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Accelerated formation of α-synuclein oligomers by concerted action of the 20S proteasome and familial Parkinson mutations

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Abstract A hallmark of Parkinson disease (PD) is the formation of intracellular protein inclusions called Lewy bodies that also contain mitochondria. α-Synuclein (αSyn) is a major protein component of Lewy bodies, where it is in an amyloid conformation and a significant fraction is truncated by poorly understood proteolytic events. Previously, we demonstrated that the 20S proteasome cleaves αSyn in vitro to produce fragments like those observed in Lewy bodies and that the fragments accelerate the formation of amyloid fibrils from full-length αSyn. Three point mutations in αSyn are associated with early-onset familial PD: A30P, E46K, and A53T. However, these mutations have very different effects on the amyloidogenicity and vesicle-binding activity of αSyn, suggesting neither of these processes directly correlate with neurodegeneration. Here, we evaluate the effect of the disease-associated mutations on the fragmentation, conformation, and association reactions of αSyn in the presence of the 20S proteasome and liposomes. The 20S proteasome produced the C-terminal fragments from both the mutant and wildtype αSyn. These truncations accelerated fibrillization of all α-synucleins, but again there was no clear correlation between the PD-associated mutations and amyloid formation in the presence of liposomes. Recent data suggests that cellular toxicity is caused by a soluble oligomeric species, which is a precursor to the amyloid form and is immunologically distinguishable from both soluble monomeric and amyloid forms of αSyn. Notably, the rate of formation of the soluble, presumptively cytotoxic oligomers correlated with the disease-associated mutations when both 20S proteasome and liposomes were present. Under these conditions, the wildtype protein was also cleaved and formed the oligomeric structures, albeit at a slower rate, suggesting that 20S-mediated truncation of αSyn may play a role in sporadic PD as well. Evaluation of the biochemical reactions of the PD-associated α-synuclein mutants in our *in vitro* system provides insight into the possible pathogenetic mechanism of both familial and sporadic PD.

Keywords 20S proteasome · Endoproteolysis · Parkinson disease · αSynuclein · Amyloid · Soluble oligomers · Cytotoxicity · Liposomes

Abbreviations

20S	20S proteasome
αSyn	α-synuclein
CD	circular dichroism
EDTA	ethylenediaminetetracetic acid
β-ME	beta-mercaptoethanol
PD	Parkinson disease

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PBS phosphate-buffered saline
POPC 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPA 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate

Background

Parkinson disease (PD) is characterized by the selective loss of dopaminergic neurons in the *substantia nigra pars compacta* of the brain and is associated with the presence of intracellular inclusion bodies termed Lewy bodies (LBs). In addition to amorphous protein aggregates that stain positive for ubiquitin, LBs contain amyloid fibrils of the protein α-synuclein (αSyn) as well as mitochondria (Bedford et al. 2008). A significant fraction of the αSyn in these structures is proteolytically processed to produce specific fragments (Baba et al. 1998; Campbell et al. 2001). While only a small fraction of PD cases have a genetic basis (Hardy et al. 2006; Lansbury and Brice 2002), several gene products have been implicated in early-onset PD, including αSyn, parkin, DJ-1, UCH-L1, PINK-1, and LRRK2 (Hague et al. 2003; Kitada et al. 1998; Leroy et al. 1998; Paisan-Ruiz et al. 2004; Polymeropoulos et al. 1997; Singleton et al. 2003; van Duijn et al. 2001). αSyn has been implicated in PD by molecular genetic data, including three point mutations: A53T, A30P, and E46K (Kruger et al. 1998; Polymeropoulos et al. 1997; Zarranz et al. 2004). The involvement of parkin and UCH-L1 suggest a critical role for the ubiquitin-proteasome system in this disease. A recent conditional knockout of the Rpt2 subunit that reduces the amount of the 26S proteasome while increasing the amount of the 20S proteasome produces mitochondrial-rich αSyn inclusions, indicating that decreased UPS or increased 20S activity can lead to Lewy body formation in the absence of mutant αSyn (Bedford et al. 2008). In an animal model expressing the A53T αSyn, increased steady state levels of the αSyn fragments are observed (Li et al. 2004). However, the relationship between the fragments, the familial αSyn mutants, the activity of the 20S proteasome, and cellular toxicity is poorly understood.

The cellular function of αSyn has not been fully elucidated. The protein has been shown to localize to presynaptic vesicles, associate with microtubules, and has been proposed to act as a vesicle-bound chaperone (Alim et al. 2004; Chandra et al. 2005; Kahle et al. 2000). Consistent with a function as a vesicle-associated protein, αSyn lacks ordered secondary structure in aqueous solution but adopts an alpha-helical structure in the presence of negatively-charged lipids or detergents (Chandra et al. 2003; Davidson et al. 1998; Eliezer et al. 2001; Ulmer et al. 2005). The 140-residue protein contains three regions: an N-terminal amphipathic region that forms a discontinuous helix on micellar surfaces (residues 1–98), a central amyloidogenic region located within the amphipathic region (residues 61–95), and a C-terminal acidic region that remains disordered even in the presence of vesicles (residues 99–140). Deletions in the C-terminal region increase the amyloidogenicity of the protein, suggesting that this region is important for maintaining solubility (Hoyer et al. 2004; Li et al. 2005; Liu et al. 2005), and may do so through long-range contacts with the N-terminus (Bernado et al. 2005; Dedmon et al. 2005b).

