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Accelerated Fibril Formation of α-Synuclein by an IF-Inserted F36V Mutant

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FK506 binding proteins (FKBPs) are molecular chaperones with the *cis-trans* peptidyl-prolyl isomerase (PPIase) activity, that are used to help nascent proteins fold correctly.[1] Some FKBPs, including FKBP12, FKBP38, FKBP52, FKBP65, Pin1, and cyclophilin A, have been shown to possess PPIase activity, and have also exhibited the potential to modulate alpha-synuclein (α-Syn) aggregation, which plays a crucial role in Parkinson's Disease (PD).[2] Among these, FKBP12 is the most potent for stimulating the aggregation of α-Syn, as has been demonstrated *in vitro*[3] as well as in a neuronal cell culture model.[4] These findings led us to develop better FKBP12-derived chaperones that can more effectively regulate the aggregation of α-Syn as targets for PD treatment.

With an aim to construct a more powerful molecular chaperone with a higher PPIase activity, we engineered the F36V system, which is similar to that reported by the Schmid group.[5,6] In a previous study, we successfully constructed an F36VIF hybrid by replacing the flap of F36V with an insertion-in-flap(IF) domain from sensitive-to-lysis D (SlyD) protein of *Escherichia coli* (*E. coli*).[7] The IF that was inserted was the 61- amino acid domain starting with AYG and ending with LKF that protrudes from a loop of the FKBP domain near the PPIase catalytic site, which is indicative of its hydrophobicity. The F36V mutant imparted a better model to fit ligands *via* this protruding structural part than FKBP12.[8] By exploiting the structural contact between them, the Wandless group established a regulation system in which the stability of a target protein fused to some mutants of F36V depends on the presence of a small molecule, such as Shield-1;[9] when fused with a destabilizing domain (DD), the target protein is believed be degraded either *via* a proteasome pathway or by another process that has not yet been identified. Applying the cleaning-up concept that uses a DD domain to degrade pre-formed aggregated proteins, which are found in many neurodegenerative diseases (NDDs), we targeted the development of an IF-domain-inserted F36V system to regulate amyloid fibril formation. Using this system, we envision that the regulation of amyloid fibril formation could be switched on and off by a small molecule.

In our previous study, we reported that F36VIF with the His_6-tag gene was obtained by site-directed mutagenesis from FKBPIF/pET28a plasmid.[5] The resulting F36VIF protein was successfully expressed in BL21(DE3) in the presence of IPTG and further separated using Ni^{2+}-affinity chromatography. The chymotrypsin-coupled assays[10] showed that the PPIase activity of F36VIF (202% of control) was remarkably higher than that of F36V itself (128% of control).[6] It is worth noting that the PPIaes activity of F36VIF even exceeds those of the positive controls, *i.e*. SlyD, from which the original IF domain was taken. To further characterize the biochemical functions of this new engineered protein, we checked the chaperone activity of F36VIF with F36V. An analogous protein, FKBPIF, was also reported to achieve improved chaperone activity when measured by the refolding of RCM-T1, which is a disulfide-reduced and S-carboxymethylated form of a variant of RNase T1.[5] In our study, we utilized the citrate synthase (CS) aggregation system, in which denatured CS can aggregate upon 200-fold dilution into a refolding buffer, resulting in light scattering at 360 nm.[5] In the presence of assisting chaperone proteins, it is expected that the light scattering decreases as the chaperone protein prevents CS from aggregating. We examined and compared the chaperone activities of FKBP12, F36V, FKBPIF and F36VIF with that of $SlyD^*$(1-165). $SlyD^*$ with the unstructured C-terminal residues 166-196 removed was used as a positive control since it possesses a higher PPIase activity than SlyD.[11] An example of the light-scattering kinetics observed for $SlyD^*$ is shown in Figure 1(a) and compared with those of a buffer control. In the presence of $SlyD^*$, light scattering dramatically decreased. Similarly, we then compared the initial rates of CS aggregation measured for 30 sec in the presence of other chaperone proteins; as expected, inhibition of light scattering was observed in the order of $SlyD^*$ >> F36VIF > FKBPIF >> FKBP > F36V ≈ control, which is indicative of the order of chaperone activity (Figure 1(b)). Moreover, the order of the chaperone activity of these proteins roughly correlates with the order of the PPIase activity, *i.e*. F36VIF > $SlyD^*$ > FKBPIF >> F36V ≈ FKBP > control, which we previously reported.[6] Combining these results, we can now conclude that the insertion of an IF domain enhances not only the PPIase activity but also the chaperone activity. To our surprise, F36V does not show any chaperone activity, which is in contrast to our previous results that the PPIase activity of F36V is comparable to that of FKBP.[6] This difference may be worth noting in order to elucidate the molecular mechanisms by which they regulate fibril formation.

