PROTEIN STRUCTURE REPORT

High-resolution crystal structure of FKBP12 from *Aedes aegypti*

Sreekanth Rajan,¹ Kai Qian Saw,¹ Quoc Toan Nguyen,¹ Kwanghee Baek,² and Ho Sup Yoon^{1,2*}

¹Division of Structural Biology and Biochemistry, School of Biological Science, Nanyang Technological University, Singapore 637551

²Department of Genetic Engineering, College of Life Sciences, Kyung Hee University, Yongin-si, Gyeonggi-do 446-701, Republic of Korea

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Abstract: Dengue is one of the most infectious viral diseases prevalent mainly in tropical countries. The virus is transmitted by *Aedes* species of mosquito, primarily *Aedes aegypti*. Dengue remains a challenging drug target for years as the virus eludes the immune responses. Currently, no vaccines or antiviral drugs are available for dengue prevention. Previous studies suggested that the immunosuppressive drug FK506 shows antimalarial activity, and its molecular target, FK506-binding protein (FKBP), was identified in the *Plasmodium* parasite. Likewise, a FKBP family protein has been identified in *A. aegypti* (AaFKBP12) in which AaFKBP12 is assumed to play a similar role in its life cycle. FKBP family proteins belong to a highly conserved class of proteins and are considered as an attractive pharmacological target. Herein, we present a high-resolution crystal structure of AaFKBP12 at 1.3 Å resolution and discuss its structural features throwing light in facilitating the design of potential antagonists against the dengue-transmitting mosquito.

Keywords: FKBP; Dengue; *Aedes aegypti*; PPIase; FK506

Introduction

Dengue is one of the most dreadful mosquito-borne viral diseases affecting nearly 50 million people worldwide every year, according to the World Health Organization. The disease is endemic in more than 100 countries, with South-east Asia and West Pacific regions being the worst affected. Despite the pro-

gressive efforts,^{1,2} a vaccine or drug is yet to be discovered. The dengue virus, consisting of four serotypes (DEN1–4), is transmitted to humans through the *Aedes* mosquito species, mainly *Aedes aegypti*.^{3,4} Thus, controlling mosquito is the first line of defense against dengue, which can be achieved by understanding the molecular mechanism of dengue transmission by these mosquitoes. Identification of the molecular target and attempts to inhibit the target might provide insights into designing novel antagonists against the dengue-transmitting mosquito. Previous studies showed that the immunosuppressive drug FK506 possesses antimalarial activity, and its molecular target, the FK506-binding protein, was found to be present in the parasite.^{5–9} FKBP12, where 12 refers to its molecular weight in kDa, is

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*Correspondence to: Ho Sup Yoon, Division of Structural Biology and Biochemistry, School of Biological Science, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551. E-mail: hsyoon@ntu.edu.sg

Figure 1. Sequence alignment and crystal structure of AaFKBP12. (A) Sequence alignment of AaFKBP12 with HsFKBP12 and PfkBD35, generated using the program ESPrpt¹⁹; the major secondary structure elements and numbering corresponding to AaFKBP12 is shown above the sequences. The highly conserved active site residues are indicated as pink colored stars. The conserved charged residues of AaFKBP12 and HsFKBP12 are shown in cyan circles, while the dissimilar residues are shown in dark blue circles. The presence of Cys77 near the active site region, replacing the Ile76 in HsFKBP12, is indicated by a black arrow. (B) Cartoon representation of AaFKBP12 crystal structure, with helices colored red, sheets with blue and loops in green. The MOPS buffer molecule in the active site is shown in stick mode. The secondary structural regions and the flexible $\beta 5/\beta 6$ loop are labeled. (C) The 2Fo-Fc electron density map contoured at 1.5 σ cutoff, showing the active site residues (purple colored density) along with the MOPS molecule (blue colored density). (D) The active site region, after superposition of the AaFKBP12 (pale green) on HsFKBP12-FK506 complex (pale pink), showing the important Cys77 (blue stick) and the neighboring Ile57 and Phe100 residues in stick mode. The other active site residues are shown as lines. This figure is generated using the program PyMOL.²⁰ [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