αSyn forms amyloid fibrils in a nucleation-dependent manner, and differences in fibrillization profiles are observed among the familial mutations. A53T-syn forms amyloid earlier and its fibrils elongate faster than wildtype αSyn (Choi et al. 2004; Conway et al. 1998, 2000b; Greenbaum et al. 2005; Li et al. 2001, 2002; Narhi et al. 1999). Data for A30P-syn are less conclusive. In general, A30P appears to form soluble higher molecular weight aggregates faster than wildtype αSyn (Conway et al. 1998; Li et al. 2002; Narhi et al. 1999), while the formation of amyloid structure is slower than wildtype (Choi et al. 2004; Conway et al. 2000b; Li et al. 2001). The relative timing of the initiation of amyloid formation by A30P-syn varies, with some methods showing a longer lag phase than wildtype (Conway et al. 2000b; Li et al. 2001) and others showing a reduced lag phase (Li et al. 2001; Narhi et al. 1999). Studies of E46K-syn have shown that amyloid is initiated and elongates more rapidly than wildtype, similar to A53T-syn (Choi et al. 2004; Greenbaum et al. 2005).

Because αSyn appears to be associated with presynaptic membranes *in vivo* (Jensen et al. 1998), the lipid-binding characteristics of wildtype and mutant αSyns have been studied. A30P-syn exhibits weaker binding to lipid vesicles both *in vivo* and *in vitro* (Choi et al. 2004; Cole et al. 2002; Jo et al. 2002; Nuscher et al. 2004). Data for A53T-syn have suggested a similar affinity as wildtype (Choi et al. 2004; Cole et al. 2002), while also perhaps interacting with vesicles differently than the wildtype protein (Jo et al. 2004). Liposome pulldown assays suggest that E46K-syn may bind liposomes more strongly than wildtype (Choi et al. 2004).

In both patients and model systems, the levels of both soluble and insoluble αSyn levels increase with age as well as disease progression (Klucken et al. 2006; Li et al. 2004; Liu et al. 2005), suggesting that the clearance of αSyn is compromised. However, the physiological clearance pathway(s) is controversial, since autophagy *via* the lysosome, the ubiquitin-proteasome system (UPS), and calcium-dependent proteases all degrade αSyn (Bennett et al. 1999; Webb et al. 2003). The relevance of the UPS is highlighted by molecular genetics, implicating both a ubiquitin hydrolase and a putative ubiquitin ligase (UCH-L1/*park5* and parkin/*park2*, respectively) (Kitada et al.

1998; Liu et al. 2002). More recently (Bedford et al. 2008), a conditional knockout of the Rpt2 subunit of the 26S proteasome has been shown to have decreased 26S activity and increased 20S activity and to accumulate protein inclusions. A model has emerged in which the lysosome is responsible for the turnover of membrane-bound αSyn, calpain acts on fibrillar protein, the 26S proteasome completely degrades unfolded and/or misfolded αSyn in a ubiquitin-dependent manner, and the 20S proteasome produces fragments in a ubiquitin-independent manner. It remains unknown which of these systems, if any, act on the nucleating and/or toxic species.

Recently, N-terminal fragments of αSyn were identified in both PD patient brain samples and mouse models (Li et al. 2005; Liu et al. 2005). Fragments of αSyn similar to those observed in PD patients are produced by calpain I (Greenbaum et al. 2005; Mishizen-Eberz et al. 2003) and by the 20S core particle of the proteasome (Liu et al. 2005), but not the 26S proteasome (Liu et al. 2006). *In vitro*, similar N-terminal fragments of αSyn form amyloid fibrils earlier and faster than wildtype and are able to seed the fibrillization of the full-length protein at sub-stoichiometric concentrations (Liu et al. 2005; Murray et al. 2003), most likely by releasing long-range interactions within αSyn to reveal highly amyloidogenic regions (Dedmon et al. 2005a; Pawar et al. 2005). The importance of the fragments to disease is further highlighted by the fact that transgenic mice expressing N-terminal fragments of human αSyn display the characteristics of PD, including selective loss of substantia nigral neurons and progressive behavioral deficits (Daher et al. 2009; Tofaris et al. 2006; Wakamatsu et al. 2006).

These data led to a model in which N-terminal fragments of αSyn contribute to early steps in PD pathology. The protease responsible for cleavage remains elusive, although several enzymes have been proposed to cleave αSyn *in vivo*. The 20S proteasome is capable of degrading proteins in a ubiquitin- and ATP-independent manner (Amici et al. 2004; Bennett et al. 1999; David et al. 2002; Di Noto et al. 2005; Liu et al. 2003; Shringarpure et al. 2003; Tofaris et al. 2001; Touitou et al. 2001). Studies have suggested that 20S particles outnumber both 26S and free regulatory particles *in vivo*, suggesting an independent role for 20S (Brooks et al. 2000; Tanahashi et al. 2000). Previous work from our lab showed that the 20S proteasome endoproteolytically cleaves αSyn *in vitro* to form truncations that are similar to those observed in tissue (Liu et al. 2005).

