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Aggregation-defective α-synuclein mutants inhibit the fibrillation of Parkinson's disease-linked α-synuclein variants

Hyun-Jung Koo [1], Min Yeong Choi, Hana Im *

Department of Molecular Biology, Sejong University, 98 Gunja-dong, Kwangjin-gu, Seoul 143-747, Republic of Korea

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ABSTRACT

α-Synuclein comprises the fibrillar core of Lewy bodies, which is one of the histologically defining lesions of Parkinson's disease. Previously, we screened for α-synuclein substitution mutants that do not form fibrils. For preventative or therapeutic uses, it is essential to suppress the oligomerization/fibrillation of the wild-type and PD-linked α-synuclein proteins. Here we have examined the effects of fibrillation-retarded α-synuclein mutants on fibril formation by wild-type and PD-linked α-synuclein molecules. Six self-aggregation-defective α-synuclein mutants completely inhibit the fibrillation of both wild-type and Parkinson's disease-linked α-synuclein variants. These results suggest future applications for gene therapy: the transplantation of a fibrillation-blocking mutant α-synuclein gene into individuals who carry an early-onset PD-associated α-synuclein allele. Short synthetic peptides derived from these mutant sequences may also serve as a lead compound for the development of therapeutics for Parkinson's disease.

Protein amyloidosis or fibrillogenesis is related to certain fatal neurodegenerative disorders [1,2], such as Alzheimer's disease, Parkinson's disease (PD), and prion diseases. PD patients exhibit characteristic movement disorders, including resting tremor, balance impairment, rigidity, and bradykinesia [3]. α-Synuclein is the major component of the intracellular protein aggregates (Lewy bodies and Lewy neurites) found in dopaminergic neurons in the substantia nigra of PD patients [4]. Aggregation of α-synuclein is also closely associated with other neurodegenerative disorders, the so-called synucleinophathies, which include the Lewy body variant of AD, dementia with Lewy body, and multiple-system atrophy [5]. Three missense mutations in the α-synuclein gene (A30P, A53T, and E46K) have been associated with PD [6–8]. That these PD-associated mutations accelerate the fibrillation or oligomerization of α-synuclein in vitro [9,10] supports the involvement of α-synuclein fibrillation in PD pathogenesis. High intracellular concentrations of wild-type α-synuclein due to gene triplication and duplication also cause inherited forms of PD [11–13]. Several studies support the linkage between concentration-dependant aggregation of α-synuclein and PD; an increased concentration of the protein facilitates fibril formation in vitro, while the overexpression of α-synuclein in transgenic flies [14] and mice [15] produces neuropathologic symptoms characteristic of PD. Therefore, the inhibition of α-synuclein aggregation may be an appropriate approach to preventing and/or treating PD and other synucleinophathies.

Despite the general belief in a non-specific protein aggregation process, protein aggregation involves specific interactions among partially folded intermediates [16,17]. The effects of protein-specific nucleation on α-synuclein fibrillation [18] and paramagnetic resonance spectroscopy of spin-labeled α-synuclein fibrils [19] also support the notion of a sequence-specific aggregation process for the α-synuclein protein [20]. The sequence specificity of α-synuclein aggregation and the discovery of missense α-synuclein mutants with altered fibrillation kinetics have motivated us to search for substitution mutants that efficiently block the aggregation of α-synuclein.

α-Synuclein is a small protein of 140 amino acids that consists of an N-terminal repeated region, a central hydrophobic non-amyloid β component (NAC) fragment, and a C-terminal highly acidic region [21]. We have previously shown that β-packing and hydrophobicity in the central region are critical for the fibrillation of α-synuclein [22]. In particular, substitution in α-synuclein of a proline residue, which is a well known secondary structure breaker, has a major impact on the propensity of the protein to form fibrils. Incorporation of charged/polar residues into the NAC region also dramatically inhibits the fibrillation of α-synuclein. We have

Abbreviations: PD, Parkinson's disease; PBS, phosphate-buffered saline; ThT, thioflavin T; AD, Alzheimer's disease; NAC, non-amyloid β component.

