfection, cells in a 10-cm plate were lysed into buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, and Complete protease inhibitor mixtures (Roche Applied Science). Lysates were centrifuged at 200 × g for 15 min at 4 °C. The pellet was collected as P1. The supernatant (S1) was further centrifuged at 16,000 × g for 30 min at 4 °C to further separate the Triton X-100-soluble (S2) and -insoluble (P2) fractions. Both P1 and P2 were washed five times with the lysis buffer and resuspended in SDS sample buffer for immunoblotting using anti-αSyn antibody.

Monitoring the Effect of αSyn Overexpression on Degradation of Endogenous Proteins in Cultured HEK293 Cells—48 h post-transfection of αSyn, cells in 35-mm plates were treated with 80 μg/ml cycloheximide to inhibit protein synthesis. To determine IκBα degradation, the cells were treated with 20 ng/ml tumor necrosis factor α to induce its rapid degradation. The cells were harvested at designated time points and lysed with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 10 mM N-ethylmaleimide, 0.2 mM sodium orthovanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and Complete protease inhibitor mixtures). The degradation of endogenous p21cip1, Ubch10, and IκBα was evaluated by immunoblotting using appropriate antibodies.

Determining αSyn Protofibrils and PA700 Interaction—To assay αSyn protofibril binding to PA700, 100 μg of PA700 was preincubated with 200 μg of αSyn protofibrils at room temperature for 30 min and then centrifuged at 16,000 × g for 5 min to pellet any insoluble aggregates prior to loading into a Superose 6 (16/60) gel filtration column equilibrated in 20 mM Tris, pH 7.2, 50 mM NaCl, and 2 mM β-mercaptoethanol. Protein separation was conducted by a fast performance liquid chromatography system at a flow rate of 0.75 ml/min and fraction size of 800 μl. 20 μl of each fraction was applied to SDS-PAGE followed by immunoblotting assays.

Size Exclusion Spin Column Assay—Micro Bio-Spin P-30 chromatography columns with an exclusion limit of 40 kDa (Bio-Rad) or homemade Sephadex G-75 spin columns with an exclusion limit of 80 kDa (matrix from Sigma and empty columns from Bio-Rad) were used to determine the interaction
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between αSyn species and the proteins of interest. Briefly, different molar ratios of αSyn monomer, dimer, or protodimers were incubated with target proteins in 20 mM Tris, pH 7.2, and 1 mM dithiothreitol at room temperature for 10 min. 60 μl of mixtures was then loaded into spin columns, and the centrifugation steps were followed according to the manufacturer’s instructions for Bio-Spin P-30 columns. The flow-throughs were collected and mixed with 5× SDS sample buffer followed by immunoblotting using appropriate antibodies. Comparable results were obtained in determining Ub, binding to αSyn protodimers when using either Bio-Spin P-30 or homemade Sephadex G-75 columns.

8-Anilino-1-naphthalenesulfonic Acid Ammonium Salt (ANS) Fluorescence—5 μM ANS was mixed with 20 μM αSyn monomer, 10 μM αSyn dimer, or 480 nM αSyn protodimers in 20 mM Tris buffer, pH 7.2. Fluorescence measurements were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path. Samples were excited at 350 nm, and emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) using a cuvette with a 0.5-cm excitation optical path.
αSyn Protofibrils Inhibit 26 S Proteasomal Degradation

Despite that the 26 S proteasome was highly active to hydrolyze the short fluorogenic peptide, purified 26 S proteasomes could not efficiently degrade αSyn monomer, dimer, or αSyn protofibrils (Fig. 2C). Because purified 26 S proteasomes efficiently degraded two other in vitro unstructured proteins, p21cip1 and securin (Fig. 2, D and E), and a polyubiquitinated protein (see Fig. 5B), the degradation resistance for the three αSyn species in Fig. 2C is not due to the inactivity of the 26 S proteasome per se. Accordingly, we concluded that αSyn monomer, dimer, and protofibrils are not efficiently degraded by purified intact 26 proteasomes.