We set out to test the hypothesis that the action of the 20S proteasome on αSyn produces N-terminal fragments that enhance the formation of the presumptive toxic species. To this end, we evaluated all known reactions of αSyn as well as the three familial PD mutations in the presence of the 20S proteasome. Although assessment of either αSyn amyloidogenicity or liposome binding has not provided a cohesive model for the role of αSyn in PD pathogenesis, these reactions compete with fragmentation and oligomerization reactions for the substrate. In this study, we evaluated the effect of proteasomal cleavage of αSyn proteins on self-association in a system that includes synthetic liposomes and allows for amyloid formation. With this *in vitro* system, we find that the reactions of αSyn degradation, self-association, and liposome binding compete with one another, but that the presence of liposomes and 20S favors the formation of the oligomeric conformation that is thought to be cytotoxic. This system therefore provides a more thorough biophysical picture of the association between αSyn familial PD point mutations and aberrant metabolism of the protein.

Results

Effects of the 20S proteasome on αSyn mutants Degradation of full-length αSyn by the 20S proteasome *in vitro* produces several N-terminal fragments (Liu et al. 2005). At a ratio of 200:1 (αSyn:20S), three prominent N-terminal fragments are produced: 1–110, 1–83, and 1–73 (Fig. 1, top panel). Under these conditions, there are no dramatic differences in the rate of degradation of full-length wildtype, A53T-syn, and A30P-syn. Full-length E46K-syn is turned over rapidly and produces a different fragmentation pattern. While wildtype αSyn primarily produces fragments consisting of residues 1–110 and 1–83 (Liu et al. 2005), E46K-syn produces the 1–73 fragment, which appears early in the timecourse, and all three fragments are clearly observed by 10 min. Consistent with previously published work (Greenbaum et al. 2005), the 1–73 fragment of E46K-syn migrates faster on the gel than the wildtype 1–73. The identity of these fragments was confirmed by mass spectrometric analysis (data not shown).

Since several sets of data suggest that the mutations can alter the affinity of αSyn for vesicles (Choi et al. 2004; Cole et al. 2002; Jo et al. 2004), and vesicle-bound αSyn is resistant to 20S degradation (Liu et al. 2005), fragmentation in the presence of liposomes was assessed. At 25 and 50 μM lipids, the conformations of all four proteins are statistically indistinguishable by circular dichroism (data not shown). The fragmentation pattern produced by the 20S proteasome was determined at the lower liposome concentration, where the majority of αSyn is unbound. At 25 μM lipids, the 20S proteasome continues to produce fragments from the full-length proteins (Fig. 1, bottom panel). Control experiments with fluorogenic peptides showed that the activity of 20S is not affected by liposomes at these concentrations (data not shown). The most prominent and long-lived fragment at both lipid concentrations, regardless

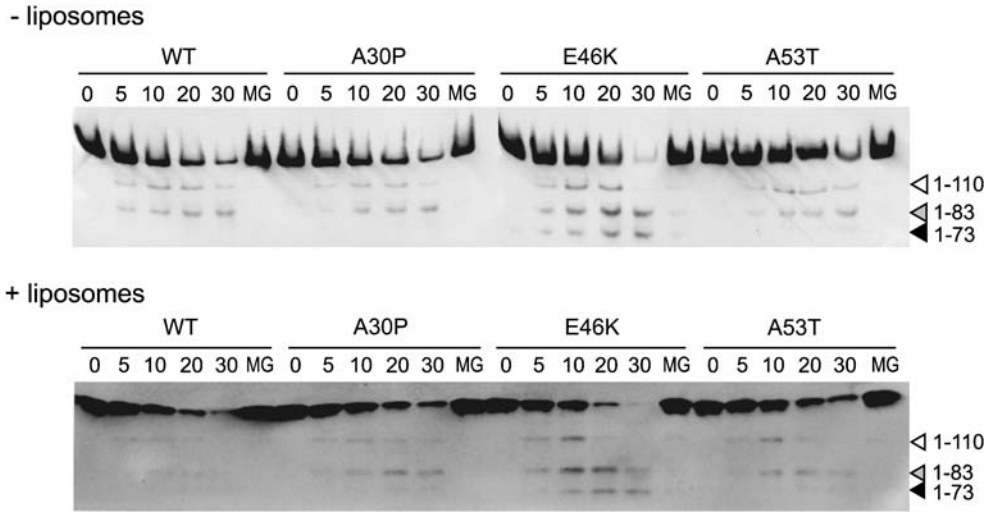


Fig. 1 Wildtype and mutant αSyn degradation by the 20S proteasome *in vitro*. *Top panel:* αSyn proteins were incubated with purified latent 20S proteasome for the times indicated (in minutes). *Bottom panel*: 20S degradation of αSyn proteins was carried out in the presence of 25 μM liposomes. For both panels, MG indicates reactions that were performed with 20S proteasome that had been preincubated with the inhibitor MG132. Specific N-terminal fragments were produced in a time-dependent manner and were identified by mass spectrometry (1–110, white triangle; 1–83, gray triangle; and 1–73, black triangle)

of mutation, is syn(1–83). Thus, fragments that are similar to those associated with the disease are produced from all four αSyn proteins by the 20S proteasome in the presence of low concentrations of liposomes.

