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Insertion of IF (insertion-in-flap) Domain into F36V to Provide Higher Activity of Peptidyl-propyl *cis-trans* Isomerase

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Molecular chaperones are required to help nascent proteins fold properly or protect cells against various stresses by means of preventing their aggregation.[1] Attempts to elucidate the molecular mechanisms by which chaperones can solubilize aggregation-prone proteins such as Aβ and α-synuclein (α-Syn) are relatively limited.[2,3] So far, just a few publications have been written on the functional roles of chaperones such as Hsp104 and FKBP12 on controlling the aggregation of α-Syn into fibrillar structures.[4,5] In our previous study, we described how functional Hsp104 protein not only inhibited α-Syn aggregation but also resolubilized aggregated protein *in vitro*. Recently, Engelborghs group has reported that FKBP12, containing the peptidyl-prolyl isomerase (PPIase) activity, accelerated fibril formation of α-Syn containing 5 Pro residues, which can be isomerized between *cis*- and *trans*-conformations.[6]

Little is known about the molecular details that explain how molecular chaperones regulate fibril formation in many neurodegenerative diseases such as Alzheimer disease[7] and Parkinson disease.[8] In pursuit of developing a candidate regulator of fibril formation, we have attempted to engineer the FKBP system to acquire a higher activity of PPIase along with the chaperone activity. The IF (insertion-in-flap) domain with 61- amino acids starting with AYG and ending with LKF was adopted from SlyD (sensitive-to-lysis) in *Escherichia coli* (*E. coli*)[9] as well as from MtFKBP17 of *Methanococcus thermolithotrophicus*.[10] The structural analysis of the IF domain, protruding from a loop of the FKBP domain near the PPIase catalytic site, indicated its hydrophobicity so that it can interact with unfolded proteins in the nonpolar region found in extended structures such as a β-sheet. Folding of the IF domain is influenced by the FKBP domain, in such a way that the guest IF domain of SlyD folds rapidly when the host FKBP domain of SlyD is folded and is unfolded when the host is unfolded.[11]

Construction of an FKBPIF hybrid replacing the flap by an IF domain was previously reported by the Schmid group.[12] The resulting hybrid FKBPIF protein exhibited strong folding activity as well as chaperone activity. For example, upon the refolding of RCM-T1 (a disulfide-reduced and S-carboxymethylated form of a variant of RNase T1), the hybrid FKBPIF showed the kcat/Km enhanced by 200 fold. In monitoring chaperone activity, FKBPIF exhibited inhibition of CS (citrate synthase) aggregation whereas FKBP did not. The insertion effect of IF in FKBPIF was also confirmed in the inhibited aggregation of insulin reduced by dithiothreitol.[9]

Upon structural analysis of the binding site of FKBP for FK506, it was envisioned that it would be surrounded by hydrophobic residues such as F36.[13] Further mutation of F36 to V36 yielded a better system in terms of fitting ligands through a protruding structural part.[14] Using the structural contact between them, the Wandless group have developed a regulation system in which the stability of a certain domain

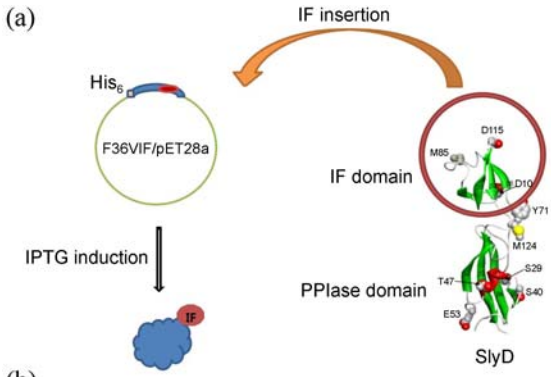

Figure 1. Scheme of constructing F36VIF mutant by IF insertion. (a) F36VIF/pET28a plasmid was constructed by inserting the IF domain of SlyD into F36V mutant in a His_6-tagging pET28a system. The illustrated structure of SlyD was taken from Zoldák *et al.*[22] (b) (1) The nucleotide sequence of the *f36vif* gene in pET28a was confirmed by DNA sequencing. Note that GTT is the codon for V36. (2) The amino acid sequence of F36VIF is shown. The sequence of the IF domain, starting with AYG and ending with GQN is shown in bold.

