A Precipitating Role for Truncated α-Synuclein and the Proteasome in α-Synuclein Aggregation

IMPLICATIONS FOR PATHOGENESIS OF PARKINSON DISEASE*

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Parkinson disease and other α-synucleinopathies are characterized by the deposition of intraneuronal α-synuclein (αSyn) inclusions. A significant fraction (about 15%) of αSyn in these pathological structures are truncated forms that have a much higher propensity than the full-length αSyn to form aggregates in vitro. However, little is known about the role of truncated αSyn species in pathogenesis or the means by which they are generated. Here, we have provided an in vitro mechanistic study demonstrating that truncated αSyns induce rapid aggregation of full-length protein at substoichiometric ratios. Co-overexpression of truncated αSyn with full-length protein increases cell vulnerability to oxidative stress in dopaminergic SH-SY5Y cells. These results suggest a precipitating role for truncated αSyn in the pathogenesis of diseases involving αSyn aggregation. In this regard, the A53T mutation found in some cases of familial Parkinson disease exacerbates the accumulation of insoluble αSyns that correlates with the onset of pathology in transgenic mice expressing human αSyn-A53T mutant. The caspase-like activity of the 20 S proteasome produces truncated fragments similar to those found in patients and animal models from degradation of unstructured αSyn. We propose a model in which incomplete degradation of αSyn, especially under overloaded proteasome capacity, produces highly amyloidogenic fragments that rapidly induce the aggregation of full-length protein. These aggregates in turn reduce proteasome activity, leading to further accumulation of fragmented and full-length αSyns, creating a vicious cycle of cytotoxicity. This model has parallels in other neurodegenerative diseases, such as Huntington disease, where coaggregation of poly(Q) fragments with full-length protein has been observed.

Formation of intraneuronal α-synuclein (αSyn)1 aggregates is a characteristic of several neurodegenerative diseases, including Parkinson disease (PD), termed α-synucleinopathies (1). For example, αSyn is the major component of Lewy bodies (LB), a pathologic marker for PD (2). A direct genetic connection between αSyn and PD has been established by evidence that three missense mutations (A30P (3), E46K (4), and A53T (5)) in αSyn underlie rare early onset hereditary PD. As the majority of the PD cases are sporadic, elevated oxidative and metabolic stresses are thought to contribute to pathogenesis (6). Impaired mitochondrial complex I elevates concentration of reactive oxygen species, which can accelerate αSyn aggregation (7). An impaired ubiquitin-proteasome degradation system may also play a role in initiating PD pathogenesis (6). Increased misfolding of proteins due to oxidative damage could overwhelm and inhibit the proteasome degradation machinery. Inefficient degradation may thus simply cause the accumulation of αSyn and, by elevating αSyn concentration, increase the rate of αSyn aggregation. Such a scenario is supported by direct genetic evidence from a familial PD with triplicate αSyn genes (8), animal models (9, 10), and a yeast model (11) in which higher concentrations of αSyn are sufficient to induce pathogenesis.

Analysis of Lewy bodies from patients revealed that they contain multiple proteins, with αSyn being predominant. Different forms of modified αSyn have been identified in these pathologic samples, including phosphorylated, nitrated, and mono-, di-, or tri-ubiquitinated αSyn (12–14). Notably, about 15% of the αSyn in these aggregates is truncated (15, 16). The tendency of truncated αSyn species to rapidly aggregate (17, 18) suggests that they may have played a role in inducing Lewy body formation. Here, we have examined the precipitating role for truncated αSyn in aggregation and cytotoxicity and have investigated the means by which they are produced. The data suggest a precipitating role of truncated αSyns in PD pathogenesis and demonstrate that the proteasome can produce truncated species via the degradation of αSyn not bound to membranes.