αSyn Protofibrils Inhibit 26 S Proteasomal Degradation of Unstructured Proteins—Recently we have demonstrated that the 26 S proteasome degrades some unstructured proteins in an ATP hydrolysis-independent manner (20). To examine whether αSyn monomer, dimer, or protofibrils affect the 26 S proteasomal degradation of unstructured proteins, we chose p21cip1 and securin as two model substrates. Neither αSyn monomer nor dimer had an obvious effect on the 26 S proteasomal degradation of these two unstructured proteins even when they were present at a level of 125-fold molar excess over the 26 S proteasome (Fig. 4A). Strikingly, αSyn protofibrils markedly inhibited the 26 S proteasome-mediated degradation of both p21cip1 and securin at 25- and 5-fold molar excesses to the 26 S proteasome, respectively (Fig. 4A). These results indicate that ubiquitin-independent 26 S proteasomal degradation of unstructured proteins is impaired selectively by αSyn protofibrils but not by αSyn monomer or dimer.

Next we hypothesized that overexpression of αSyn in cells could impair the degradation of endogenous p21cip1, which has been found previously to be degraded by the proteasome with no need of polyubiquitination in cells (50–52). It has been reported that overexpression of αSyn in cells causes αSyn aggregation (53). Consistently, we found that overexpression of αSyn in HEK293 cells led to accumulation of Triton X-100-insoluble αSyn aggregates that were pelleted from both 200 × g (P1) and 16,000 × g (P2) centrifugations, but these Triton X-100-insoluble aggregates were SDS-soluble as revealed by the fact that they were disrupted into the monomeric form in SDS-PAGE (Fig. 4B). Next we monitored endogenous p21cip1 degradation by a cycloheximide-directed chase experiment. In mock-transfected cells, p21cip1 was degraded with a half-life of about 1.94 h consistent with previous reports (54, 55). Remarkably degradation of p21cip1 in αSyn-overexpressing HEK293 cells was impaired, consistent with our in vitro observations.

αSyn Monomer, Dimer, and Protofibrils Do Not Inhibit the Peptidase Activities of the 26 S Proteasome—Fluorogenic peptides have been developed to monitor individual hydrolytic activities of the proteasome. Next we examined whether the peptidase activities of the 26 S proteasome are directly affected by αSyn monomer, dimer, or protofibrils. In these assays, appropriate concentrations of tested αSyn species were preincubated with purified 26 S proteasome prior to being mixed with short peptide substrates. We found that none of the three αSyn species impaired the activity of 26 S proteasome to degrade these short peptide substrates (Fig. 3, A–C). Interestingly, concerted slight stimulations of all three peptidase activities were observed when high concentrations of αSyn monomer or dimer even lower concentrations of αSyn protofibrils co-existed. These results demonstrate that αSyn monomer, dimer, and protofibrils do not block the translocation of short peptides into the degradation chamber of the 26 S proteasome nor do they directly compete with short peptides for any of the three catalytic activities.

Syn Protofibrils Inhibit Ubiquitin-independent Proteasomal Degradation of Unstructured Proteins—Recently we have demonstrated that the 26 S proteasome degrades some unstructured proteins in an ATP hydrolysis-independent manner (20). To examine whether αSyn monomer, dimer, or protofibrils affect the 26 S proteasomal degradation of unstructured proteins, we chose p21cip1 and securin as two model substrates. Neither αSyn monomer nor dimer had an obvious effect on the 26 S proteasomal degradation of these two unstructured proteins even when they were present at a level of 125-fold molar excess over the 26 S proteasome (Fig. 4A). Strikingly, αSyn protofibrils markedly inhibited the 26 S proteasome-mediated degradation of both p21cip1 and securin at 25- and 5-fold molar excesses to the 26 S proteasome, respectively (Fig. 4A). These results indicate that ubiquitin-independent 26 S proteasomal degradation of unstructured proteins is impaired selectively by αSyn protofibrils but not by αSyn monomer or dimer.