Abbreviations: α-Syn, α-synuclein; IF, insertion-in-flap; CS, citrate synthase; ThT, thioflavin T; TEM, transmission electron microscopy

As candidates for regulating amyloid fibril formation in many NDDs, some chaperone proteins, such as Hsp27, Hsp104, and FKBP12, have recently been studied by several groups. Accordingly, it was demonstrated that the level of Hsp27 increased in dementia with Lewy bodies (DLB), and Hsp27 not only reduced α-Syn aggregation but also protected cells from α-Syn-induced toxicity.[12] In a previous study, we described how the functional Hsp104 protein not only inhibites α-Syn aggregation but also resolubilizes aggregated proteins *in vitro*.[13] The amyloid precursor protein (APP) intracellular domain (AICD) in Alzheimer disease (AD) is another target that was identified for the regulation of fibril formation.[14] An interaction of FKBP12 with APP was reported using a yeast two- hybrid system[15] and the dimerization of APP that was induced by the APP-FKBP chimera resulted in decreased Aβ production.[16] Engelborghs and coworkers reported that FKBP12 accelerated the fibril formation of α-Syn.[3,4] The modulation of α-Syn aggregation by FKBP12 with the PPIase activity by altering the conformation of the Pro residues was subsequently reported.[17] With the aim of acquiring a more powerful regulator of fibril formation, we designed a more powerful chaperone, F36VIF, that exhibits the higher PPIase activity than F36V. In the present study, we examined the effect of F36V and F36VIF on the fibril formation of α-Syn using thioflavinT (ThT) fluorescence. For this purpose, we constructed α-Syn/pET28a plasmids and His-tagged α-Syn protein was purified by Ni^{2+}-affinity chromatography. SDS-PAGE analysis of purified α-Syn confirmed the purified α-Syn protein, but several minor bands were also detected (data not shown). Accordingly, the subsequent separation by anion exchange chromatography was carried out to remove the impurities, yielding a single band corresponding to α-Syn protein. Kinetic experiments performed using a ThT fluorescence assay to identify α-Syn aggregation showed that the rate of fibril formation significantly increased when α-Syn was incubated with either F36V or F36VIF. The effect of the F36VIF protein was dramatically higher than that of F36V (Figure 2(a)). From the previous study, we know that both hybrid F36VIF and F36V have remarkable PPIase activities of 202% and 128% compared to the control, respectively.[5] In contrast, in this