EXPERIMENTAL PROCEDURES

Antibodies—SNL-4 and SNL-1 are rabbit antibodies raised against synthetic peptides corresponding to amino acids 2–12 and 104–119 in αSyn, respectively. Syn204 and Syn211 are mouse monoclonal antibodies specific for human αSyn (19). The αSyn mouse monoclonal antibody from BD Transduction Laboratories recognizes an epitope between 83 and 100 amino acids determined using C-terminal-truncated αSyn proteins. Mouse anti-β-actin monoclonal antibody was from Chemicon. Mouse anti-c-Myc monoclonal antibody was from Santa Cruz Biotechnology.
Fractionation and Characterization of Human and Mouse Brain Tissues—Sample sources were: cingulate cortex from three controls (ages 73, 76, and 83 years old) and three dementia with Lewy bodies (DLB) patients (ages 77, 79, and 89 years old); substantia nigra from two Alzheimer disease (AD) patients (ages 85 and 86) as controls, and one PD patient (age 75); and cortex and brain stem from different ages of transgenic mice expressing human α-Syn or human α-Syn A53T mutant. Samples were sequentially fractionated as follows: ground tissue was homogenized on ice in 10 ml of buffer A (20 mM Tris-HCl, pH 7.6 at 4 °C, 20 mM NaCl) with complete Escherichia coli proteins were removed by centrifugation, and the supernatant was then isolated on a Superdex 200 size exclusion column (Amersham Biosciences). To assess whether hybrid protofibrillar population in the supernatant was then purified on a hydrophobic interaction (butyl-Sepharose) column and an anion exchange (DEAE) column. Protein purity in relevant fractions was 95% as determined (Amersham Biosciences). A panel of α-Syn antibodies was used to identify both full-length and truncated α-Syn species.

α-Syn Aggregation Assay—α-Syn proteins were expressed in Escherichia coli BL21(DE3). Cells were lysed in buffer D (20 mM Tris-HCl, pH 7.6 at 4 °C, 20 mM NaCl) with complete protease inhibitor mixture. After centrifugation, the supernatant was boiled at 100 °C for 10 min. Aggregated bacterial proteins were removed by centrifugation, and α-Syn in the supernatant was purified on a hydrophobic interaction (butyl-Sepharose) column and an anion exchange (DEAE) column. Protein purity in relevant fractions was >95% as determined by Coomassie blue-stained SDS-PAGE. For aggregation assay, proteins were equilibrated in buffer E (20 mM phosphate, pH 7.1 at 37 °C, 20 mM NaCl, 5 mM EDTA) with complete protease inhibitor mixture (Roche Diagnostics)) for each gram of tissue. The homogenates were centrifuged at 100,000 × g for 30 min at 4 °C. The supernatants were the high salt (HS)-soluble fraction. The HS pellets were rinsed twice with buffer A and then sonicated within 5 ml of buffer B (20 mM Tris-HCl, pH 7.6 at 4 °C, 4% SDS with complete protease inhibitor mixture) for each gram of tissue. Samples were centrifuged at 100,000 × g for 30 min at 25 °C. The supernatants were the SDS-soluble fraction. The SDS pellets were rinsed twice in buffer B at room temperature and extracted by sonication in 2.5 ml of buffer C (20 mM Tris-HCl, pH 7.6 at 4 °C, 8 M urea, and 4% SDS) for each gram of tissue. After 30 min of centrifugation at 100,000 × g at 25 °C, the supernatants were the urea-soluble fraction. 3 μl of HS-soluble, 6 μl of SDS-soluble, and 12 μl of urea-soluble fractions were diluted to 40 μl with 1× SDS sample buffer, separated by electrophoresis in 12.5% SDS-polyacrylamide gels, and transferred to nitrocellulose (Millipore). A panel of α-Syn antibodies was used to identify both full-length and truncated α-Syn species.

In Vitro Cell Death Assay for Cytotoxicity of Proteofibrils—SH-SY5Y cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained in 5% CO2 at 37 °C. Cells were plated at a density of about 10,000 cells/well on a 96-well culture plate (Greiner Bio-one) in 100 μl of fresh medium. After overnight incubation, the medium was exchanged with 100 μl of Dulbecco’s modified Eagle’s medium/10% fetal bovine serum without phenol red and supplied with 50 mM purified proteofibrils. After 4 h of incubation, cell death was assayed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction kit (Sigma) according to the manufacturer’s instructions.

Co-overexpression of Full-length and Truncated α-Syns in Cell Culture Models—Full-length α-Syn, α-Syn110, and α-Syn120 cDNA were subcloned into the mammalian expression vector pcDNA3.1(+)(Invitrogen). SH-SY5Y cells were split into 12-well plates at 50% confluence. After overnight incubation, cells were transfected with 1.0 μg/well total DNA using a 4:1 FuGENE (Roche Applied Science) to DNA ratio. Mixed DNA samples were as follows: (1) 1 μg/μl pcDNA3.1(+) vector, (2) 0.5 μg/μl α-Syn + 0.5 μg/μl pcDNA3.1(+) vector, (3) 0.35 μg/μl α-Syn + 0.65 μg/μl α-Syn110, and (4) 0.35 μg/μl α-Syn + 0.5 μg/μl α-Syn120 + 0.15 μg/μl pcDNA3.1(+) vector. 16 h later, cells were transfected a second time to evaluate transgene expression. 48 h after the first transfection, cells were challenged with 0.75 mM H2O2 for a 45-min incubation, H2O2 was removed, and cells were allowed to recover overnight in fresh medium. For Western blot analysis of protein expression, cells were lysed with 0.3 ml/well radioligand precipitation assay buffer (1× phosphate-buffered saline containing 10% fetal bovine serum) without phenol red and supplied with 50 nM/well purified proteofibrils. After 4 h of incubation, cell death was evaluated by counting ethidium homodimer-1-positive cells against total cells.