* Corresponding author. Fax: +82 2 3408 4336.
E-mail address: hanaim@sejong.ac.kr (H. Im).
[1] Present address: Kyung Hee University, School of Medicine, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea.

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identified more than 20 single-amino acid substitutions that block self-aggregation, even during prolonged incubation. Consistent with the results, deletion of a 12-amino acid motif in the middle of the central region (residues 71–82) abolishes α-synuclein fibrillation [23]. Deletion of an overlapping sequence (residues 66–74) also abolishes the oligomerization and filamentation of α-synuclein [24]. In contrast, deletion of the N-terminal region [6] or truncation of the highly charged C-terminal tail [25] accelerates the formation of β-sheet-rich oligomers and fibrils, possibly by increasing the overall hydrophobicity of the α-synuclein protein. Increasing the number of negative charges in the N-terminal region also interferes with the filamentation of α-synuclein [22].

To have therapeutic values, it is essential to suppress the oligomerization/fibrillation of the wild-type and PD-linked α-synuclein proteins. In the present study, we tested whether our self-fibrillation-defective α-synuclein mutants were able to prevent the polymerization of wild-type and/or PD-linked α-synuclein variants. The results can be used to design small β-sheet breaker peptides that inhibit α-synuclein fibrillation, implicated in pathology of PD.

Materials and methods

Expression and purification of α-synuclein mutant proteins. The cDNA encoding for human α-synuclein was cloned on a plasmid for the expression in *Escherichia coli* generating pSyn [22]. *E. coli* BL21(DE3) harboring pSyn was grown at 37 °C in Luria–Bertani broth containing 100 μg/ml ampicillin. When A_{600} reaches about 0.6, IPTG was added to the final concentration of 0.1 mM to induce α-synuclein production. After further incubation at 37 °C for 3 h, cells were harvested by centrifugation and resuspended in the lysis buffer (50 mM Tris–Cl, 50 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 8.0). Cells were disrupted using a HD2200 sonicator (Bandelin, Germany) with a TT13 tip at 50% cycle (1 min × 3 times), and cell lysate was clarified by high-speed centrifugation at 9800*g* for 30 min at 4 °C. Supernatant fraction was heat-treated at 95 °C for 10 min in the presence of 0.5 M NaCl. Insoluble aggregates were removed by centrifugation at 9800*g* for 30 min. The supernatant was dialyzed against the loading buffer (10 mM phosphate, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 6.5), and α-synuclein was purified by anion exchange column chromatography on Q-Sepharose (Amersham Pharmacia Biotech.). Purified α-synuclein was dialyzed against phosphate-buffered saline (PBS; 1.76 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, and 137 mM NaCl, pH 7.4). Purified protein was analyzed on 15% SDS–polyacrylamide gels, and visualized by staining with Coomassie Brilliant Blue. Protein concentration was measured using Bio-Rad DC protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), using bovine serum albumin as a standard.

Table 1
The effects of fibrillation-retarded mutant α-synucleins on fibrillation kinetics of the wild-type molecules.