Effect of mutations, liposomes, and 20S fragmentation on amyloidogenesis Liposome binding slows the degradation of αSyn by the 20S proteasome (Liu et al. 2005), and the disease-associated mutations have been demonstrated to alter the affinity of αSyn for vesicle surfaces (Choi et al. 2004). To understand how the liposome binding together with 20S-mediated fragmentation affects amyloid formation, the fibrillization of αSyn into amyloid in the presence of liposomes and/or 20S proteasome was monitored by Thioflavin T fluorescence (Fig. 2). In samples with only αSyn protein, the lag phase of polymerization of the A30P mutant prior to fibril initation is slightly longer than that of wildtype αSyn. Both E46K-syn and A53T-syn form amyloid fibrils earlier and elongate faster than wildtype, consistent with previous reports (Choi et al. 2004; Conway et al. 1998, 2000b; Greenbaum et al. 2005; Li et al. 2001, 2002; Narhi et al. 1999). The addition of liposomes lengthened the lag phase and slowed the rate of fibrillization of both E46K-syn and A53T-syn, but did not appear to measurably affect A30P-syn at a ratio of 2.5:1 lipids:αSyn (Fig. 2a). These results indicate that binding to liposomes counteracts the accelerating affect of the E46K and A53T mutations on amyloid formation.

Previously, we established that the 20S proteasome cleaves wildtype αSyn into amyloidogenic fragments that seed the aggregation of full-length protein (Liu et al. 2005). In this study, 20S proteasome was added to amyloid formation reactions to create sub-stoichiometric amounts of amyloidogenic fragments (less than 1% of the total synuclein in the reaction, less than that reported to be present in Lewy bodies, data not shown). As previously observed for wildtype αSyn, proteasome-produced fragments also accelerated amyloid formation of the full-length mutant proteins as measured by the lag phase before amyloid fibrils were detected by Thioflavin T fluorescence (Fig. 2c). This confirms that the fragments produced by proteasomal cleavage of the mutant αSyn proteins are also amyloidogenic.

The αSyn reactions of lipid-binding and proteasomal degradation are coupled as binding to membranes induces structural changes in αSyn that alter 20S degradation. Therefore, we evaluated the effects of the mutations on fibrillization under conditions where the competing lipid binding and 20S degradation reactions occur. In the presence of both liposomes and 20S proteasome, all four αSyn proteins began forming amyloid fibrils at earlier times than when only liposomes were present, although the lag times are still longer than when only 20S was present (Fig. 2c). Importantly, there was no observed correlation between the fibrillization kinetics and any of the three disease-associated mutants under any condition. If there is a common mechanism of cytotoxicity exerted by the disease-associated mutations, it must be reflected by a biochemical characteristic other than lipid binding or amyloid fibril formation.

Evaluation of oligomer formation in the in vitro *system* A growing body of evidence indicates that the cytotoxic conformation of αSyn is not amyloid fibrils, but is instead a