In Vitro 20 S Proteasomal Degradation of α-Syn—20 S proteasome was purified from bovine red blood cells (21). Proteasomal degradation was carried out in buffer F (20 mM Tris-HCl, pH 7.1 at 37 °C, 20 mM NaCl, 1 mM EDTA). 200-μl reactions were set up at 37 °C containing 20 nM 20 S proteasome and either 0.4 or 4 μM α-Syn. At each time point, 40- or 4-μl samples were withdrawn, and reactions were stopped by the addition of SDS sample buffer. Degradation was assayed on 12.5% SDS-polyacrylamide gel by direct Coomassie staining or Western blotting analysis. Site-specific inhibitors, as stated in the figure legends, were used to identify which activity is responsible for the production of truncated α-Syn. To identify the fragments, a 500-μl reaction containing 20 nM 20 S proteasome and 100 μM α-Syn-His6 in buffer F was incubated for 30 min at 37 °C. 20 S proteasome was removed with a YM-100 (Millipore) spin column, and the flow-through containing the remaining α-Syn-His and degradation fragments was incubated with 25 μl of Ni-NTA-agarose and rocked at 4 °C for 1 h. Beads were washed by centrifugation with the supernatant containing non-His-bound fraction. Beads were washed three times with buffer F plus 0.5 mM NaCl, and the His-bound fraction was eluted with 0.2 M imidazole. Both frac-
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We first identified truncated α-Syn species in both PD and dementia with Lewy bodies (DLB) patients using a spectrum of α-Syn antibodies. Brain samples from PD and DLB patients and controls (age-matched normal brains or AD patients) were sequentially extracted with a high salt buffer (HS), a 4% SDS buffer, and an urea buffer to obtain soluble fractions, respectively, by sequential extraction with a high salt buffer (buffer A), a 4% SDS buffer (buffer B), and an 8 M urea buffer (buffer C). Two C-terminal-truncated fragments and one internal fragment of α-Syn were identified using a spectrum of α-Syn-specific antibodies recognizing different epitopes.

RESULTS

Two C-terminal-truncated α-Syns and One Central NAC Fragment Coaggregate with Full-length α-Syn—We first identified truncated α-Syn species in both PD and dementia with Lewy bodies (DLB) patients using a spectrum of α-Syn antibodies. Brain samples from PD and DLB patients and controls (age-matched normal brains or AD patients) were sequentially extracted with a HS buffer, a 4% SDS buffer, and an 8 M urea buffer to obtain soluble α-Syn, SDS-soluble aggregates, and urea-soluble/SDS-insoluble aggregates, respectively. Two C-terminal-truncated forms of α-Syn, H1 and H2, were detected in the HS-soluble fractions of controls, PD, and DLB patient brains (Fig. 1, A, B, and D). A small amount of H1 was also observed in the SDS-soluble fractions of the control samples. However, only PD and DLB patient brains contained appreciable amounts of α-Syn truncations, as well as higher molecular weight species, in both the SDS- and urea-soluble fractions. These fractions also were the only ones to contain a third fragment (H3) that is both N- and C-terminal truncated and also contains the highly amyloidogenic NAC region of α-Syn (Fig. 1, A, B, and D), possibly explaining why this hydrophobic fragment was detected only in aggregates.

Truncated α-Syn species were also observed in transgenic (Tg) mice expressing human α-Syn (22, 23). The human α-Syn-A53T mutant Tg mice develop an age-dependent pathological phenotype (complex motor neuron impairment leading to paralysis and death) associated with the accumulation of pathological α-Syn inclusions (22, 23). To examine whether truncated α-Syns were also associated with pathology, we analyzed brain stem samples from α-Syn-A53T Tg mice not yet exhibiting pathology (4-month old, normal behavior) and exhibiting pathology (11-month old, displaying quadriparesis, stiff back, and impaired movement). HS-soluble α-Syn truncations were identified in both 4- and 11-month-old mice, but animals showing pathology had a greater amount of α-Syn present in SDS-soluble, aggregated forms (Fig. 1, C, M1, M2, and M3), indicating that truncated species accumulate in pathological inclusions.

