Parkinson’s Disease-associated α-Synuclein Is More Fibrillogenic than β- and γ-Synuclein and Cannot Cross-seed Its Homologs*

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Parkinson’s disease (PD) is a neurodegenerative disorder that is pathologically characterized by the presence of intracytoplasmic Lewy bodies. Recently, two point mutations in α-synuclein were found to be associated with familial PD, but as of yet no mutations have been described in the homologous genes β- and γ-synuclein. α-Synuclein forms the major fibrillar component of Lewy bodies, but these do not stain for β- or γ-synuclein. This result is very surprising, given the extent of sequence conservation and the high similarity in expression and subcellular localization, in particular between α- and β-synuclein. Here we compare in vitro fibrillogenesis of all three purified synucleins. We show that fresh solutions of α-, β-, and γ-synuclein show the same natively unfolded structure. While over time α-synuclein forms the previously described fibrils, no fibrils could be detected for β- and γ-synuclein under the same conditions. Most importantly, β- and γ-synuclein could not be cross-seeded with α-synuclein fibrils. However, under conditions that drastically accelerate aggregation, γ-synuclein can form fibrils with a lag phase roughly three times longer than α-synuclein. These results indicate that β- and γ-synuclein are intrinsically less fibrillogenic than α-synuclein and cannot form mixed fibrils with α-synuclein, which may explain why they do not appear in the pathological hallmarks of PD, although they are closely related to α-synuclein and are also abundant in brain.

Parkinson’s disease (PD)† is a neurodegenerative disorder that predominantly affects dopaminergic neurons in the nigrostriatal system but also affects several other regions of the brain. Pathological hallmarks of PD are Lewy bodies and Lewy neurites (1–3), which also accumulate in dementia with Lewy bodies (4) but not in a variety of other neurodegenerative disorders. Recently, two dominant mutations in α-synuclein have been linked to familial early onset PD (5, 6). Interestingly, Lewy bodies and Lewy neurites do not stain for β- or γ-synuclein (4, 14–18), a very puzzling result given that Lewy bodies and Lewy neurites are both associated with intracytoplasmic inclusions. An unrelated disorder, multiple system atrophy, shows α-synuclein pathology in the form of glial cytoplasmic inclusions (18, 19), and these inclusions also stain only for α-synuclein (20).

α-Synuclein has been identified as the major filamentous component of Lewy bodies and Lewy neurites in all cases of PD (4, 14), suggesting that Lewy bodies or earlier stage components contribute mechanistically to the degeneration of neurons in PD. Interestingly, Lewy bodies and Lewy neurites do not stain for β- or γ-synuclein (4, 15–18), a very puzzling result given the sequence homology and the overlapping expression of α-, β-, and γ-synuclein in affected regions like the substantia nigra and the overlapping cellular localization of α- and β-synuclein. An unrelated disorder, multiple system atrophy, shows α-synuclein pathology in the form of glial cytoplasmic inclusions (18, 19), and these inclusions also stain only for α- but not β- or γ-synuclein (20).

In vitro studies have shown that recombinant α-synuclein can form Lewy body-like fibrils (21–25) by a nucleation-dependent mechanism (26), and oligomeric intermediates have been described in vitro (21, 27, 28). Most importantly, PD-linked α-synuclein mutations accelerate this aggregation process (24, 25), which suggests that such in vitro studies can have relevance for explaining aspects of PD pathogenesis. We decided to compare the aggregation behavior of α-, β-, and γ-synuclein in a detailed time course to address whether there are fundamental differences among the three homologs that could offer insight into why β- and γ-synuclein are not found in Lewy bodies and Lewy neurites.

Here we show that fresh solutions of β- and γ-synuclein exhibit the same natively unfolded structure as α-synuclein; however, both proteins are not only intrinsically less fibrillogenic than α-synuclein, but they are also not seedable by α-synuclein fibrils. This may explain why they do not appear to make a major contribution to the pathogenesis of Parkinson’s disease.

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‡ The abbreviations used are: PD, Parkinson’s disease; TBS, Tris-buffered saline; FTIR, Fourier transform infrared.