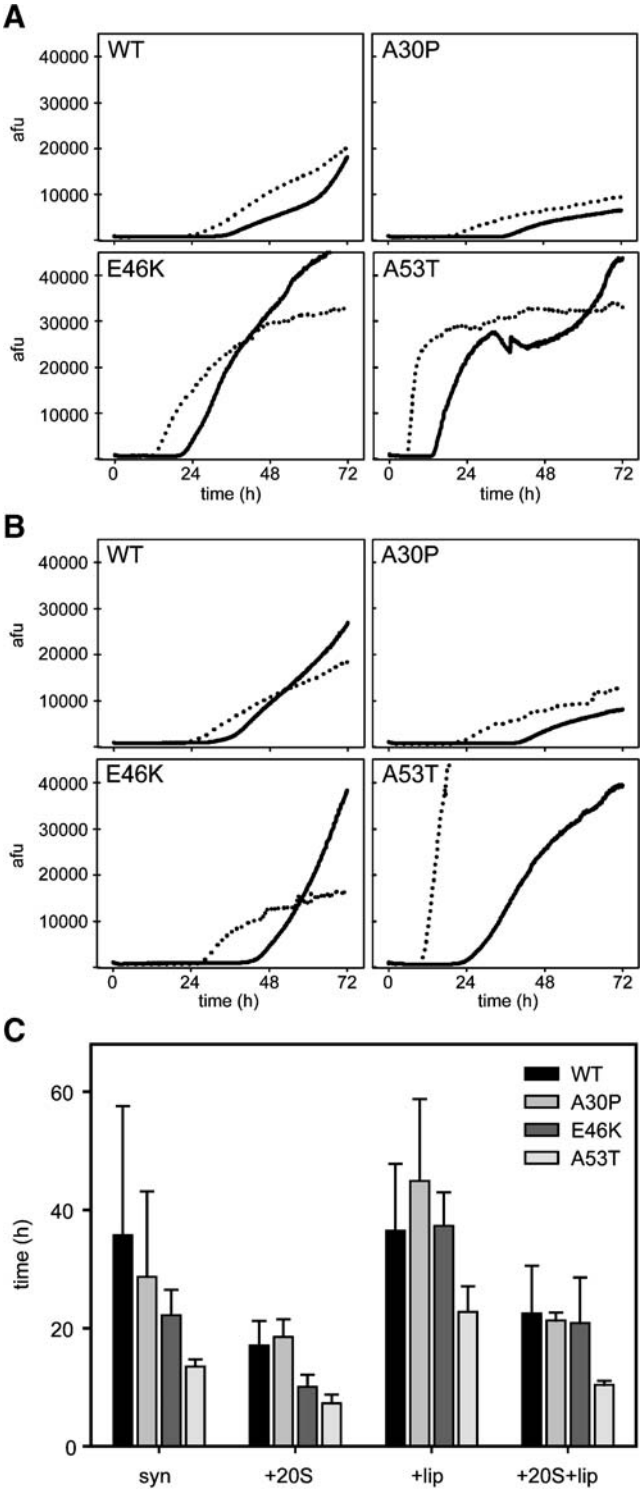


Fig. 2 Fibrillization of αSyn proteins. Representative traces of αSyn fibrillization. 100 μM αSyn protein in 20 mM phosphate buffer (pH 7.2) was measured utilizing Thioflavin T fluorescence to monitor amyloid fibril formation. **a** Samples were prepared with either protein alone (solid lines) or preincubated for 10 min with 20 nM purified latent 20S proteasome (broken lines). **b** As in **a**, except all samples also contained POPA/POPC liposomes. **c** The average length of the lag phase for amyloid formation for each protein. Error bars indicate the standard deviation. Abscissa labels: syn, αSyn protein alone; +20S, αSyn protein and 20S; +lip, αSyn protein and liposomes; +20S + lip, αSyn protein with both liposomes and 20S

pre-amyloid oligomeric conformation (Kayed et al. 2003; Necula et al. 2007). Antibodies that specifically recognize the soluble oligomeric species of αSyn and other aggregation-prone proteins, but not the monomeric or the amyloid forms of α-synuclein, have been used to establish a correlation between the presence of the oligomers and cytotoxicity (Kayed et al. 2007; Kostka et al. 2008). Although the basis of this toxicity still remains unclear, several studies suggest the oligomers perturb membranes (Kayed et al. 2004; Sokolov et al. 2006). To test the hypothesis that the 20S proteasome and liposomes promote the formation of the potentially cytotoxic oligomeric species from the mutant αSyn proteins, a dot-blot analysis of oligomer formation was performed using the oligomer specific antibodies. Thus, for this analysis, an "oligomer" was defined as any species that was immunoreactive with the anti-oligomer antibody I-11 (Kayed et al. 2007). To validate the assay, we evaluated oligomer formation from two recombinant C-terminal truncations of αSyn, αSyn119 and αSyn110, that were previously demonstrated to exert cytotoxicity in cell culture (Liu et al. 2005). Aliquots were removed at regular intervals from a fibrillization assay plate for dot-blot analysis with I-11 (Fig. 3). The truncated αSyn proteins formed oligomers more quickly than the wildtype protein. Assays were then performed on the disease-associated mutants under the same conditions as used for the fibrillization assessment above (Fig. 4). In samples that contained only αSyn (Fig. 4, top panel), oligomers were only observed in E46K-syn and A53T-syn over the course of the experiment. These two mutant proteins also readily produce oligomers in the presence of 20S, liposomes, or both 20S and liposomes (Fig. 4, bottom panel). Notably, immunoreactive oligomers of the third disease-associated mutant, A30P-syn, are only observed when both 20S and liposomes are present (Fig. 4, bottom panel). Therefore, a correlation exists between the rate formation of the putative cytotoxic oligomeric species and the presence of the three disease-associated mutations. It should be noted that wild-type αSyn produced small amounts of oligomers when incubated with both 20S proteasome and liposomes (Fig. 4, bottom panel), conditions that most closely relate to those in the cell. These results demonstrate for all three disease-associated αSyn proteins, oligomer formation is promoted by the fragments produced by the 20S proteasome in the presence of liposomes.