Mutant[a]	Lag time (Day)[b]	Max time (Day)[c]	F_{max}[d]	Mixture	Lag time (Day)[b]	Max time (Day)[c]	F_{max}[d]
(1) Mutants that interact with the WT α-synuclein and are incorporated into the fibrils							
WT[e]	1.75 ± 0.3	3 ± 0.2	250 ± 32.7	WT/2	3.5 ± 0.2	5 ± 0.4	100 ± 7.1
V16P	5 ± 0.7	8.5 ± 0.7	140 ± 18.4	V16P/2 + WT/2	3 ± 0.4	6.5 ± 0.4	150 ± 25.5
A18P	4 ± 0.5	9 ± 0.6	80 ± 10.6	A18P/2 + WT/2	2.75 ± 0.2	5.5 ± 0.4	75 ± 3.5
K23E	5.5 ± 0.6	9 ± 0.4	300 ± 47	K23E/2 + WT/2	1.75 ± 0.2	4 ± 0.4	270 ± 28.3
V26P	4.5 ± 0.8	7.75 ± 0.9	25 ± 3.2	V26P/2 + WT/2	2.75 ± 0.2	3.75 ± 0.5	100 ± 17.7
A27P	4.5 ± 0.5	6 ± 0.4	300 ± 28.3	A27P/2 + WT/2	2.5 ± 0.2	4.75 ± 0.5	270 ± 38.9
A29P	2.75 ± 0.4	7.5 ± 0.6	250 ± 35.4	A29P/2 + WT/2	2 ± 0.4	4.75 ± 0.7	210 ± 14.1
T64P	>50	>50	0	T64P/2 + WT/2	2.75 ± 0.2	3.75 ± 0.2	130 ± 24.7
(2) Mutants that do not interact with the WT α-synuclein							
V37P	>20	>20	0	V37P/2 + WT/2	3.75 ± 0.2	7.5 ± 0.4	100 ± 7.1
V49P	>15	>15	0	V49P/2 + WT/2	3.75 ± 0.2	6 ± 0.2	110 ± 24.7
V63G	>20	>20	0	V63G/2 + WT/2	3.75 ± 0.2	5.5 ± 0.4	145 ± 10.6
N65P	>50	>50	0	N65P/2 + WT/2	3 ± 0.4	4.5 ± 0.2	120 ± 28.3
V70E	>20	>20	0	V70E/2 + WT/2	3 ± 0.7	4.75 ± 0.5	130 ± 22.6
V70P	15 ± 0.6	19 ± 0.9	135 ± 14.1	V70P/2 + WT/2	3.75 ± 0.2	5.5 ± 0.4	160 ± 7.1
T72E	>20	>20	0	T72E/2 + WT/2	3.75 ± 0.4	6 ± 0.4	110 ± 28.3
K80Q	>25	>25	0	K80Q/2 + WT/2	3.5 ± 0.4	5.5 ± 0.4	120 ± 7.1
(3) Mutants that retard fibrillation of the WT α-synuclein							
K45E	8.5 ± 0.5	14.5 ± 1.2	120 ± 10	K45E/2 + WT/2	7 ± 0.4	9.5 ± 0.4	130 ± 10.6
T59P	12.5 ± 0.2	14 ± 0.3	55 ± 5.4	T59P/2 + WT/2	8.75 ± 0.5	11.5 ± 1.1	60 ± 3.5
V63E	>20	>20	0	V63E/2 + WT/2	5 ± 0.4	8.75 ± 0.2	150 ± 14.1
V63P	>30	>30	0	V63P/2 + WT/2	5.5 ± 0.4	7.5 ± 0.9	130 ± 17.7
G68P	>49	>49	0	G68P/2 + WT/2	5 ± 0.5	6.75 ± 0.5	140 ± 14.1
V70G	>26	>26	0	V70G/2 + WT/2	4.75 ± 0.2	11 ± 0.7	120 ± 3.5
T81P	>25	>25	0	T81P/2 + WT/2	4.5 ± 0.5	9 ± 0.5	95 ± 3.5
E83P	>25	>25	0	E83P/2 + WT/2	4.75 ± 0.2	6 ± 0.7	110 ± 10.6
A89P	>21	>21	0	A89P/2 + WT/2	5 ± 0.4	6.5 ± 0.7	100 ± 14.1
V48P	>25	>25	0	V48P/2 + WT/2	6 ± 0.4	8 ± 0.4	30 ± 3.5
G67P	>30	>30	0	G67P/2 + WT/2	5.5 ± 0.4	7.5 ± 0.4	45 ± 3.5
(4) Mutants that completely inhibit fibrillation of the WT α-synuclein							
V66S	>21	>21	0	V66S/2 + WT/2	>10	>10	0
V66P	>30	>30	0	V66P/2 + WT/2	>10	>10	0
T72P	>53	>53	0	T72P/2 + WT/2	>10	>10	0
V74E	>20	>20	0	V74E/2 + WT/2	>10	>10	0
V74G	>26	>26	0	V74G/2 + WT/2	>10	>10	0
T75P	>46	>46	0	T75P/2 + WT/2	>10	>10	0