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**FIGURE 4.** αSyn protofibrils impair the 26 S proteasomal degradation of unstructured proteins. A, αSyn protofibrils, but not monomer and dimer, significantly inhibit the 26 S proteasomal degradation of unstructured proteins in vitro. Reactions contained 1 μM p21cip1 or 250 nM securin and 5 nM 26 S proteasome in the absence or presence of different molar ratios of αSyn monomer, dimer, or protofibrils. Reactions were stopped by adding SDS sample buffer after 15 min of incubation and assayed by immunoblotting. The values of densitometric quantitation of the remaining p21cip1 or securin in each reaction are shown under the panels. B, overexpression of αSyn in cells produces αSyn aggregates. HEK293 cells overexpressing αSyn were lysed into buffer containing 1% Triton X-100 (TX) and subjected to fractionation by centrifugation as shown in the scheme. αSyn in different fractions was dissolved in SDS sample buffer and detected by immunoblotting against anti-αSyn antibody. C, overexpression of αSyn in cells inhibits endogenous p21cip1 degradation. Degradation of p21cip1 was assayed in mock-transfected and αSyn-overexpressing HEK293 cells (upper panel). Overexpression of αSyn was confirmed by immunoblotting (middle panel). The loading control was evaluated by immunoblotting against β-actin (lower panel). The right panel shows the quantitated data for p21cip1 degradation from three independent experiments. Error bars represent the S.D.

cells was inhibited (Fig. 4C). This result suggests that overexpression of αSyn impairs ubiquitin-independent degradation in cells.

**αSyn Protofibrils Inhibit Proteasomal Degradation of Polyubiquitinated Folded Proteins**—In contrast to unstructured proteins, degradation of folded proteins requires polyubiquitination and coordinated actions including substrate engagement, substrate unfolding, substrate deubiquitination, substrate translocation, and ATP hydrolysis (20). To evaluate whether αSyn monomer, dimer, or protofibrils have an effect on the 26 S proteasome-mediated degradation of polyubiquitinated proteins, we chose Ubch10 as a model substrate. Ubch10 is a *bona fide* proteasomal substrate because the ubiquitination-dependent degradation of Ubch10 is critical for triggering the G1-S phase transition in cell cycle progression (56). We conjugated Lys-48-linked Ub4 chain to Ubch10 using immunoprecipitated Xenopus anaphase-promoting complex/cyclosome as the E3 Ub ligase (57). After the polyubiquitination reaction, about 70% of Ubch10 was conjugated with a Ub4 chain, whereas some Ubch10 remained non-ubiquitinated (Fig. 5A). Ub4-Ubch10 was less sensitive than Ubch10 toward an anti-Ubch10 antibody as revealed by immunoblotting (Fig. 5B) presumably because the epitope of Ubch10 was partially masked by the conjugated Ub4 chain. We have showed previously that Ubch10 itself is not a substrate of the 26 S proteasome, whereas Ub4-Ubch10 is rapidly degraded (20). Degradation of Ub4-Ubch10 was judged by comparing the reactions without and with a proteasome inhibitor (Fig. 5B, lane 2 versus lane 3). Non-ubiquitinated Ubch10 accumulated when the 26 S proteasome was inhibited by adding epoxomicin because deubiquitination was still allowed (lane 3). In contrast, no obvious accumulation of Ubch10 was present in the reaction without epoxomicin (lane 2), indicating degradation. Notably, some Ub4-Ubch10 still remained when the proteasome was inhibited by epoxomicin (Fig. 5B, lane 3); this is consistent with our earlier report that proteasome inhibition can partially block deubiquitination (20).