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EXPERIMENTAL PROCEDURES

Cloning, Bacterial Expression, and Purification of Synucleins—Recombinant purified wild type human α-synuclein was generated as described previously (25). Human β-synuclein and γ-synuclein cDNA were obtained by polymerase chain reaction amplification from an adult human cDNA library using the following primers: β-synuclein, CAC AAG ACA TAT GGA CCT GPT CAT GAA GGG CTT GTC CA and TCA CTC GAG TTA CGC CTC TGG CTC ATA CTC CTG ATA TTC; γ-synuclein, CAC AAG ACA TAT GGA CCT GPT CAT GAA GGG CTT GTC CAT CG and ACA CTC GAG TTA GTC TCC CCC ACT CTG GGC CTC CTC TGC CAC TT. The correct DNA sequence was confirmed by DNA sequencing, and Escherichia coli expression and purification were performed as described for α-synuclein (25). The final preparations were >99% pure. α- and β-synuclein proteins ran as single bands on SDS-polyacrylamide gel electrophoresis (Fig. 2). γ-Synuclein ran as a closely spaced doublet, containing full-length protein and a form truncated during bacterial expression after the fifth amino acid (data not shown), which we were not able to separate from the full-length form on a preparative scale.

Aggregation of Synuclein—Purified α-, β-, and γ-synuclein samples were concentrated to the indicated starting concentrations using Centricon-3 spin filters (Amicon). Following concentration, the samples were centrifuged for 10 min at 100,000 × g to remove any aggregates that could have formed during the concentration step. The supernatants were all adjusted to a final concentration of 7 mg/ml using Tris-buffered saline (TBS), which consists of 20 mM Tris, pH 7.5, and 0.2 M NaCl. The samples were then dispersed into 1.5-ml Beckman ultracentrifuge microtubes and were incubated at 37 °C. At various time points, the samples were centrifuged at 100,000 × g for 10 min, and 11 μl of their supernatants were removed and diluted to 110 μl with TBS. These dilutions were then analyzed by their absorbance at 280 nm. The material was stored frozen at 20 °C until needed. In seeded aggregation experiments incubations of soluble α-, β-, and γ-synuclein at concentrations ranging from 2 to 7 mg/ml in TBS + 0.05% sodium azide were spiked with various amounts of pre-formed α-synuclein aggregates to serve as nuclei for fibril formation. The final concentration of seed is reported as a percentage of the soluble synuclein in the incubation (e.g. a 2 mg/ml incubation seeded at a level of 10% contains 0.2 mg/ml seed). Loss of soluble synuclein is measured by A280 of soluble material following ultracentrifugation as described above. For accelerated aggregation experiments with continuous shaking, samples were incubated at 37 °C in an Eppendorf Thermomixer shaken at high speed. At the indicated time points aliquots of 50 μl were subjected to ultracentrifugation as described above, and 20 μl of the resulting supernatant were diluted with 110 μl buffer to determine A280.

Circular Dichroism—CD spectra were determined at 20 °C on a Jasco J-715 Spectropolarimeter, using water-jacketed cuvettes with a path length of either 0.01 (for the far UV region, 250-190 nm, secondary structure) or 1 cm (for the near UV region, 340-240 nm, tertiary structure). Molar ellipticity was calculated using the protein concentration determined as above, and a mean residue weight of 103 for α-, 106.7 for β-, and 104.7 for γ-synuclein.

FTIR Measurement and Analysis—FTIR spectra of protein solutions and aggregates were recorded at room temperature with a Nicolet Magna 550 series II Fourier transform infrared spectrometer, equipped with a DTGS detector. Protein solutions were prepared for infrared measurement in a sample cell (SpectraTech FT04–036) that employed CaF2 windows separated by a 6-μm Mylar spacer. Reference spectra were recorded under identical conditions with appropriate buffer blank in the cell. The spectra for liquid and gaseous water were subtracted from the protein spectra, according to criteria previously established. Aggregate samples were centrifuged at 13,000 rpm for 10 min. The pellet was washed 3 times with distilled water, and the slurries were spread on a 3M disposable polyethylene IR card. After air drying, the infrared spectra were recorded. The spectra for the corresponding film and gaseous water were subtracted from the protein spectrum as appropriate. For each spectrum, a 256-scan interferogram was collected in a single beam mode, with 4 cm⁻¹ resolution. Second-derivative IR spectra were obtained with the derivative function of the Omnic soft-
ware (Nicolet). Derivative spectroscopy was chosen over deconvolution (Fourier self-deconvolution) as a mathematical band-narrowing technique due to its complete objectiveness (29, 30). A major drawback of the Fourier self-deconvolution method is that the choice of values for half-bandwidth and enhancement factor is arbitrary and highly subjective because of the lack of knowledge of the real values, as well as because the component bands may have unequal half-bandwidths.