[a] The fibrillation patterns for mutants only samples are from the previous study [22].
[b] The incubation time during which no change in ThT fluorescence occurred, indicating that no fibrils were formed.
[c] The incubation time at which the ThT fluorescence signal reached its maximum intensity and remained constant.
[d] The maximum ThT fluorescence value (arbitrary units) at Max Time.
[e] WT: wild-type.

Inhibition of α-synuclein fibril formation by mutant α-synuclein proteins. Monomeric wild-type or PD-linked α-synuclein proteins in PBS were incubated at 37 °C with shaking at 150 rpm (1205 SWI Shaking Waterbath, Vision Co.), either alone or with self-aggregation-defective α-synuclein mutants at a molar ratios of 1:1, making the total concentration of α-synuclein to 350 μM. To monitor the degree of fibril formation, 20 μl of samples were taken at each time points and added to 3 ml of 20 μM thioflavin T solution, pH 7.4 (ThT; Sigma Co.). Fluorescence emission was measured using a spectrophotometer (Shimadzu RF-5301PC) with excitation wavelength at 450 nm (with slit width of 5 nm) and emission at 482 nm (with slit width of 3 nm). Fluorescence readings were fitted to a two state model. The degrees of fibrillation were also monitored by analyzing the amounts of remaining soluble α-synuclein proteins on 15% SDS–polyacrylamide gels.

Results

Fibril formation by the wild-type α-synuclein proteins can be blocked by self-fibrillation-defective α-synuclein mutants

Previously, we screened for α-synuclein substitutions that efficiently delayed or blocked self-aggregation. For preventative or therapeutic uses, it is essential to suppress the oligomerization/fibrillation of the wild-type and PD-linked α-synuclein proteins. The effects of fibrillation-retarded or -blocked α-synuclein mutants on fibril formation by wild-type molecules were examined by co-incubation of mutant synuclein with wild-type molecules in fibrillation reactions. Fibril formation was monitored based on the binding to the amyloid-specific fluorescence dye ThT. Typically, ThT fluorescence did not change significantly during the initial lag phase. Thereafter, it increased exponentially for a period, and then remained almost constant. Consistent with previous studies, fibrillation of wild-type α-synuclein started at ~1.75 day and reached maximum fluorescence (F_{max}) at ~3 days. For the co-incubation studies, equimolar amounts of the mutant and wild-type α-synuclein were combined to a final concentration of 350 μM. Since protein aggregation is a concentration-dependent process, lowering the protein concentration in the fibrillation reaction by half delayed the aggregation kinetics and reduced the eventual levels of fibrils, as evidenced by the low F_{max} value for the half-concentration of wild-type α-synuclein alone (WT/2 in Table 1, Fig. 1A).

The effects of fibrillation-retarded mutants on the fibrillation of wild-type α-synuclein can be categorized into four groups (Table 1). (1) Mutants that facilitate the fibrillation of the wild-type