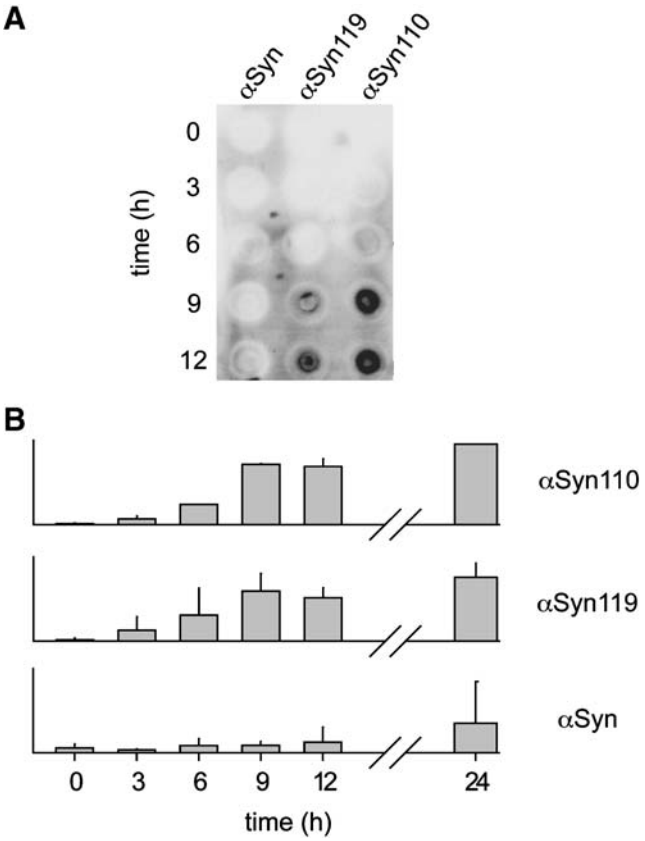
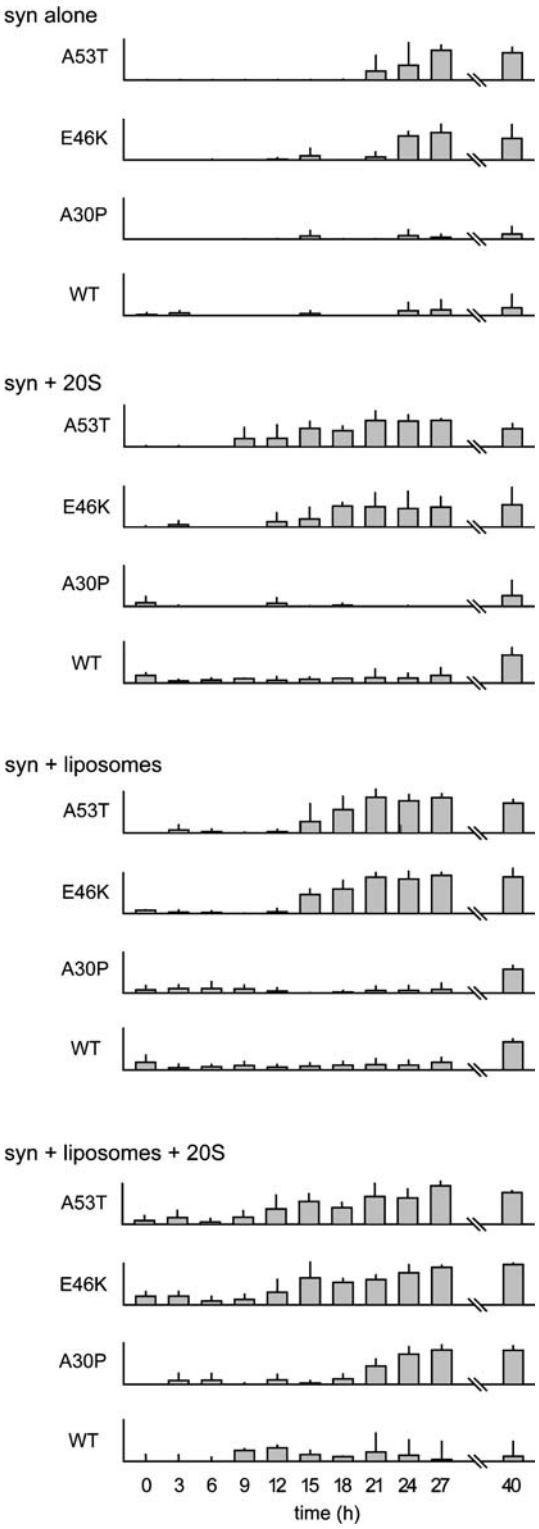


Fig. 3 Oligomer formation of truncated αSyn proteins. 100 μM recombinant αSyn protein (full-length, αSyn119, or αSyn110) was incubated in 20 mM phosphate buffer (pH 7.2) at 37°C with shaking. Aliquots were removed at the indicated time points and assayed for oligomer content using the anti-oligomer antibody I-11, which does not cross-react with either monomeric or amyloid αSyn (Kayed et al. 2007). **a** Representative blot. **b** The data from two independent experiments was first quantitated, and then normalized to a range of 0 to 1 before being averaged. The height of the Y-axis is 1. The height of the gray bars indicates the mean and error bars show the SEM