**Atomic Force Microscopy**—Aggregated γ-synuclein was resuspended in phosphate-buffered saline, and this suspension was vortexed for 10 s. Excess liquid was removed, and the sample on the mica was then imaged under 40× magnification after 3 min. Excess liquid was removed, and the sample on the mica was then imaged under 40× magnification using a Digital Instruments Nanoscope III atomic force microscope. The probe used for imaging was an oxide-sharpened silicon nitride twin tip with a nominal spring constant of 0.58 N/m. The image was obtained in “tapping mode” in fluid using a drive frequency of 8.88 kHz, a drive amplitude of 250 mV, and a set point voltage of 0.378 V.

**RESULTS**

To compare the aggregation properties of α-, β-, and γ-synuclein, we cloned β- and γ-synuclein cDNAs (8, 31) in addition to the previously described α-synuclein cDNA (32), and we generated bacterial expression constructs. Fig. 2 shows an SDS-polyacrylamide gel of the three purified proteins, which are >99% pure. To address whether α-, β-, and γ-synuclein differ in their conformation, we performed CD and FTIR spectroscopy; fresh solutions of all three proteins showed the same natively unfolded structure with identical near and far UV CD spectra (Fig. 3A), confirming the far UV CD results of Serpell et al. (33). The titration curves of the α-helix inducing agent trifluoroethanol for β- and γ-synuclein are identical to that for the α-synuclein, demonstrating that under conditions that strongly favor α-helix these proteins have the same propensity for helical formation (data not shown). We then subjected α-, β-, and γ-synuclein to FTIR spectroscopy, which is more sensitive for β-sheet structure than CD spectroscopy. The FTIR spectra are nearly identical with the amide I absorption maximum for all three proteins at 1650 cm⁻¹, indicating that they contain primarily random coil structure.

In our previously established in vitro system α-synuclein forms fibrillar aggregates during extended incubations by a nucleation-dependent mechanism, and both PD mutations accelerate this aggregation (to a different extent) (25, 26). We now followed a similar aggregation time course to compare α-, β-, and γ-synuclein in parallel at the same concentrations (Fig. 4A). Under the experimental conditions we reproduced the previously reported loss of soluble α-synuclein, which is accompanied by the formation of fibrils (25); the depletion of soluble monomer continues until the critical concentration is reached (26). In contrast, we did not observe a loss of soluble material for either β- or γ-synuclein at the same concentration (Fig. 4A).

However, in a nucleation-dependent process like α-synuclein aggregation, the rate-limiting step is the formation of nuclei. Thus, while β- and γ-synuclein do not spontaneously form fibrils, under the conditions of our experiment, they could still be aggregation-competent and participate in fibril elongation once a seed is provided. This could have pathological relevance, if the more rapidly aggregating α-synuclein was able to cross-seed β- or γ-synuclein. To address this possibility, we performed cross-seeding experiments in which we tried to seed α-, β-, and γ-synuclein with α-synuclein seeds at 1% of the monomer concentration. We have previously shown that under these conditions α-synuclein fibrillogenesis is strongly accelerated (26), and we reproduce this finding here (Fig. 4B). However, no fibril formation of β- or γ-synuclein is detectable (Fig. 4B), indicating that neither of these proteins can be cross-seeded by α-synuclein.

To test whether β- and γ-synuclein can form fibrils on their own at all, we forced the conditions toward aggregation by performing incubations with continuous shaking at 37 °C and 3 mg/ml (200 μM). Under these conditions a solution of α-synuclein monomers is converted into fibrils within 1 day (versus 10 days in the non-shaken paradigm) and shows a lag phase of 7–10 h (Fig. 5). When β-synuclein was incubated with continuous shaking, we did not observe a reduction in soluble material even after several days (Fig. 5). We extended the incubation to several weeks and still did not detect loss of β-synuclein (data not shown). However, γ-synuclein in solution did decrease after an initial lag phase of about 1 day (approx-
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Approximately three times that of α-synuclein) and reached steady state at about 2.5 days (Fig. 5). Paralleling the loss of soluble γ-synuclein, insoluble material could be precipitated by centrifugation. When we analyzed the γ-synuclein pellet by atomic force microscopy, we could clearly detect fibrils (Fig. 6A); their average height was ~7 nm. To gain structural information, we performed FTIR spectroscopy of the γ-synuclein precipitate.