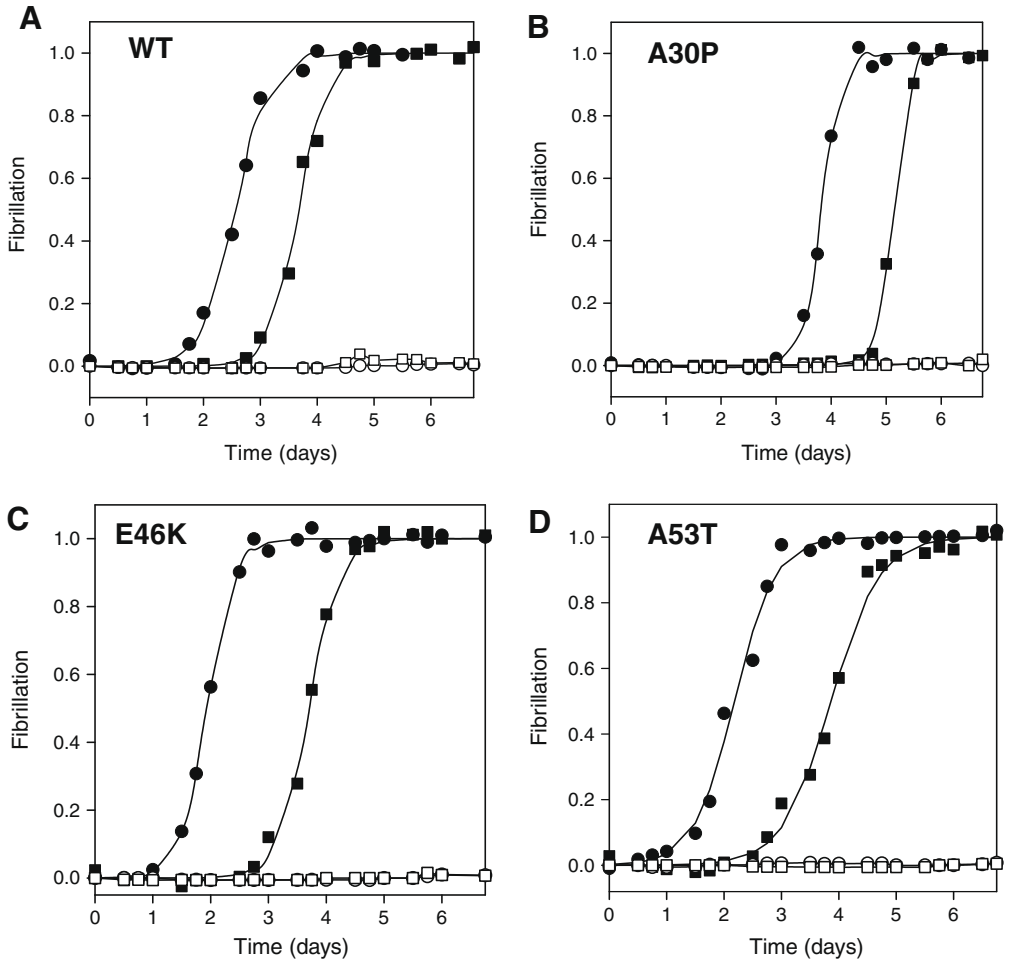

Fig. 1. Suppression of wild-type and PD-linked α-synuclein fibrillation by self-fibrillation-defective α-synuclein mutants. Fibrillation-blocking mutants, T72P and T75P, were added separately to the wild-type (A) or PD-linked mutant α-synucleins A30P (B), E46K (C), and A53T (D) at a molar ratio of 1:1. Fibrillation of α-synuclein was followed by fluorescence emission at 482 nm following binding to the amyloid-specific fluorescence dye ThT. The maximum ThT fluorescence of wild-type and PD-linked α-synuclein without the added fibrillation defective α-synuclein mutant was taken as 1.0. Symbols: ●, wild-type or PD-linked α-synuclein alone; ■, half-concentration of wild-type or PD-linked α-synuclein alone; ○, a half-concentration of wild-type or PD-linked α-synuclein incubated with a half-concentration of T72P α-synuclein mutant; □, a half-concentration of wild-type or PD-linked α-synuclein incubated with a half-concentration of T75P α-synuclein mutant.