We next examined the effects of αSyn monomer, dimer, or protofibrils on the 26 S proteasomal degradation of Ub4-Ubch10. Most strikingly, Ub4-Ubch10 degradation was completely abolished when αSyn protofibrils were added at 5-fold or higher molar excess relative to the 26 S proteasome (Fig. 5B, lanes 10–12). In contrast, αSyn monomer and dimer did not obviously inhibit Ub4-Ubch10 degradation when present at 5-fold molar excess to the 26 S proteasome (Fig. 5B, lanes 4 and 7). However, partial accumulation of non-ubiquitinated Ubch10 was observed when αSyn monomer or dimer was added at 25-fold or higher molar excess to the 26 S proteasome (Fig. 5B, lanes 5, 6, 8, and 9), and slight accumulation of Ub4-Ubch10 was observed when the concentration of dimer was 125-fold more than the 26 S proteasome (Fig. 5B, lane 9). Obviously, when compared with the inhibited degradation in the presence of epoxomicin (Fig. 5B, lane 3), 125-fold more αSyn monomer or dimer was not able to completely block the proteasomal degradation of Ub4-Ubch10 (Fig. 5B, lanes 6 and 9). These results demonstrate that αSyn monomer and dimer have a mild inhibitory effect on 26 S proteasomal degradation when they are at 25-fold or higher molar excess to the 26 S proteasome, whereas αSyn protofibrils exhibit a dramatic inhibitory effect on proteasomal degradation of polyu-
Syn protofibrils inhibit the 26 S proteasomal degradation of polyubiquitinated proteins. A, Coomassie-stained SDS-PAGE of 1 μg of Ub$_4$-UbcH10. B, αSyn monomer, dimer, and protofibrils inhibit Ub$_4$-UbcH10 degradation. Reactions contained 100 nM Ub$_4$-UbcH10 and 15 nM 26 S proteasome in the absence or presence of different molar ratios of αSyn monomer, dimer, or protofibrils. The lane denoted epox. indicates that the 26 S proteasome was preincubated with 100 μM epoxomicin, a potent proteasome-specific inhibitor. Reactions were stopped after 15 min of incubation, and degradations were assayed by immunoblotting using an anti-UbcH10 antibody. The values of densitometric quantitation of the remaining Ub$_4$-UbcH10 and UbcH10 in each reaction are shown in the lower panel. C, αSyn protofibrils do not inhibit the deubiquitinating activity of the 26 S proteasome. Deubiquitination of 500 nM Ub-Amc by 5 nM 26 S proteasome was assayed in the absence (○) and in the presence of 250 nM αSyn protofibrils (▼). c.p.s., counts/s. D and E, overexpression of αSyn in HEK293 cells inhibits endogenous Ubch10 and tumor necrosis factor α (TNFα)-induced IκBα degradation. Degradations were assayed analogously to those in Fig. 4C. The right panels show the quantitated data for Ubch10 or IκBα degradation from three independent experiments. Error bars represent the S.D.

Protein deubiquitination is tightly coupled with degradation, and inhibition of the proteasome-residing deubiquitination enzymes blocks the degradation of polyubiquitinated proteins (20, 58, 59). We next asked whether the deubiquitination activity of the 26 S proteasome per se is inhibited by αSyn protofibrils; this could directly contribute to the observed inhibitory effect of αSyn protofibrils on degradation of Ub$_4$-UbcH10 as shown in Fig. 5B. Surprisingly, we found that purified 26 S proteasomes deubiquitinated Ub-Amc equally well in the absence and in the presence of 50-fold excess amounts of αSyn protofibrils (Fig. 5C). These results indicate that the overall deubiquitination activity of the 26 S proteasome is not inhibited by αSyn protofibrils. Certainly we cannot exclude the possibility that deubiquitination of polyubiquitinated proteins and Ub-Amc might be catalyzed differently by the 26 S proteasome.

Next we examined whether ubiquitin-dependent proteasomal degradation is affected by overexpressing αSyn in cells. To test this, we chose UbcH10 and IκBα as substrates. In response to tumor necrosis factor α stimulation, IκBα is phosphorylated and then ubiquitinated for rapid degradation (60). We found that degradation of both UbcH10 and IκBα was significantly inhibited in αSyn-overexpressing HEK293 cells as compared with mock-transfected cells (Fig. 5, D and E). Thus, αSyn overexpression impairs ubiquitin-dependent proteasomal degradation in vivo.
αSyn Prototubifics Directly Bind the 26 S Proteasome—αSyn was reported to bind the S6’ (also called Rpt5) ATPase subunit of the 26 S proteasome (34, 61); therefore, it is interesting to examine whether αSyn monomer, dimer, or prototubifics bind the intact 26 S proteasome complex. To do this, we used gel filtration to monitor whether PA700 co-migrates with αSyn monomer, dimer, or prototubifics. We chose to use PA700 instead of the 26 S proteasome because the 2.5-MDa 26 S proteasome had a similar retention time with αSyn prototubifics, but the 700-kDa PA700 and αSyn prototubifics had different retention times on a 120-ml Superpose 6 column. As shown in Fig. 6, PA700 co-migrated with αSyn prototubifics and eluted about 10 fractions earlier than PA700 alone, indicating a direct interaction of αSyn prototubifics with the 26 S proteasome. Although αSyn was found to directly interact with the S6’ ATPase subunit of the 26 S proteasome (34, 61), we did not detect co-migration of αSyn monomer or dimer with the PA700 complex by using a similar gel filtration assay even when αSyn monomer or dimer was present at 10-fold molar excess to PA700 (data not shown). Presumably it reflects the difference between the PA700 complex and the individual S6’ subunit. Nevertheless these results suggest that αSyn prototubifics have a higher binding affinity to PA700 or the 26 S proteasome than does αSyn monomer or dimer.