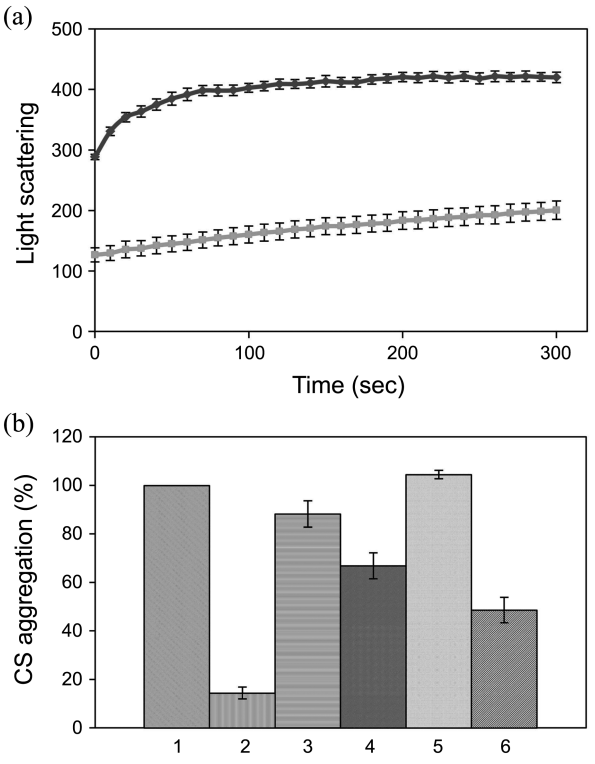

Figure 1. Measurement of chaperone activity by CS aggregation. (a) 30 μM denatured CS was diluted 200-fold with buffer A containing 30 mM GdmCl in the presence of 4.2 μM $SlyD^*$. The kinetics of light scattering were measured for 300 sec at 360 nm in the absence (curve 1) and presence (curve 2) of $SlyD^*$ (1-165). (b) Aggregation rates were measured in the presence of chaperones, *i.e.* $SlyD^*$ (1-165) (lane 2), FKBP (lane 3), FKBPIF (lane 4), F36V (lane 5) and F36VIF (lane 6), and compared with that of the buffer control (lane 1), which was set as 100%.

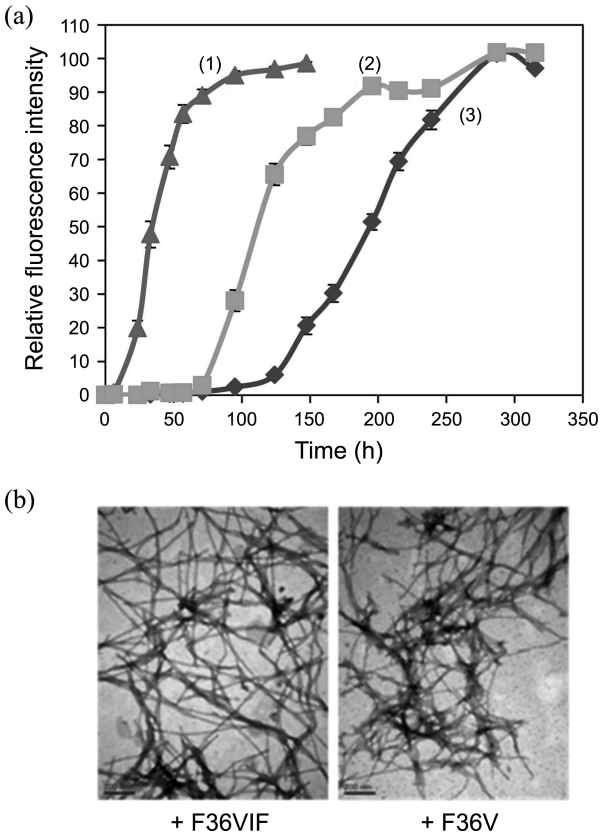

Figure 2. Formation of amyloid fibrils in the presence of F36V and F36VIF. (a) The formation of amyloid fibrils was measured using ThT fluorescence. α-Syn (70 μM) and chaperone proteins (10 μM) were incubated at 37 °C with shaking at 200 rpm. Aliquots were taken every 10 h and mixed with 20 μM ThT solution; the fluorescence intensities of the samples were then measured for 30 sec with excitation at 450 nm and emission at 482 nm. The percentage of ThT fluorescence was calculated by dividing the arbitrary fluorescence intensities by the maximum value for each sample. The chaperone proteins included in this Figure are F36VIF (curve 1) and F36V (curve 2); for comparison, the fluorescence data for α-Syn alone (curve 3) is included as a control. (b) TEM images were recorded for 70 μM α-Syn after incubation in the presence of 10 μM F36V or F36VIF hybrid proteins at 37 °C for 240 h with continuous shaking.

study we found that only F36VIF functions as a molecular chaperone whereas F36V does not (Figure 1(b)). Based on these observations, we envision that the PPIase activities of both proteins play more crucial roles in accelerating the fibril formation of α-Syn than the chaperoning activities. *Cis-trans* isomerization of the Pro residues of α-Syn may affect the conformational change so that the extended α-Syn readily forms the amyloid fibril. Chaperoning does not seem to be involved in accelerating α-Syn aggregation since the F36V protein, even without chaperone activity, triggered faster fibril formation. It is also interesting to note that the fibril formation of α-Syn was accelerated by SlyD* although its effect was less than that of FKBPIF (data not shown). Finally, in order to check the morphology of the aggregated α-Syn formed in the presence of either F36V or F36VIF, transmission electron microscopy (TEM) images were taken of the samples, which were incubated for 240 h at 37 °C. The presence of amyloid fibrils in both F36V and F36VIF-treated α-Syn samples was thus confirmed, and their shapes are very similar to those found in the buffer control (Figure 2(b)). No significant difference between them was observed.