Fig. 4. Comparison of α-, β-, and γ-synuclein aggregation. A, aggregate formation of α-synuclein (■), β-synuclein (○), and γ-synuclein (□) at 5.5 mg/ml as monitored by A280 of soluble material following ultracentrifugation (see “Experimental Procedures”). Values shown are average of triplicate incubations ± S.E. B, aggregation of α- (■), β- (○), and γ-synuclein (□) at 5.5 mg/ml seeded with 1% α-synuclein seeds. A non-seeded α-synuclein control incubation (■) is also shown for comparison. Values shown are the average of triplicate incubations ± S.E.

Fig. 5. Aggregation of α- (■), β- (○), and γ-synuclein (□) at 3 mg/ml with continuous shaking during the incubation. Values shown are means of 12 incubations ± S.E.

Fig. 6A shows the second derivative FTIR spectrum; the strong low wave number β-sheet band at 1629 cm⁻¹ together with a weak high wave number β-sheet band at 1696 cm⁻¹ indicates the presence of predominantly antiparallel and intermolecular...
β-sheet structure. The weak bands at 1644 (β-sheet), 1650 (unordered), 1654 (α-helix), and 1661 (turn) and the band at 1673 cm⁻¹ (turn) suggest the existence of a small amount of other secondary structures as well. The fact that the frequencies of the low wave number β-sheet bands of α- and γ-synuclein are the same suggests that the intermolecular arrangements of the β-sheet structures are similar between α- and γ-synuclein (Fig. 6B). We have shown in Fig. 4, A and B, that under our standard non-shaken conditions γ-synuclein does not aggregate and cannot be cross-seeded by α-synuclein. However, forcing aggregation by continuous shaking showed that γ-synuclein does not only have the intrinsic capacity to form fibrils (Fig. 5), but in doing so shows a pronounced lag phase, which is an indication of nucleation-dependent aggregation (34). To test for this mechanism, we added preformed γ-synuclein aggregates as seeds to a fresh γ-synuclein solution. This resulted in bypass of the lag phase and initiated aggregation (Fig. 6C), with the rate of fibrillogenesis being seed concentration-dependent. Thus, γ-synuclein fibril formation, like α-synuclein fibril formation, is nucleation-dependent. In summary, we could not detect fibrillogenesis of β-synuclein in any of our paradigms, but under extreme conditions γ-synuclein can be forced to form fibrils by a nucleation-dependent mechanism; however, γ-synuclein cannot be cross-seeded by α-synuclein.

DISCUSSION

Here we compared the in vitro aggregation properties of α-, β-, and γ-synuclein. We reproduce our previous results on the in vitro fibril formation of α-synuclein by a nucleation-dependent mechanism. β- and γ-synuclein incubated at the same concentrations under the same conditions do not form fibrils within the same time frame as α-synuclein. Although the latter starts to form fibrils around day 6, incubation of β- and γ-synuclein for a period three times longer did not result in loss of soluble material. However, if the incubation at 37 °C was extended over 4 weeks, we did occasionally, but not reproducibly, observe a loss in soluble β-synuclein, and we could spin out an amorphous precipitate of different appearance than the α- and γ-synuclein fibrils. Atomic force microscopy analysis of this precipitate did not reveal fibrils or protofibrillar intermediates, and providing preformed precipitate as a seed could not accelerate its formation (data not shown). Therefore, we conclude that this material does not form ordered fibrils but is instead an unstructured precipitate, due to protein denaturation.