αSyn Prototubifics Directly Bind Some Proteasomal Substrates—During αSyn fibrillation, formed prototubifics associate with αSyn monomer to develop fibrils. Therefore, it would be of interest to examine whether αSyn prototubifics bind other proteins as well. To do this, we used a size exclusion spin column assay to examine whether αSyn prototubifics bind proteasomal substrates. αSyn monomer (14 kDa) has an apparent molecular mass of about 50 kDa on a gel filtration column because of its unfolded structure. We detected that αSyn monomer was excluded by the matrix of the P-30 spin column, which has an exclusion limit of 40 kDa (data not shown). Similarly αSyn dimer and prototubifics were also excluded by the P-30 spin column (data not shown). In contrast, p21cip1 or securin was trapped inside the column as evidenced by the fact that they were not detected in the flow-throughs after centrifugation (Fig. 7A and data not shown). However, p21cip1 was co-eluted with αSyn prototubifics in the flow-through when incubated with an equal molar ratio of αSyn prototubifics but not with 40-fold molar excess of αSyn monomer or 20-fold molar excess of αSyn dimer (Fig. 7A), demonstrating that p21cip1 binds αSyn prototubifics. Using this size exclusion spin column assay and a gel filtration assay, we also detected αSyn prototubific binding to securin (supplemental Fig. S1A and data not shown).

FIGURE 6. αSyn prototubifics interact with PA700, the regulatory complex of the 26 S proteasome. A 10 μg of PA700 was preincubated with 200 μg of αSyn prototubifics at room temperature for 30 min before injection into a Superose 6 (16/60) gel filtration column. Fractions were immunoblotted with anti-p31, a subunit of PA700, or anti-αSyn antibody. PA700 or prototubifics alone were run as a control under the same condition.

FIGURE 7. αSyn prototubifics interact with substrates of the 26 S proteasome. A, αSyn prototubifics bind p21cip1 and Ub2. 60 nm p21cip1 or Ub2 was incubated with 2.4 μM αSyn monomer, 1.2 μM dimer, or 60 nm prototubifics at room temperature for 10 min before loading to Micro Bio-Spin P30 chromatography columns. After centrifugation, flow-throughs were subjected to immunoblotting with an anti-p21cip1 or anti-ubiquitin antibody. B, Ub does not interact with αSyn prototubifics. 240 nm His6-Ub or 60 nm Ub2 was incubated with different molar ratios of αSyn prototubifics at room temperature for 10 min before loading to Micro Bio-Spin P30 chromatography columns. After centrifugation, flow-throughs were subjected to immunoblotting with an anti-His6, or anti-ubiquitin antibody. C, salt facilitates the binding of Ub2 to αSyn prototubifics. 60 nm Ub2 was incubated with 60 nm αSyn prototubifics in the absence or presence of 0.5 M (NH4)2SO4 prior to being applied to the Sephadex G-75 spin columns. Values in C are the means ± S.D. of four independent experiments. D, αSyn prototubifics, but not monomer and dimer, have exposed hydrophobic surfaces. ANS fluorescence of 20 μM αSyn monomer (short dashed line), 10 μM αSyn dimer (dotted line), or 480 nm αSyn prototubifics (solid line), or 480 nm αSyn prototubifics with 0.5 M (NH4)2SO4 (long dashed line). c.p.s., counts/s.
(Fig. 7C) whereas only 5% of Ub₄ bound to αSyn protofibrils (Fig. 7B) when Ub₄ or Ub₅ was incubated at a 1:1 molar ratio with αSyn protofibrils. Interaction between polyUb chains and αSyn protofibrils was further confirmed by gel filtration (supplemental Fig. S1B). Interestingly, binding of Ub₅ to αSyn protofibrils was markedly increased when 0.5 M (NH₄)₂SO₄ was added (Fig. 7C), suggesting that binding of polyUb chains to αSyn protofibrils is mediated by a hydrophobic-hydrophobic interaction. Indeed as determined by an ANS binding assay, αSyn protofibrils gained an exposed hydrophobic surface(s) when compared with αSyn monomer and dimer (Fig. 7D). Consistent with its increased capacity to bind Ub₅ in the presence of 0.5 M (NH₄)₂SO₄, ANS fluorescence of αSyn protofibrils increased 4-fold when 0.5 M (NH₄)₂SO₄ was added in the mixture (Fig. 7D). Taken together, these results indicate that αSyn protofibrils bind both unstructured and polyubiquitinated substrates of the 26 S proteasome likely through a hydrophobic-hydrophobic interaction.