Abbreviations: IF, insertion in flap; SDM, site directed mutagenesis; IPTG, isopropyl β-D-1-thiogalactopyranoside; PPIase, peptidyl-propyl *cis-trans* isomerase

when fused to some mutants of F36V depends on the presence of a small molecule, such as Shield-1.[15] In the present study, with the aim of engineering a better molecular chaperone, we have designed the insertion hybrids by putting the IF domain into FKBP12 or F36V mutant (Fig. 1(a)), whose function can be then regulated by FK506 or Shield-1, respectively.[16,17] Chaperones tested as controls here were SlyD or SlyD*(1-165) from which the IF domain was taken. At first, the gene encoding FKBP was cloned into pET28a by polymerase chains reactions (PCR) with two primers F1 (5'-CCCATATGCGAGTGCAGGTGGAAACC-3', Nde1 site) and R1 (5'-CCCTCGAGCTATTCCAGTTTT-AGAAGCTCCACATCG-3', Xho1site). The resulting plasmid was named FKBP/pET28a. The second plasmid F36V/pET28a was obtained by site directed mutagenesis (SDM) of the FKBP/pET28a plasmid with two primers F2 (CTTGAA-GATGGAAAGAAA**G**TTGATTCCTCCCGGGACA) and R2 (TGTCCCGGGAGGAATCAA**C**TTTCTTTCCATCTTCAAG) by the Kunkel method.[18] A PCR reaction was carried out at 95 °C for 30 sec, 55 °C for 1 min and 72 °C for 6 min 30 sec. After repeating for 15 cycles, the PCR products were treated with Dpn1(NEB) at 37 °C for 1 hr to get rid of the original plasmid. In order to obtain FKBPIF, the IF encoding gene was inserted into the *fkbp* gene to provide the *fkbpif* gene with 486 bp in length using solid phase oligonucleotide synthesis (Cosmo Genetech). Using F1 and R1 in PCR reactions, an Nde1/Xho1 digest of the PCR product was ligated into pET28a to give FKBPIF/pET28a. Finally, F36VIF/pET28a plasmid was similarly obtained by SDM with F2 and R2 primers, as described above. The nucleotide sequence and amino acid sequence of its insert in the final F36VIF/pET28a plasmid was confirmed by DNA sequencing (Cosmo Genetech) and translation tool (expasy.org), as demonstrated in Figure 1(b). The underlined GTT corresponded to mutated Val36 and the presence of an IF encoding region marked in bold characters was clearly confirmed. As a positive control, SlyD*(1-165)/pET28a was constructed from the SlyD/pET28a plasmid using PCR amplification with a set of primers [F3 with Nde1 site (GGCAGCCATATGAAAGTA-GCAAAAAGAC) and R3 with HindIII site (GACCAAG-CTTGCCATTAGTCGTGGTCGTG)].

In order to check if the resulting plasmids can be expressed in *E. coli*, BL21(DE3) transformed with each plasmid was cultured until the mid-log phase and then further incubated in the presence of IPTG. The presence of IPTG induced F36V and F36VIF proteins were confirmed in SDS-PAGE analysis (lane 2 and 4 in Fig. 2). Overexpressed F36V and F36VIF proteins were further purified into a homogeneous fraction by Ni^{2+} column affinity chromatography since they have His_6 tags attached at the N-terminus.[19] The single bands eluted with 400 mM imidazole were pooled and buffer-exchanged and concentrated to 2.0 mg/mL by Amicon filtration. The yields of the purified proteins were between 6.5 and 8.0 mg/L culture. The molecular weights of the recombinant proteins were estimated as 12 kDa for F36V and 20 kDa for F36VIF in SDS-PAGE, respectively (lane 3 and 6 in Fig. 2).