One notable difference between human patients and α-Syn-A53T Tg mice is that the Tg mice do not produce urea-soluble aggregates. α-Syn epitope mapping (Fig. 1D) showed that the truncated α-Syns produced by human α-Syn-A53T Tg mice are very similar to those found in human PD and DLB patients. Thus, both patient samples and pathological Tg mouse brains contain at least three truncated α-Syn forms: two C-terminal-truncated forms (approximately ending between amino acid residues 102–125 and 83–110, respectively) and one central fragment that contains the NAC domain. Similar fragments were also detected by the same spectrum of α-Syn antibodies in Neuro 2a cells when full-length human α-Syn was overexpressed (data not shown). A study that appeared during the review of this report observed a nearly identical pattern of fragments in patient and animal model samples (24).

C-terminal-truncated α-Syns Induce Rapid Aggregation of Full-length Protein at Substoichiometric Ratios—To explore the possible mechanism underlying the coaggregation of truncated α-Syn with full-length protein as observed in patients and Tg mice, the effect of two C-terminal-truncated fragments, α-Syn1–120 (α-Syn110) and α-Syn1–120 (α-Syn120), on the rate of fibrillization of full-length protein was determined. In vitro, recombinant α-Syn110 and α-Syn120 adopt a random coil conformation indistinguishable from the full-length protein as detected by circular dichroism spectroscopy (data not shown).

α-Syn aggregation is a nucleation-dependent process (25) involving at least three steps: monomer → oligomer (seed) → protofibril → fibril. TTF binding, a fluorescent dye assay that...
specifically reports amyloid fibril formation, showed that both model C-terminal-truncated aSyns aggregate much more rapidly than the full-length protein, as previously described (17, 18). Significantly, substoichiometric amounts (5%) of truncated aSyn110 and aSyn120 also dramatically accelerate full-length aSyn aggregation (Fig. 2A), suggesting a possible scenario in which truncated fragments nucleate full-length aSyn aggregation by formation of hybrid protofibrils and then fibrils. C, purification of protofibril mixture. Protofibrils were prepared from mixed samples of equimolar aSyn-His₆ and aSyn110 and purified with a Superdex 200 gel filtration column. Protofibrils eluted in the void volume. Protofibrils were dissolved in 6 M urea before being subjected to SDS-PAGE (Fig. 2B). These results demonstrate that formation of hybrid protofibrils does occur in vitro. Furthermore, TIT binding assays demonstrate that purified protofibril mixtures can effectively nucleate full-length aSyn aggregation (Fig. 2E). These data support a model by which C-terminal-truncated aSyns induce protein aggregation by forming hybrid protofibrils with full-length aSyn, which subsequently accelerates aggregation of the remaining proteins (Fig. 2B).

Coexistence of Full-length and Truncated aSyns Increases Cellular Vulnerability to Oxidative Stress—The pathology of α-synucleinopathies is accompanied by the formation of aSyn inclusions, but whether these aggregates are the species that cause neuronal cell death is as yet unclear. Recent evidence suggests that the aggregation intermediates (soluble oligomers) may be the major cytotoxic species causing neuronal cell death (26, 27) and that mature fibrils may have a neuronal protective effect (28). We used a well established in vitro cell death assay to test whether purified aSyn protofibril mixtures are cytotoxic. We added 50 nM protofibrils or 5 μM monomeric aSyn to the cell culture medium. After 4 h, about 30% of human neuroblastoma SH-SYSY cells treated with protofibrils died, as assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction. In contrast, no cell death was observed after treatment with either 5 μM monomeric aSyn alone or a mixture of monomeric truncated and full-length aSyn (Fig. 3A). The cellular toxicity of purified protofibril mixtures suggests that C-terminal-truncated aSyns may promote cell death by accelerating protofibril formation and accumulation when promoting full-length protein aggregation.

To further examine the role of truncated aSyns in development of cellular toxicity, truncated aSyns were co-overexpressed with full-length protein in SH-SYSY cells. Overexpression of full-length or C-terminal-truncated aSyns increased neuronal cell vulnerability to oxidative stress (29, 30). When truncated aSyns were co-overexpressed with full-length protein at a ratio of about 1:10, cellular vulnerability to oxidative stress was significantly increased as compared with the presence of equivalent amounts of full-length pro-
tein alone (Fig. 3B). These results suggest that substoichiometric amounts of C-terminal-truncated αSyns exacerbate αSyn-dependent cytotoxicity.