In conclusion, we discovered that the F36VIF hybrid protein with an inserted IF domain possesses strong chaperone activity as well as high PPIase activity. The enhanced PPIase activity of the F36VIF protein triggers the conformational change of α-Syn to a more extended structure thereby accelerating the kinetics of α-Syn amyloid fibril formation. The expected accelerated aggregation rate of α-Syn in the presence of F36V and F36VIF was revealed by ThT fluorescence and their morphologies were confirmed to be amyloid fibrils *via* TEM. Continuing studies on the structural roles of the five Pro residues in α-Syn are in progress in order to elucidate the mechanism by which increased PPIase activity regulates the process of amyloid fibril formation. It is of significant interest to fully explore the effects of small molecules, such as Shield-1,[9] that are being developed as on-and-off switches on the acceleration of the fibril formation of α-Syn.

Experimental Sections

Measurement of Chaperone Activity by CS Aggregation. Citrate synthase (30 μM; CS, Sigma) was dissolved in denaturation buffer (6.0 M guanidium chloride (GdmCl), 50 mM Tris-Cl (pH 8.0), and 20 mM DTT). The mixture was then incubated at 37 °C for 1 h. The denatured CS was diluted 200-fold with buffer A (50 mM Tris-Cl (pH 8.0), 0.1 mM DTT, 30 mM GdmCl, and 4.2 μM chaperone protein), then added to a 3 mL sample to achieve a final concentration of 0.15 μM. The light scattering was measured for 30 sec *via* fluorescence spectroscopy (SHIMADZU RF-5301 PC) with both excitation and emission at 360 nm.

Expression and Purification of α-Syn Protein. To construct the expression plasmid of a α-Syn/pET28a with a His_6-tag, the encoding gene was amplified from α-Syn/AED4 *via* the polymerase chain reaction (PCR) using primers (F1: ggccatatggatgtattcatgaaaggactttcaaag and R1: gccctcgagctaggcttcaggttcgtagtcttgata). The resulting α-Syn/pET28a plasmid was transformed into BL21(DE3) and grown until an optical density of 0.5 at 600 nm was achieved. At this point, 0.5 mM IPTG was added and the mixture was further incubated at 37 °C for 3 h. The expressed His_6-α-Syn protein was purified by Ni^{2+}-affinity chromatography (His GraviTrap™, GE Healthcare) using a binding buffer (50 mM Tris-Cl (pH 7.9), 250 mM NaCl, and 8 mM Imidazole), washing buffer (50 mM Tris-Cl (pH 7.9), 250 mM NaCl, 60 mM Imidazole] and elution buffer (50 mM Tris-Cl (pH 7.9), 250 mM NaCl, and 250 mM Imidazole). The partially purified His_6-α-Syn proteins were subjected to anion exchange chromatography (HiTrap™ Q HP, GE Healthcare) with 300 mM NaCl eluent followed by Amicon-10 filtration. The concentrations of the purified proteins were measured using the Bradford method (Bio-Rad protein DC assay).[18] The purity of the His_6-α-Syn protein was confirmed by 12% SDS-PAGE.

Fibril Formation of α-Syn Measured by ThT Fluorescence. The formation of amyloid fibril was measured by ThT fluorescence assay as follows: Lyophilized α-Syn was dissolved in buffer B (20 mM Tris-Cl (pH 7.8), 150 mM NaCl, and 0.02% NaN_3) and filtered through a 0.45 μm filter syringe before the measurement of its concentration. α-Syn (70 μM) and chaperone proteins (10 μM; F36V and F36VIF) were dissolved in buffer B to a final volume of 700 μL. During incubation at 37 °C with shaking at 200 rpm, 20 μL aliquots were taken at 10-12 h intervals and mixed with 3 mL of 20 μM ThT solution. The fluorescence intensity of these samples was measured for 30 sec with excitation at 450 nm and emission at 482 nm.