Discussion

As observed in our previous studies, αSyn is fragmented by the 20S proteasome *in vitro.* The N-terminal fragments of αSyn produced by 20S accelerate amyloid fibril formation (Liu et al. 2003, 2005). These data led to a model of sporadic PD pathogenesis in which a misprocessing of

Fig. 4 Oligomer formation of mutant αSyn proteins. αSyn proteins were incubated in 20 mM phosphate buffer (pH 7.2) at 37°C with shaking. Where indicated, 20S proteasome and/or liposomes were also included in the incubation mixture. Aliquots were removed at the indicated times, and assayed for oligomer content using the anti-oligomer antibody I-11, which does not cross-react with either monomeric or amyloid αSyn (Kayed et al. 2007). The data from three independent experiments were quantitated, normalized to a range of 0 to 1, and averaged. The Y-axis has a height of 1. Gray bars indicate the mean, and error bars show the standard deviation

αSyn by the 20S proteasome accelerates the formation of a cytotoxic conformer (Fig. 5). In this study, we produced simple, reconstituted system for evaluation of the biochemical effects of the three point mutations in αSyn associated with early-onset familial PD. The *in vitro* system is comprised of αSyn, the 20S proteasome, and synthetic liposomes to mimic the lipid vesicles with which αSyn associates *in vivo*. The model predicted that the formation of the cytotoxic species would be accelerated in this simple reconstituted system by the early-onset mutations PD mutations, by contrast to the lack of correlation observed when the reactions were examined in isolation.

The individual reactions composing the *in vitro* system were first evaluated independently. The point mutations did not have a significant effect on either endoproteolysis or turnover by 20S (Fig. 5, "endoproteolysis" and "turnover") nor did they have a correlated effect on either liposomal binding or amyloid formation. However, the binding of αSyn to a liposomal surface affects the rate of degradation by 20S (Liu et al. 2005). Therefore, a variant that affects liposome binding affinity or orientation may also affect 20S degradation by altering the availability of αSyn substrate. Liposomes were added to the degradation reaction to evaluate the effect of mutations when the reactions of liposome binding and 20S proteolysis are in direct competition (Fig. 5, "liposome binding", "endoproteolysis", and "degradation"). Even when liposomes are present, the 20S proteasome produces the similar fragments, which are similar to those previously identified in patients, from all four αSyn proteins (Fig. 1, bottom panel) (Liu et al. 2005).

The most frequently studied biochemical characteristic of αSyn is its propensity to fibrillize into amyloid (Fig. 5, "fibrillization"). As shown in Fig. 2, this property is affected by liposomes ("liposome binding" + "fibrillization") and by fragments produced by 20S ("endo" + "degradation" + "fibrillization"). However, again none of these reactions reveal a clear correlation to the presence of disease-associated mutations. Liposomes considerably slow the initiation of amyloid formation of E46K- and A53T-syn, but only slightly delay that of the A30P mutant. There is a slight increase in fibrillization of wildtype αSyn, which may be due to a local concentration effect on vesicle surfaces (Necula et al. 2003). As expected, the presence of 20S-produced fragments promotes the formation of amyloid and fibril elongation for all four proteins (Fig. 5). This indicates that the three mutant αSyn proteins are susceptible to seeding by those amyloidogenic fragments, as observed for the wildtype protein (Liu et al. 2005). By measuring fibrillization in the presence of both liposomes and 20S proteasome, we evaluated the end result of multiple competing reactions: binding, endoproteolysis, fibril initiation, and fibril elongation. Each of these reactions is affected by the PD-associated mutations in different ways when evaluated independently. However, when these reactions were combined in a system with both 20S and liposomes, still no correlation was found between the disease-associated mutations and amyloid fibril formation, despite the fact that the presence of amyloidogenic fragments dramatically accelerates both the initiation of amyloid formation and fibril elongation.

Recent work by others has strongly implicated a pre-amyloid conformation as being responsible for cytotoxicity (Danzer et al. 2009). To that end, the acceleration of oligomerization, rather than amyloid formation, has been suggested as the common link between the mutants (Conway et al. 2000a, b). To directly test this hypothesis, the rate of oligomer formation in the presence of 20S-produced fragments and liposome binding was assessed for the wildtype and the three PD-associated mutations. Here the "oligomer" is operationally defined as any conformation of αSyn that was detected by the anti-oligomer antibody I-11(Kayed et al. 2003). When the reaction contained only αSyn protein, oligomers were observed only for E46K-syn and A53T-syn (Fig. 4, top panel). The addition of either