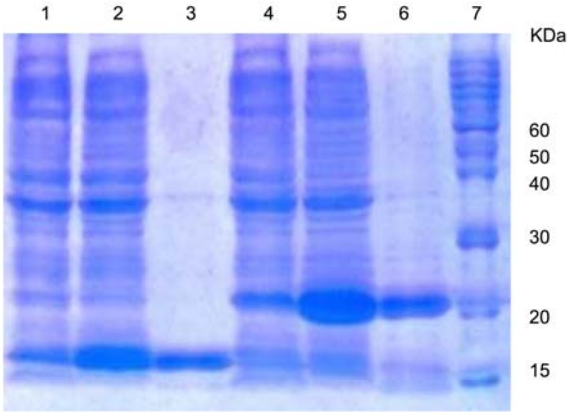

Figure 2. Induction and purification of F36V and F36VIF hybrid proteins. BL21 (DE3) cells were transformed with F36V/pET28a (lane 1 and 2) and F36VIF/pET28a (lane 4 and 5). Induction experiments were carried out at 37 °C for 3 hrs. Lysates of non-induced cells (lane 1 and 4) and induced cells (lane 2 and 5) were compared in 12% SDS-PAGE. The total proteins form ITPG-induced cells were subject to Ni^{2+}-affinity chromatography using a binding buffer containing 8 mM imidazole, a wash buffer containing 60 mM imidazole and an elution buffer containing 400 mM imidazole. Eluted proteins, F36V (lane 3) and F36VIF (lane 6), were also compared with protein size markers (lane 7) in 12% SDS-PAGE.

Since IF-inserted hybrid FKBPIF was proven to display a higher activity of PPIase by the Schmid group,[12] we have asked if the F36VIF protein can be expressed and folded properly to present the functional PPIase activity. In order to measure this activity, we carried out a chymotrypsin-coupled assay.[20] Since the FKBP chaperone can be also extensively cleaved off by chymotrypsin, we had to modify the method by adding chymotrypsin right before absorbance measurements. As we expected, the burst phase in A390 nm was observed within 30 sec as a monitor of released *p*-nitroaniline. The difference of absorbance between t = 0 and t = 30 sec was compared both with and without each chaperone protein (Fig. 3). Consistent with the previous report, we were able to demonstrate that the PPIase activity of FKBPIF (181%) was also higher than that of FKBP (118%) in our modified method. More significantly, the hybrid F36VIF (202%) showed an even better PPIase activity than F36V (128%), by 58%. As a positive control, we compared the PPIase activities with that of SlyD (178%) and SlyD* (191%). It is worthy of note that the PPIaes activity of F36VIIF even exceeds those of positive controls SlyD and SlyD*, which originally harbored the IF domain. At this point, we can envision that the 5 Pro residues in α-Syn can be more efficiently isomerized between *cis* and *trans* to induce the more significant conformational change at the C-terminus of α-Syn in the presence of F36VIF. The higher PPIase activity of F36VIF may thus result in an accelerated fibril formation of α-Syn when coincubated, so that more powerful inhibitor than Shield-1[15] may be required to regulate fibrillization processes. Other small molecules as potent inhibitors of fibril formation should be investigated in a following study.

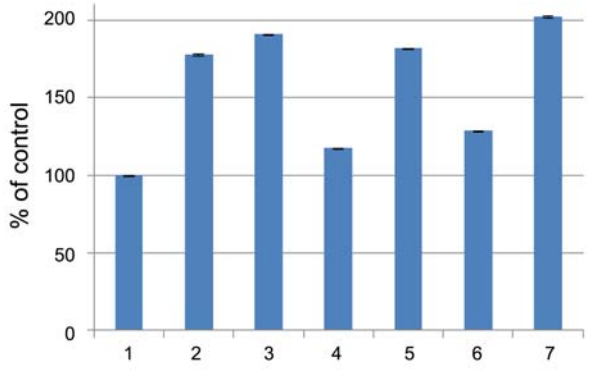

Figure 3. PPIase activities of F36V and F36VIF. Purified chaperone proteins (4.2 μM) were incubated with suc-Ala-Phe-Pro-Phe-p-nitroaniline and chymotrypsin. The percentage of PPIase activity in the presence of each chaperone was compared with that of a control measured in the absence of any chaperone (lane 1). The chaperones tested were SlyD (lane 2), SlyD* (1-165) (lane 3), FKBP (lane 4), FKBPIF (lane 5), F36V (lane 6) and F36VIF (lane 7).