The A53T Mutation Exacerbates the Effect of Truncated αSyns on the Aggregation of Full-length Protein—The A53T mutation in αSyn causes an early onset familial PD (5). In vitro, αSyn-A53T aggregates more rapidly than the wild type αSyn (31). The cross-seeding mechanism (Fig. 2B) predicts that αSyn-A53T truncations may also promote the aggregation of full-length αSyn-A53T. Consistent with this hypothesis, C-terminal-truncated αSyn110-A53T and αSyn120-A53T mutants aggregate much more rapidly than full-length αSyn-A53T (data not shown). Significantly, C-terminal-truncated αSyn120-A53T mutants promote aggregation of full-length αSyn-A53T much more rapidly (a much shorter lag phase) than parallel combinations without the mutation (Fig. 4, A and B). This dramatic acceleration of seeding and aggregation would be predicted to contribute to the early appearance of αSyn inclusions in patients with the A53T mutation and in αSyn-A53T Tg mice, thereby causing pathology.

To evaluate this hypothesis, the age-dependent accumulation of truncated αSyn species was assessed in Tg mice expressing human αSyn-A53T mutant (line M83, which develops an age-dependent severe and complex motor disorder leading to paralysis and death). These studies were conducted in parallel with Tg mice expressing equivalent levels of wild-type human αSyn (line M7, which does not develop pathological inclusions or any abnormal phenotype) (23). Immunoblot analyses were performed to evaluate both full-length and truncated αSyns in their aggregated (SDS-soluble) and HS-soluble forms of age-matched M83 and M7 Tg mice (Fig. 4, C and D). Samples from 11-month-old M83 Tg mice exhibiting pathology were also included. The early onset and enhanced extent of deposition of both truncated and full-length αSyn aggregates (SDS-soluble) in the M83 mice parallel the pathology (Fig. 4C). The amount of soluble full-length αSyn extracted by HS buffer remains constant between 1 and 8 months of age for both A53T mutant and wild type αSyn Tg mice (Fig. 4D); however, the production and accumulation of truncated αSyns in both M83 and M7 lines are age dependent. The M83 line reaches a steady state of about 20% of total αSyn being soluble truncations at month 8, correlating well with the average age of disease onset (7 months) (23). Line M7 expressing wild type αSyn accumulates the same level of truncations even earlier (2 months), but no pathology phenotype was observed. These results are consistent with the reduced ability of the wild type αSyn fragments to form aggregates in vitro compared with αSyn-A53T fragments as seen in Fig. 4, A and B.

The 20 S Proteasome Produces C-terminal-truncated αSyn Fragments from the Degradation of Unstructured, but Not Vesicle-bound, αSyn—The possible role of αSyn truncations in pathogenesis necessitates the discovery of the mechanism by which they are produced. The model predicts that inhibition of this activity could retard the formation of intracellular αSyn aggregates. The 20 S proteasome is capable of degrading natively unfolded, non-ubiquitinated αSyn in vitro (32, 33) except in the case of the minor fraction of O-glycosylated αSyn that is a substrate for the E3 ubiquitin ligase, Parkin (34).

We performed in vitro proteasomal degradation assays to examine whether the proteasome produces fragments of αSyn as it degrades the full-length protein. Purified 20 S proteasome degraded full-length αSyn to produce C-terminal-truncated fragments as detected by an αSyn N terminus antibody, with fragment accumulation more substantial at higher ratios of αSyn to 20 S proteasome (Fig. 5A). In cells, αSyn may bind on the surface of vesicles and form α-helical conformation (35–37). As the 20 S proteasome is capable of degrading unfolded or damaged proteins, but not stable proteins (38), we hypothesized that the vesicle-bound αSyn would not be a good substrate for the 20 S proteasome. Consistent with the hypothesis, vesicle-bound αSyn is predominantly α-helical as confirmed by CD spectroscopic analysis (data not shown) and is not degraded by the 20 S proteasome (Fig. 5B). The vesicles themselves did not inhibit activity of the proteasome against a control flurogenic substrate (data not shown). This result suggests that only a fraction of intracellular αSyn, which is not bound to vesicles, is a substrate for the 20 S proteasome and that loss of vesicle binding because of mutation (39) or oxidative damage (7, 40) may result in enhanced 20 proteasome access to αSyn and the production of truncated fragments.