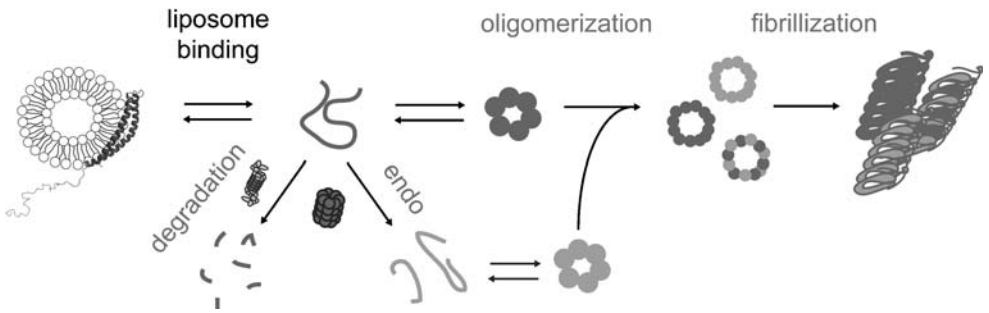


Fig. 5 A dynamic model of Parkinson disease pathogenesis. The point mutations in αSyn that are associated with early-onset Parkinson disease have variable effects on independent reactions which exist within the cell as an interconnected pathway. The *in vitro* system of this study evaluated the effects of the disease-associated mutations on each of these reactions independently and in combination. A correlation was found between all three disease-associated mutations and formation of the presumptive toxic oligomeric species only in the presence of both liposomes and 20S

20S or liposomes failed to induce appreciable oligomer formation in either A30P-syn or wildtype (Fig. 4, middle panels). By contrast, the presence of both liposomes and 20S produced considerable amounts of oligomers from all three disease-associated mutant proteins: A30P-syn, E46K-syn, and A53T-syn (Fig. 4, bottom panel). Therefore, all of the known disease-causing mutations in αSyn accelerate the formation of oligomers only when liposomes and the 20S proteasome are present. Interestingly, under the same conditions (both 20S and liposomes), low levels of oligomers formed from the wildtype protein (Fig. 4, bottom panel). Presumably, gene duplication or other defects that lead to increased levels of wildtype αSyn, such as the Rpt2 knockout (Bedford et al. 2008), would accelerate the concentration-dependent oligomerization. Therefore, these results also suggest a mechanism for αSyn-mediated pathogenesis in the absence of familial mutations, as observed in the majority of PD patients.

Methods

Antibodies Syn303 is a mouse monoclonal antibody specific for amino acids 2-4 of human αSyn (Duda et al. 2002). The secondary antibody used was horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA).

Purification of proteins 20S proteasome was purified from bovine red blood cells essentially as described (McGuire and DeMartino 1986). αSyn proteins were expressed and purified as described (Liu et al. 2005).

Preparation of liposomes Liposomes were prepared essentially as described (Eliezer et al. 2001). Equimolar amounts of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (monosodium salt) (POPA) in chloroform (Avanti Polar Lipids, Inc., Alabaster, AL, USA) were combined, evaporated under nitrogen, and lyophilized for a minimum of 3 h. Following resuspension to a final concentration of 2.5 mM in lipid buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.4), the solution was sonicated for 4 cycles of 2 min each (power 3, 40% duty cycle).

In vitro *αSyn degradation assay* 20S proteasomal degradation of αSyn was carried out as previously described (Liu et al. 2005).

αSyn fibrillization assays 500 μL reactions of 100 μM αSyn were prepared as previously described, with some modification (Liu et al. 2005). Samples also contained either lipid buffer or 250 μM lipids in the form of vesicles. Where present, 20S proteasome was added at a final concentration of 20 nM. Samples were incubated at 37°C for 10 min followed by the addition of the proteasomal inhibitor MG132 at a final concentration of 100 μM. After mixing, the sample was centrifuged at 16,000x*g* for 30 sec at room temperature prior to initiating the ThioflavinT assay. The aggregation reaction was performed in triplicate in microtiter plates with a Teflon bead, essentially as described (Liu et al. 2005). The plates were sealed with an ABI-PRISM optical adhesive cover (Applied Biosystems, Carlsbad, CA, USA), and shaken continuously for 8 min 20 sec of every 10 min at 37°C. Thioflavin T fluorescence was monitored at 450 nm excitation/482 nm emission on a Molecular Devices fluorescence plate reader. An aliquot of the reaction mix was reserved for analysis by western blot. Lag times for initiation of fibril formation were determined by the time at which the Thioflavin T fluorescence signal exceeded twice the noise in the data acquired to that point.

αSyn oligomerization assays 650 μL reactions containing 100 μM αSyn were prepared as for the fibrillization reactions described above. At appointed times, a 10 μL aliquot was removed and spotted 2 μL at a time onto a 0.45 μm nitrocellulose membrane. The membrane was blotted in TBS with 0.05% Tween-20 (TBS-LowTween) with a 1:8,000 dilution of I-11 anti-oligomer antibody ((Kayed et al. 2007), generous gift of R. Kayed, UTMB), and a 1:10,000 dilution of anti-rabbit secondary (Jackson Immuno-Research, #111-035-144; resuspended in water and stored in aliquots at -20°C). Detection was performed with SuperSignal West Dura Extended Duration Substrate (Pierce, #34075). Data from each of two (αSyn truncations) or three (αSyn point mutants) independent experiments were quantitated by densitometry using ImageQuantTL (GE Biosciences) and normalized to a range of 0-1, following which the mean and SEM (truncations) or standard deviation (mutants) were determined.

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Competing Interests The authors declare that they have no competing interests.

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