Fibril Morphology Examined by TEM. Transmission electron microscopy images were taken of 70 μM α-Syn after incubation in the absence and presence of 10 μM F36V and F36VIF hybrid proteins at 37 °C for over 240 h with continuous shaking. The samples were adsorbed onto carbon grids for 1 min and then negatively stained with 1% uranyl acetate for 10 sec. The samples were examined using a JEM1010 transmission electron microscope (JEM1010, NICEM, South Korea) operating at 80 kV.

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References

1. Siekierka, J. J.; Hung, S. H. Y.; Poe, M.; Lin, C. S.; Sigal, N. H. *Nature* **1989**, *341*, 755.
2. Deleersnijder, A.; Van Rompuy, A. S.; Desender, L.; Pottel, H.; Buée, L.; Debyser, Z.; Baekelandt, V.; Gerard, M. *J. Biol. Chem.* **2011**, *286*, 26687.
3. Gerard, M.; Debyser, Z.; Desender, L.; Kahle, P. J.; Baert, J.; Baekelandt, V.; Engelborghs, Y. *FASEB J.* **2006**, *20*, 524.

4. Gerard, M.; Deleersnijder, A.; Daniëls, V.; Schreurs, S.; Munck, S.; Reumers, V.; Pottel, H.; Engelborghs, Y.; Van den Haute, C.; Taymans, J. M.; Debyser, Z.; Baekelandt, V. *J. Neurosci.* **2010**, *30*, 2454.
5. Knappe, T. A.; Eckert, B.; Schaarschmidt, P.; Scholz, C.; Schmid, F. X. *J. Mol. Biol.* **2007**, *368*, 1458.
6. Shin, E.; Lee, K. *Bull. Chem. Korean Soc*. **2012**, *33*, 281.
7. Weininger, U.; Haupt, C.; Schweimer, K.; Graubner, W.; Kovermann, M.; Bruser, T.; Scholz, C.; Schaarschmidt, P.; Zoldak, G.; Schmid, F. X.; Balbach, J. *J. Mol. Biol*. **2009**, *387*, 295.
8. Yang, W.; Rozamus, L. W.; Narula, S.; Rollins, C. T.; Yuan, R.; Andrade, L. J.; Ram, M. K.; Phillips, T. B.; van Schravendijk, M. R.; Dalgarno, D.; Clackson, T.; Holt, D. A. *J. Med. Chem.* **2000**, *43*, 1135.
9. Banaszynski, L. A.; Chen, L. C.; Maynard-Smith, L. A.; Ooi, A. G.; Wandless, T. J. *Cell* **2006**, *126*, 995.
10. Küllertz, G.; Lüthe, S.; Fischer, G. *Clin. Chem.* **1998**, *44*, 502.
11. Hottenrott, S.; Schumann, T.; Pluckthun, A.; Fischer, G.; Rahfeld, U. *J. Biol. Chem*. **1997**, *272*, 15697.
12. Outeiro, T. F.; Klucken, J.; Strathearn, K. E.; Liu, F.; Nguyen, P.; Rochet, J. C.; Hyman, B. T.; McLean, P. J. *Biochem. Biophys. Res. Commun.* **2006**, *351*, 631.
13. Kong, B.; Chae, Y. K.; Lee, K. *Protein. Pept. Lett*. **2003**, *10*, 491.
14. Selkoe, D. J. *Annu. Rev. Neurosci*. **1989**, *12*, 463.
15. Liu, F. L.; Liu, P. H.; Shao, H. W.; Kung, F. L. *Biochem. Biophys. Res. Commun.* **2006**, *350*, 472.
16. Eggert, S.; Midthune, B.; Cottrell, B.; Koo, E. H. *J. Biol. Chem.* **2009**, *284*, 28943.
17. Meuvis J.; Gerard, M.; Desender, L.; Baekelandt, V.; Engelborghs, Y. *Biochemistry* **2010**, *49*, 9345.
18. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.