The Caspase-like Activity of the Proteasome Is Responsible for Fragment Production—We used inhibitors specific for the in-
FIG. 4. The A53T mutation in αSyn exacerbates the precipitating effect of truncated αSyn in inducing inclusion formation, which correlates with the pathology of human αSyn-A53T Tg mice. A and B, mixtures of subtochiometric amounts of truncated αSyn-A53T and full-length αSyn-A53T aggregate much more rapidly than the combinations without the A53T mutation. A, aggregation of 20 μM αSyn-A53T mutant + 5 μM αSyn120/110-A53T mutant (curve 1), 20 μM αSyn + 5 μM αSyn120 (curve 2), 25 μM αSyn-A53T mutant (curve 3), and 25 μM αSyn (curve 4). B, aggregation of 20 μM αSyn-A53T mutant + 5 μM αSyn110-A53T mutant (curve 1), 20 μM αSyn + 5 μM αSyn110 (curve 2), 25 μM αSyn-A53T mutant (curve 3), and 25 μM αSyn (curve 4). As controls, aggregation of 5 μM αSyn120/110-A53T mutant (curve 5), αSyn120/110 (curve 6), αSyn-A53T (curve 7), and αSyn (curve 8) in buffer E (20 mM phosphate, pH 7.4) with 0.02% NaN₃ and 20 μM thiorflavin T are also shown. C, human αSyn-A53T mutant Tg mice deposit both full-length and truncated αSyn aggregates earlier and to a greater extent than Tg mice expressing wild type αSyn. Immunoblot analysis of the SDS-soluble extracts from 1- to 8-month-old A53T mutant and wild type Tg mice. 11-month-old A53T mutant and wild type Tg mice. 11-month-old pathological A53T mutant Tg mice were also evaluated. D, accumulation of soluble truncated αSynes in both wild type and A53T mutant Tg mice is age dependent. Immunoblot analysis of HS-soluble extracts from 1- to 8-month-old A53T mutant and wild type Tg mice. 11-month-old A53T mutant Tg mice exhibiting pathology are also included. Bands a and b mark the full-length and the predominant truncated form of αSyn, respectively.

FIG. 5. Production of C-terminal-truncated αSyn fragments by the 20 S proteasomal degradation of unstructured αSyn. A, in vitro 20 S proteasomal degradation of recombinant αSyn-His₆ produces C-terminal-truncated fragments. Time course of degradation of 400 or 4000 nM recombinant αSyn-His₆ by 20 nM purified 20 S proteasome at 37 °C in buffer F (20 mM Tris-HCl, pH 7.1, at 37 °C, 20 mM NaCl, 1 mM EDTA). The lanes with MG132 indicate that the proteasome was pre-treated with 100 μM MG132 for 20 min prior to the addition of substrate. B, vesicle-bound αSyn is not a substrate for the 20 S proteasome. 3 μM αSyn-His₆ was preincubated in 20 mM Tris-HCl, pH 7.6, 20 mM NaCl or in the presence of 750 μM 1-palmitoyl 2-oleoyl phosphatidic acid/1-palmitoyl 2-oleoyl phosphatidylcholine vesicles (37). 20 nM proteasome was added, and the time course of degradation was assayed by Western blotting.

dividual catalytic sites of the proteasome to identify which activity was responsible for fragment production. YU-102, an inhibitor of the caspase-like activity (41), retards fragment production as determined by Western blotting (Fig. 6A). Notably, a larger fragment accumulated at a 1:5000 ratio of protea-
some to αSyn (Fig. 6A), and inhibition of trypsin-like activity increased fragment production (Fig. 6A). To identify the preferred proteasomal cleavage sites, degradation products of αSyn-His₆ were separated into Ni-NTA-bound and Ni-NTA flow-through fractions. Mass spectrometric analysis of the NTA flow-through fraction identified three predominant fragments: αSyn (1–119), αSyn (1–110), and αSyn (1–83) (Fig. 6B). Interestingly, corresponding fragments αSyn (120–149), αSyn (111–149), and αSyn (84–149) are present in the NTA-bound fraction, suggesting that the endoproteolytic activity of the proteasome identified previously (33) may be responsible for production of the fragments. The occurrence of all three scissors bonds after an acidic residue (Asp-119, Glu-110, and Glu-83) is consistent with the results of the inhibition assay, which implicated the caspase-like activity of the proteasome in fragment production (Fig. 6A). It is also notable that the larger fragments observed in vitro (e.g. 1–119, 1–110) are very similar to those observed in PD patients (H1, H2) and in animal models (M1, M2) (Fig. 1) (24).

To examine whether the fragments produced from the 20 S proteasomal degradation are capable of inducing full-length protein aggregation, mixtures of αSyn from proteasomal degradation were assessed for fragment production by Western blotting (Fig. 6C, inset) and rate of fibril formation. TTF binding demonstrated that fragments produced by the caspase-like activity of the 20 S proteasome induced earlier initiation of the aggregation (Fig. 6C).