α-synuclein. These mutants may increase the concentration of α-synuclein molecules to be incorporated into the fibrils. Fibrillation kinetics of co-incubated mixtures was accelerated compared to that of the half-concentration of wild-type α-synuclein alone (WT/2). For most of the fibrillation-retarded mutants and a self-fibrillation-defective mutant (T64P), the addition of a mutant protein to the wild-type molecule resulted in accelerated fibrillation kinetics, which suggests that they interact with the wild-type protein molecules and are incorporated into the fibrils. (2) Mutants that do not interact with the wild-type α-synuclein. In this case, the presence of mutant α-synuclein should not affect fibrillation kinetics of the wild-type α-synuclein. Some self-fibrillation-defective mutants (V37P, V49P, V63G, N65P, V70E, T72E and K80Q) did not seem to interact with the wild-type molecules and remained self-fibrillation-defective, as the fibrillation kinetics of the mixtures appeared similar to that of the half-concentration of wild-type α-synuclein alone. V70P, a fibrillation-extremely retarded mutant, also did not affect fibrillation of the wild-type molecules. (3) Mutants that retard the fibrillation of the wild-type α-synuclein. Many of the self-fibrillation-defective mutants (i.e., V63E, V63P, G68P, V70G, T81P, E83P, A89P, V48P, and G67P) and two fibrillation-retarded mutants (K45E and T59P) appeared to interact with the wild-type molecule and impede the fibril formation, as revealed by the delayed fibrillation kinetics of the co-incubated mixtures. Although the suppression of the wild-type fibrillation was not complete, two self-fibrillation-defective mutants (V48P and G67P) formed only low levels of fibrils with delayed kinetics. (4) Mutants that interact with the wild-type molecule and completely inhibit the fibrillation of the wild-type α-synuclein. The rate of fibrillation and the amount of fibrils should be reduced. The most exciting results were that six self-fibrillation-defective mutants (V66S, V66P, T72P, V74E, V74G, and T75P) completely suppressed fibrillation of the wild-type α-synuclein molecule (Table 1; Fig. 1A for T72P and T75P).

Some self-fibrillation-defective α-synuclein mutants also block fibril formation of PD-linked α-synuclein proteins

The question of whether these fibrillation-blocking mutants are able to prevent the aggregation of early onset familial PD-linked α-synuclein variants is also addressed. Consistent with previous studies, in the absence of the addition of other mutants, the fibrillation rates of A53T and E46K were accelerated, while that of A30P was slightly retarded, as compared to that of the wild-type α-synuclein [9,26]. When the self-fibrillation-defective mutants T72P and T75P were added separately to PD-linked α-synuclein variants at a molar ratio of 1:1, fibril formation by all three PD-linked missense mutants was suppressed (Fig. 1B–D). Amyloid formation was also monitored by measuring the decrease in the amount of soluble monomers or oligomers in the reaction solution (Fig. 2). Consistent with the above ThT fluorescence results, the amount of soluble α-synuclein decreased quickly when the wild-type and PD-linked α-synuclein variants were incubated without any fibrillation-blocking mutant. However, the disappearance of soluble α-synuclein molecules was inhibited when a fibrillation-suppressing mutant (T72P or T75P) was co-incubated with these proteins, and most α-synuclein molecules remained soluble. Indeed, there was a good correlation between the increase in ThT fluorescence and the decrease in soluble α-synuclein protein.