We further tried to force aggregation by continuous shaking and increased concentration, and we observed dramatically accelerated aggregation of α-synuclein, as shown in a 15–20-fold reduction in lag time (7–10 h versus 6 days). Under these conditions we can generate atomic force microscopy-detectable γ-synuclein fibrils of ~7 nm height, which show a cross-β-sheet structure by FTIR. Although in our standard, non-shaken incubation paradigm γ-synuclein does not aggregate for up to 4 weeks, it can be seeded by the addition of exogenous preformed γ-synuclein seeds. These results establish that γ-synuclein can form well structured fibrils of similar height as α-synuclein filaments and that fibrillogenesis occurs by a nucleation-dependent mechanism as in α-synuclein. Again, even under these accelerated conditions, we cannot induce β-synuclein aggregation, even if the experiment is extended to several weeks. Along this line, a study investigating the induction of α-synuclein aggregation by iron reports no ferric ion effect on β-synuclein (27). However, Serpell et al. (33) report “small numbers of filaments” detectable by electron microscopy after 5 weeks of shaken incubation for γ- as well as β-synuclein. Finally, the most important aspect of our study addresses the in vitro situation of synuclein coexpression and the possibility of heterogeneous seeding. We show that neither β- nor γ-synuclein can be seeded by α-synuclein.

These results should be useful to get a first hint at the exact regions of α-synuclein that are critical for fibril formation; the fact that β-synuclein completely fails to fibrillize under our conditions, although it has a higher overall homology to α-synuclein, than γ- has to α-synuclein, suggests that the region between amino acids 72 and 84, which is lacking in β- but not γ-synuclein, may contain critical fibrillation determinators. Consistent with this idea, two independent standard secondary structure prediction programs (35, 36) both suggest that within the α-synuclein protein this particular region has the highest propensity to form β-sheets (Fig. 1B). Interestingly, the Chou-Fasman program predicts a tendency for β-sheet in the corresponding region of γ-synuclein, spanning amino acids 74–81, but not β-synuclein, consistent with the idea that this region may be critical for fibril formation. The pathogenic A53T mutation in α-synuclein even completely abolishes the α-helical stretch from amino acids 51–66 and results in an additional β-sheet spanning amino acids 51–58 (Chou-Fasman). Thus, the disease-associated A53T mutation induces a nearly continuous β-sheet stretch in α-synuclein between amino acids 51 and 81 and shows significantly accelerated fibrillogenesis in our in vitro assays (25). Unlike β-synuclein, γ-synuclein carries a threonine at position 53 (see Fig. 1A) and, consequently, shows a somewhat extended β-sheet in this area. However, the effect is small and, in contrast to the pathogenic A53T α-synuclein mutation, has nearly no consequence for the helical properties, which in both cases still dominate the overall structure. We feel that the differences in fibrillogenesis between β- and γ-synuclein cannot be attributed to just the threonine at position 53 alone but are most probably associated with the existing β-sheet at position 74–81, stabilized or extended by β-sheet-favoring sequences, e.g. Ala → Thr, or α-helix breakers like A30P.

Lewy bodies and Lewy neurites contain α-synuclein as their major fibrillar component and are defining pathological hallmarks of Parkinson’s disease. It is unknown if and how the Lewy bodies and/or Lewy neurites cause neuronal degeneration. The finding that two α-synuclein mutations cause familial autosomal dominant PD and that both mutations enhance aggregation of α-synuclein in vitro suggests a simple working model according to which at least some cases of PD could be due to enhanced aggregation of α-synuclein, leading to the formation of Lewy bodies and Lewy neurites and somehow causing the observed neuronal loss.

β- and γ-synuclein are abundant in brain; both are similar to α-synuclein (78 and 60%, respectively), and both are expressed in regions of Lewy body formation and PD pathology. However, they have not been linked to PD genetically, and although a recent study describes novel hippocampal axonal lesions involving all synucleins (37), β- and γ-synuclein are conspicuously absent from Lewy bodies and Lewy neurites as well as from the glial cytoplasmic inclusions of multiple system atrophy. This study offers a simple explanation for this conundrum by demonstrating the following. (i) In two aggregation paradigms (with and without continuous shaking) both β- and γ-synuclein are intrinsically much less aggregation-competent than α-synuclein. (ii) Despite their high sequence homology neither β- nor γ-synuclein can be cross-seeded by α-synuclein. This may explain why Lewy bodies contain α-synuclein fibrils but no β- or γ-synuclein fibrils. The intrinsically lower aggregation propensity of β- and γ-synuclein compared with α-synuclein may also account for the failure to detect PD mutations within these genes. Pathogenic mutations in β- and γ-synuclein would need to have drastic effects to accelerate aggregation to even the level of wild type α-synuclein. The
consistency between the low aggregation propensity of β- and γ-synuclein and their lack of involvement in fibrillary synuclein pathology provide additional evidence for the hypothesis that α-synuclein aggregation plays a specific and critical role in neurodegeneration.

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