In conclusion, we were able to construct a hybrid plasmid encoding for the F36V protein with an IF domain inserted. Overexpression of the F36VIF protein by IPTG induction followed by subsequent Ni^{2+}-affinity chromatography was successfully carried out. The resulting F36VIF protein contained a better PPIase activity than the F36V mutant itself. Further research into a regulatory role of the F36VIF hybrid protein by a small molecule in regulating fibril formation is in progress.

Experimental Sections

Construction of F36VIF/pET28a Plasmid. In order to introduce the IF domain into F36V, we prepared the FKBPIF encoding gene as follows. pDNR-FKBP1A plasmid (HscD00003325, from PLASMID, Harvard) was used as a template for PCR with an annealing temperature of 55 °C. Two primers used here were F1 (5'-CCCATATGCGAGTGCAGGTGGAAACC-3') containing an Nde1 site and R1 (5'-CCCTCGAGCTATTCCAGTTTTAGAAGCTCCACATCG-3') containing an Xho1 site. The Nde1/Xho1 digested FKBP product was ligated into pET28a vector for expression with an N-terminal His_6 tag, FKBP/pET28a. The FKBPIF/pET28a plasmid was constructed similarly except that the insert was amplified from FKBPIF/pUC18, in which the insert *fkbpif* (486 bp) was previously prepared by solid phase synthesis (Genetech). The two primers used to amplify the *fkbpif* gene were F4 (5'-CCCATATGGAGTGCAGGTGGAAACC-3') and R4 (5'-CCCTCGAG CTATT CCAGTTTTAGAAGCTCCACATCG-3'). The *fkbpif* insert was confirmed by DNA sequencing. Finally the F36VIF/pET28a plasmid was obtained by site directed mutagenesis (SDM) of FKBP/pET28a using the Kunkel method, in which Dpn1 was used to eliminate the original DNA template. The primers for SDM were F2 (5'-CTTGAAGATGGAAAGAAA**G**TTGATTCCTCCCGGGACA-3') and R2 (5'-TGTCCCGGGAGGAATCAA**C**TTTCTTTCCATCTTCAAG-3'). For constructing the SlyD*(1-165)/pET28a plasmid, the encoding gene for SlyD* was amplified from the SlyD/pET28a plasmid (donated by Minsun Sung) with primers F5 (5'-GGCGTCCATATGAAAGTAGCAAAAGAC-3') and R5 (5'-GCCGAATTCCTAGTCGTGGTCGTG-3') and ligated into a pET28a vector.

Induction and Purification of Hybrid Proteins. BL21 (DE3) competent cells were transformed with the hybrid plasmids by heat shock at 42 °C. Positive clones were cultured in LB^{kan} until the O.D. of 600 nm reached 0.5. At this point, the cells were incubated with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at 37 °C for another 3 hrs. Cell lysates containing overexpressed hybrid proteins were analyzed on 12% SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining. His_6-tagged hybrid proteins were further purified by Ni^{2+}-column chromatography (His GraviTrap, GE healthcare) using a binding buffer [50 mM Tris-Cl (pH 7.9), 250 mM NaCl, 8 mM imidazole], wash 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, 400 mM imidazole]. The purified proteins were examined in 12% SDS-PAFE and the concentrations of purified proteins were measured by the Bradford method (Bio-Rad protein DC assay).[21] Eluted proteins were concentrated to a 2 mg/mL concentration using Amicon-10 filtration.

Assay of PPIase. The PPIase activity was indirectly measured by protease-coupled assay. In brief, 7 μL of suc-Ala-Phe-Pro-Phe-*p*-nitroaniline (20 mg/mL stock in DMSO, BACHEM) was added to 35 mM HEPES (pH 7.8) containing chaperone proteins at 4.2 μM. Right after adding 7 μL of chymotrypsin (3.58 mg/mL stock in 35 mM HEPES (pH 7.8)), absorbance values at 390 nm (A_{390nm}) were read for 30 sec to measure the amount of released *p*-nitroaniline. The PPIase activity was calculated as $\Delta A_{chaperone}/\Delta A_{no\ chaperone} \times 100$ (%) where ΔA was defined as $A_{30s}-A_{0s}$.

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