DISCUSSION

Although truncated αSynes are one of the major modified αSyn species in Lewy bodies of PD, little is known about their role in pathogenesis or the mechanism of their production (42). We first identified one central NAC-containing and two C-
terminal-truncated α-Syn fragments in the Lewy bodies of PD and DLB patients and in inclusions of human α-Syn-A53T Tg mice exhibiting pathology (Fig. 1). These fragments are analogous to those recently identified by Li et al. (24) in patients, animal models, and cell culture systems. Truncated α-Syns are not unique to PD/DLB patients and human α-Syn-A53T Tg diseased mice, as they also are present as soluble forms in non-pathology age-matched controls, AD patients, and non-pathology human α-Syn-A53T Tg mice (Figs. 1 and 4D). In addition, data from Li et al. suggested that C-terminal-truncated α-Syn fragments are generated by the normal cellular processing of the full-length α-Syn (24).

Notably, aggregated α-Syn truncations are unique to PD/DLB patients, as fragments in age-matched controls are present only in soluble forms (Fig. 1). Because of the greater tendency of the C-terminal-truncated α-Syns to aggregate as compared with the full-length protein (17, 18), we suspected that truncated α-Syns may play a role in PD pathogenesis and other α-synucleinopathies. To explore the mechanism underlying the coaggregation of truncated and full-length α-Syns in PD/DLB brains, we performed in vitro aggregation assays demonstrating that truncated α-Syns induce rapid aggregation of the full-length α-Syn at very low substoichiometric ratios, possibly by a seeding mechanism in which more aggregation-prone truncated α-Syns function as seeds to nucleate the full-length protein aggregation as we tested in vitro (Fig. 2). Others (18, 24) also observed acceleration of aggregation by similar C-terminal-truncated α-Syns but did not investigate the mechanism by which the rate was enhanced. Together, these results suggest that truncated α-Syns may play a precipitating role in inducing the formation of inclusions in PD/DLB brains.

Promoting α-Syn aggregation with substoichiometric amounts of truncated α-Syn results in earlier formation and accumulation of aggregation intermediates (protofibrils) and, subsequently, final mature fibrils. Recent data suggest that these or related aggregation intermediates are the cytotoxic species that cause neuronal cell death (26, 27), whereas mature fibrils may have a neuronal protective effect (28). A well-established in vitro cell death assay (26, 27) demonstrated that purified soluble protofibrils, but not monomers, from mixtures of truncated and the full-length α-Syn cause SH-SY5Y cell death (Fig. 3A). Although the mechanism underlying the cytotoxicity of the protofibrils is not clear, earlier studies showed that α-Syn protofibrils disrupt synthetic vesicles in vitro (43, 44), similar to the mechanism utilized by cellular toxins (45), causing imbalance of cellular ions and fluid, and thus, cell

FIG. 6. Production of the major C-terminal-truncated α-Syns by the caspase-like activity of the proteasome. A, a specific inhibitor of the caspase-like activity prevents the production of C-terminal-truncated α-Syns. 20 nM proteasome was preincubated with inhibitors (0.4 μM β-lactacystin for chymotrypsin-like activity, 100 μM leupeptin for trypsin-like activity, 20 μM YU102 for caspase-like activity) for 20 min prior to the addition of 5 or 100 μM α-Syn-His6. Reactions were stopped at 10 min, and Western blotting was used to detect fragment production. B, identification of α-Syn fragments from 20 S proteasomal degradation. Mass spectroscopy analysis were used to identify the fragments produced from 20 nM 20 S proteasome degradation of 100 μM α-Syn-His or 5 μM α-Syn-His in the presence of YU102 inhibitor. The schematics represent both Ni-NTA-bound and unbound fragments. αSyn119 and αSyn110 were not produced by the 20 S proteasome inhibited by YU102, and αSyn84 was generated instead of αSyn83. C, αSyn fragments produced by 20 S proteasomal degradation promote αSyn aggregation. After 10 min of incubation of 500 μl of 100 μM α-Syn-His6 with 20 nM purified 20 S proteasome, the proteasome was removed by a YM-100 cutoff spin column. The flow-through from the YM-100 column was used to set up the TF binding aggregation experiment as described under “Experimental Procedures”: (1) 100 μM αSyn, (2) 100 μM αSyn + 20 nM 20 S proteasome, (3) 100 μM αSyn + 20 nM 20 S proteasome + 20 μM YU102. The inset shows the protein mixtures applied for aggregation assay.
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density. Consistent with this mechanism, in these studies the toxic conformers of cytoplasmic proteins were toxic even when administered extracellularly. Because αSyn is an intracellular protein, we also examined the consequences of coexpression of small fractions of truncated αSyn with the full-length αSyn in the cytosol by transiently co-overexpressing ~10% of truncated αSyn110 or αSyn120 with the full-length αSyn in SH-SY5Y cells. Cell death assays demonstrated that the coexpression of truncated αSyns with full-length αSyn increased cell death during an oxidative challenge (Fig. 3B). As elevated oxidative stress is one of the events arising from impaired mitochondria function and perhaps plays a role in PD pathogenesis (6), these results suggest that the induction of rapid αSyn aggregation by truncated forms could accelerate neuronal cell death.