**DISCUSSION**

**Inhibition of Proteasomal Degradation by αSyn Protofibrils**—The impaired proteasomal function is believed to be one of the main reasons for PD pathogenesis (14). In this report, we examined the effects of three soluble αSyn species (αSyn monomer, dimer, and protofibrils) on the protein degradation activity of the 26 S proteasome. αSyn has been reported to be a substrate of both the proteasome (31, 32) and the lysosome (62, 63). Interestingly, we found that purified intact 26 S proteasomes degraded αSyn monomer and dimer at much slower rates as compared with unstructured p21cip1 and securin (Fig. 2, C and E). Mechanistically, it is unclear why unstructured proteins have different access to the catalytic sites of the 26 S proteasome. The 26 S proteasome efficiently degrades some unstructured proteins without the requirement of ATP hydrolysis (20), indicating that translocation of these unstructured proteins (with no need for further unfolding) into the degradation chamber does not consume energy from ATP hydrolysis. However, the degradation distinctions between αSyn and p21cip1 or securin suggest that the 26 S proteasome possesses a substrate recognition step for screening unstructured protein substrates.

The 26 S proteasome has three distinct peptidase activities located in the central β channel of the 20 S proteasome. We did not detect any inhibitory effect of αSyn monomer, dimer, and protofibrils on the peptidase activities of the 26 S proteasome using short fluorogenic peptides as substrates (Fig. 3), demonstrating that they do not block the translocation of small peptides nor directly compete for the catalytic sites. Because αSyn is a substrate of the 20 S proteasome (22, 32), the inhibitory effect of αSyn on the 20 S proteasome found in two earlier studies (34, 38) using short fluorogenic peptides as substrates could simply be due to their competition for the catalytic sites. In striking contrast, αSyn protofibrils, but not the higher concentrations of monomer and dimer we examined, inhibited 26 S proteasomal degradation of p21cip1 and securin (Fig. 4A). This inhibition cannot be due to a direct competition toward the catalytic sites because αSyn protofibrils with an average size of 2–4 nm cannot pass through the narrow substrate translocation channel of the proteasome (see the discussion below). Furthermore we found that αSyn protofibrils had a marked inhibitory effect on the degradation of Ub₅-UbcH10 that occurred at the deubiquitination level (Fig. 5B). In the presence of high concentrations of αSyn monomer and dimer (at 25-fold or higher molar excess to the proteasome), degradation of Ub₅-UbcH10 by the 26 S proteasome was mildly inhibited as observed by the accumulation of UbcH10 (Fig. 5B). This result suggests that the inhibitory effect of high concentrations of αSyn monomer and dimer comes from impaired substrate translocation and/or unfolding. Consistent with our in vitro studies, we found that both ubiquitin-independent and ubiquitin-dependent proteasomal degradation were impaired in cells when αSyn was overexpressed (Figs. 4C and 5, D and E). Although the formation of αSyn aggregates was detected in αSyn-overexpressing cells (Fig. 4B), it is unknown whether the inhibited proteasomal degradation in αSyn-overexpressing cells results directly from the aggregated αSyn. A previous report showed that αSyn overexpression in PC12 cells has no effect on proteasome-mediated basal turnover of IκBα (64). This discrepancy is currently unclear, but we monitored the signal-induced rapid IκBα degradation.