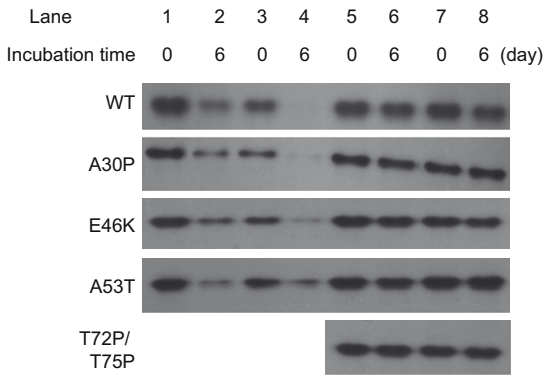

Fig. 2. The wild-type and PD-linked α-synuclein molecules remained soluble by addition of self-fibrillation-defective α-synuclein mutants. Monomeric wild-type or PD-linked α-synuclein proteins in PBS were incubated at 37 °C with shaking, either alone or with self-aggregation-defective α-synuclein mutants at a molar ratios of 1:1. The total concentration of α-synuclein was 350 μM. The amount of soluble α-synuclein protein remaining was monitored by 15% SDS–PAGE. Fibril formation was assessed by the amount of α-synuclein protein disappeared from the soluble fraction. Lanes 1 and 2, wild-type or PD-linked α-synuclein alone; lanes 3 and 4, half-concentration of wild-type or PD-linked α-synuclein alone; lanes 5 and 6, a half-concentration of wild-type or PD-linked α-synuclein incubated with a half-concentration of T72P α-synuclein mutant; lanes 7 and 8, a half-concentration of wild-type or PD-linked α-synuclein incubated with a half-concentration of T75P α-synuclein mutant. Bottom gel shows either T72P (left two lanes) or T75P (right two lanes) α-synuclein proteins incubated alone.

Discussion

We have hoped to use specific protein–protein interactions to prevent fibrillation of α-synuclein, the aggregated form of which has been implicated in the etiology of PD. In contrast to the general belief in a non-specific protein aggregation, fibrillation of α-synuclein molecules has been suggested to be a sequence-specific aggregation process [20]. To select most effective protein sequences, we tested whether self-fibrillation-defective α-synuclein mutants were able to suppress the fibrillation of wild-type or PD-linked missense α-synuclein molecules. Most of the fibrillation-delayed mutants interacted with the wild-type molecules and accelerated fibrillation rates compared to the wild-type alone samples, possibly by increasing concentration of α-synuclein molecules (Table 1). Some of the self-fibrillation-defective α-synuclein mutants appeared to lack the ability to interact with wild-type α-synuclein molecules, as revealed by the fibrillation kinetics, which was very similar to that of the wild-type α-synuclein alone. Meanwhile, other self-fibrillation-defective α-synuclein mutants significantly delayed the fibrillation of wild-type α-synuclein molecules. These mutants appear to retain the factors critical for inter-molecular interactions, although the structural modifications caused by the mutation may not be sufficiently strong to block the fibrillation of α-synuclein. Interestingly, we identified six α-synuclein mutants that effectively block fibrillation of the wild-type α-synuclein molecules. Those mutants are likely to retain residues critical for annealing to the wild-type molecules while undergoing structural modifications that are sufficient to prevent further growth to larger oligomers, protofibrils or fibrils. All six fibrillation-blocking mutants contain substitutions at residues 66–75. Our results are consistent with those of previous studies, in which the central hydrophobic region, particularly residues 61–78, was found to be essential for the fibrillation of α-synuclein [24,27]. Our mutants also prevented the fibrillation of PD-associated α-synuclein missense mutants (Fig. 1 and 2). Although safety issues and technical problems remain to be resolved, these results suggest future applications for gene therapy, which might be, for example, the transplantation of a fibrillation-blocking mutant α-synuclein gene into individuals who carry an early-onset PD-associated α-synuclein allele, so as to suppress facile oligomerization/fibrillation of the protein.

Six fibrillation-blocking α-synuclein mutant sequences can be applied to design β-sheet-breaking peptides to block fibrillation of α-synuclein. Several small molecules, such as curcumin and tan-

nic acid, prevent in a non-specific manner the fibrillogenesis of amyloidogenic proteins, such as α-synuclein and amyloid β-protein [28]. However, it is desirable to target a specific amyloidogenic protein rather than all fibrillation-prone proteins, to avoid unexpected side effects, considering the currently-unknown functions of the proteins involved in conformation-related diseases. β-Sheet breaker peptides that originate from a specific protein sequence are likely to anneal to the corresponding region of the target protein in a specific fashion, as full-length α-synuclein proteins do in fibrils [19]. Therefore, in addition to the ability to block pathologic conformational changes, β-sheet breaker peptides offer the possibility of generating therapeutic compounds that are targeted to each of these conformation-related diseases. Indeed, β-sheet breaker peptides derived from amyloid β-protein cross the blood–brain barrier and inhibit amyloid deposition and brain damage in transgenic mouse models of AD [29]. Similar approaches can be taken for the development of therapeutic compounds for PD, for which no effective cure is currently available.

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