Point mutations (A30P, A53T, and E46K) in αSyn cause inheritable early onset PD (3–5). Human αSyn-A53T Tg mice, but not wild type human αSyn Tg mice, develop a pathological phenotype similar to human patients (22, 23). The greater propensity for aggregation of the αSyn-A53T mutant compared with the wild type protein (31) likely contributes to the dramatic differences between the A53T mutant and the wild type Tg mice. Age-dependent coaggregation of truncated αSyns with the full-length αSyn was found to correlate with the development of pathology in A53T mutant Tg mice (Fig. 4). These fragments are very similar to those deposited in human patients as detected by αSyn epitope mapping (Fig. 1) and putative mass spectroscopic identification (24). Furthermore, accumulation of soluble αSyn fragments in A53T mice is age dependent whereas full-length protein level remains constant, and only the former correlates with the average disease onset time of this Tg line (7 months) (Fig. 4D). The in vitro aggregation assay demonstrated that the A53T mutant exacerbates the accelerating effect of truncated αSyns on rapid aggregation of the full-length protein (Fig. 4, A and B). Together, these results suggest that truncated αSyns in human αSyn-A53T Tg mice may play a precipitating role in inclusion formation in this Tg line and, thus, accelerate pathogenesis.

Partial proteolysis has a role in several neurodegenerative diseases (42). In AD, the highly amyloidogenic Aβ peptide is generated by the sequential action of β-secretase and γ-secretase on amyloid precursor protein (46), whereas in Huntington disease, toxic truncated N-terminal fragments of the Hunting- ton protein that contain an expanded polyglutamine repeat are deposited in the inclusions of Huntington disease postmortem tissue (47, 48). Our data indicate a possible role for truncated αSyns in inducing inclusion formation. Moreover, they impli- cate the critical role of the 20 S proteasome in fragment pro- duction. Several proteases and degradation pathways have been shown to regulate the degradation of various forms of αSyn. In vitro and in vivo data support the involvement of the proteasomal (32, 33, 49) and lysosomal pathways (50, 51), and in vitro data suggest a role for calpain I in degradation of aggregated protein (52). Because the physiological function of αSyn may require its association with lipid vesicles where it assumes an α-helical conformation, the metabolism of the vesicle-bound αSyn may occur through the lysosomal degradation pathway, such a model is in agreement with the relatively long half-life of this protein of about 20 h in certain cell cultures (50, 51). Structural studies show that αSyn binding to vesicles is dynamic (53) and dissociation from the vesicle could be pro- moted by mutation (39) and oxidative stress (7, 40). Our data demonstrate that the 20 S proteasome degrades this free, un- structured αSyn, but not membrane-bound, α-helical αSyn. Furthermore, the caspase-like activity of the proteasome gener- ates C-terminal-truncated αSyn species similar to those de- posited in human patients that promote the aggregation of full-length αSyn. This acceleration is abolished by inhibition of fragment production by the caspase-like activity inhibitor YU102. Finally, production of C-terminal-truncated αSyn frag- ments is dramatically affected by the proteasome capacity, as greater amounts of fragments are observed under higher sub- strate to proteasome ratios as well as by inhibition of the trypsin-like activity of the proteasome. Thus, because aggreg- ated αSyn has been shown to inhibit some proteasome activ- ity, this mechanism could result in the accumulation of both truncated and full-length αSyn and an even further elevation of the concentration of aggregation-prone truncated αSyn that, in turn, could initiate the aggregation of full-length αSyn, espe- cially in the crowded cellular environment (54).

In summary, our data suggest a model in which incomplete degradation of αSyn produces highly amyloidogenic fragments that rapidly aggregate and seed the aggregation of full-length protein. Subsequently, proteasomal activity could be inhibited by further attempts to dispose of these more proteolysis-resis- tant fragments and aggregates (55). This inhibition would, in turn, cause further accumulation of both full-length and frag- mented αSyn species, thus creating a vicious cycle of cytotoxicity. This model makes the paradoxical prediction that early interference in the proteolysis of full-length αSyn may actually retard aggregate formation by reducing the production of truncated αSyn.

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