Interestingly, αSyn protofibrils, but not monomer and dimer, had strong binding affinity to both the 26 S proteasome and proteasomal substrates (unstructured proteins and polyubiquitinated proteins) (Figs. 6 and 7). These results might provide mechanistic explanations for αSyn protofibrils to inhibit proteasomal degradation: by binding on PA700, presumably on the ATPase ring because it has a chaperone-like activity to bind denatured proteins with exposed hydrophobic surface(s) like αSyn protofibrils (Fig. 7), the large oligomeric αSyn protofibrils could block the narrow substrate translocation channel of the proteasome (the narrowest part is 13 Å), thus inhibiting degradation by impeding entry of protein substrates (but not short peptides) into the proteolytic chamber (Fig. 8). This scenario is supported by the evidence that the degradation of p21cip1 was still completely inhibited when increasing the concentration of p21cip1 while keeping αSyn protofibrils at 25-fold molar excess to the proteasome (data not shown). On the other hand, by
binding proteasomal substrates, αSyn protofibrils could sequester substrates from the proteasome, therefore blocking degradation (Fig. 8). This mechanism could, at least partially, account for the observed inhibitory effect for αSyn protofibrils on the degradation of Ub4-UbcH10 at the deubiquitination level. Because the deubiquitination activity of the 26 S proteasome per se was not impaired by αSyn protofibrils as revealed by the Ub-Amc deubiquitination assay (Fig. 5C), the inhibited Ub4-UbcH10 deubiquitination, as well as degradation, might come from substrate sequestration by αSyn protofibrils. Certainly these two possibilities for αSyn protofibrils to inhibit proteasomal degradation are not mutually excluded, but a certain model is favored depending on the affinity between protofibrils binding to the 26 S proteasome and binding to the proteasomal substrates.

A Possible Generality for Protofibrils in the Pathogenesis of Neurodegenerative Diseases by Inhibiting Proteasomal Activity—One of the potential cytotoxic mechanisms for protofibrils is that they form a pore-like structure that enables them to penetrate the cell membrane, thus causing cell leaking (48, 65, 66). In the current study, our data suggest a novel potential mechanism for αSyn protofibrils to exert their cytotoxic effect: through interacting with other intracellular proteins αSyn protofibrils might disrupt important cellular pathways including the ubiquitin–proteasome pathway. Even worse, the formation of αSyn protofibrils and the impairment of proteasome activity might be mutually exacerbated processes. Loss of proteasomal activity could cause the accumulation of αSyn in cells (31, 32, 67), which may eventually result in αSyn aggregation and the production of protofibrils. Conversely, accumulating αSyn protofibrils impairs the proteasomal function by directly binding to the proteasome or sequestering proteasomal substrates as found in this study. Interestingly, oligomeric forms of other disease-related proteins have been found to impair proteasomal activity. For example, soluble oligomeric amyloid β peptide (Aβ), but not monomer, inhibits proteasomal activity in vitro (68). In 3XtG-AD mice brains, the impaired proteasomal function occurs in parallel with the appearance of soluble amyloid β peptide oligomers, and the impairment is relieved as soluble amyloid β peptide oligomers are converted into insoluble aggregates (68). Inhibition of proteasomal activities by disease-associated prion protein (PrPsc) is abolished by anti-oligomer-specific antibody consistent with the notion that oligomeric species mediate proteasome dysfunction (69).

Therefore, we propose that a vicious cycle between the impaired proteasomal activity and the promoted protein aggregation (production of high molecular weight oligomers) might be a common feature involved in the pathogenesis of neurodegenerative diseases. Actually soluble oligomers formed from different amyloidogenic proteins, including αSyn, amyloid β peptide, prion protein, and polyglutamine, share a common structure regardless of the protein sequences (40). Therapeutically small molecules and antibodies that inhibit oligomer formation or eliminate formed oligomers might be useful therapeutical reagents for these neurodegenerative diseases.

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