known to mediate the immunosuppressive activity of FK506 by recruiting and inactivating the serine/threonine phosphatase calcineurin, resulting in the blockage of the signaling pathway mediated by calcineurin.¹⁰ As FKBP is highly conserved from plants to mammals and are ubiquitous in almost all mammalian cell types, its physiological role is possibly very important. This moonlighting protein primarily possesses peptidyl-prolyl cis-trans isomerase (PPIase) and FK506-binding activities, apart from regulating various functions like protein folding, stability,^{11,12} chaperonic activity,^{12,13} receptor signaling,^{14,15} calcium homeostasis,^{15,16} and neuroprotective and neurotrophic activities.^{17,18} Within the family, the FK506 drug binds to the PPIase domain that is roughly 100 amino acids in length, leading to inhibition of the PPIase. Thus, inhibition of PPIase activity of FKBP may be a potential antiparasite mechanism. The AaFKBP12 shows high similarity with Human FKBP12 (HsFKBP12) and *Plasmodium falciparum* FKBD35 (PfkBD35) [Fig. 1(A)], suggesting that AaFKBP12 may also have a similar function against the dengue-transmitting mosquito. Currently, no systematic study has been performed to characterize AaFKBP12 and its biochemical properties. As a starting point, we present here the high-

resolution crystal structure of AaFKBP12 solved at 1.3 Å resolution, which can serve as the basis for designing new antagonists against the dengue-transmitting mosquito.

Results and Discussion

Overall structure

The 1.3 Å high-resolution crystal structure of AaFKBP12 revealed a topology [Fig. 1(A,B)] consisting of six beta strands with a traversing alpha helix ($\beta 1$ - $\beta 2$ - $\beta 3$ - $\beta 4$ - $\alpha 1$ - $\beta 5$ - $\beta 6$). The crystal belonged to orthorhombic space group $P2_12_12_1$ with one molecule in the asymmetric unit containing 108 amino acids and a total of 133 water molecules. One MOPS buffer molecule [Fig. 1(B,C)], present in the crystallization solution, was observed in the active site pocket [Fig. 1(C)].

Comparison of *A. aegypti* and human FKBP12

There is a 71% sequence identity present between AaFKBP12 and HsFKBP12 [Fig. 1(A)]. The AaFKBP12 (108 residues) has an additional methionine residue at the N-terminus compared with HsFKBP12 (107 residues) [Fig. 1(A)]. The dissimilar residues, 12 in total, were mostly surface exposed

with an average surface accessibility per residue of 12.5 Å² (calculated using the program AREAIMOL implemented in the CCP4 suite²¹ of programs) compared with 7 Å², the average accessibility for the entire protein. Of these 12 residues, only one (Cys77) was found near the active site region. This Cys77 replaces the Ile76 [Fig. 1(A,D)] of *HsFKBP12* and can be exploited in designing inhibitors that can target the sulfur atom specific to this species. There were a total of 30 charged residues, of which 23 remain charged in both *Hs* and *Aa* FKBP12 [Fig. 1(A)] and five out of the remaining seven (Val6, Ala14, Ala19, Ala32, Val36, Thr42, and Asn95) were mutated to hydrophobic residues in *AaFKBP12*. In addition, Gly12 and Thr85 of *HsFKBP12* were mutated to charged residues Glu13 and Arg86, respectively. Interestingly, these mutations resulted in the elimination of a salt bridge between residues Lys35 and Asp41, observed in *HsFKBP12* (Supporting Information Fig. S1). In conjunction, it has been shown that the regions His87 to Ile90 and Asp37 to Phe46 are involved in calcineurin binding²² and activity.²³ Therefore, while looking closer into this region (Supporting Information Fig. S1), we observed changes of three other charged residues (Glu31 to Ala32; Thr85 to Arg86; and His94 to Asn95) apart from the loss of the Lys 35–Asp41 salt bridge. Mutational studies on charged surface residues of *HsFKBP12*,²³ such as Lys35Val, Thr85Arg, and His94Asn, coincides with those observed naturally in *AaFKBP12*. However, none of these mutations affected the calcineurin activity of *HsFKBP12*.²³ Nonetheless, it will be of interest to study their effect on *AaFKBP12*, as there are multiple mutations observed here.

Active site

An overall root-mean-square deviation (r.m.s.d.) of 0.43 Å for C α atoms with subtle changes in the conformation of the loops (Fig. 2) was observed from structure superposition with *HsFKBP12*. But, the active site pocket of *AaFKBP12* [Fig. 1(C)], consisting of highly conserved [Fig. 1(A,C)] residues (Tyr27, Phe37, Asp38, Arg43, Phe47, Glu55, Val56, Ile57, Trp60, Ala82, Tyr83, His88, Ile92, and Phe100), exhibits a topology identical to its human counterpart. Despite the similarity, a Cys77 in *AaFKBP12*, replacing Ile76 in *HsFKBP12* [Fig. 1(A)], could be targeted to gain specificity toward this species. On superposition of the *HsFKBP12*–FK506 complex (PDB ID 1FKJ) on *AaFKBP12*, the Cys77 was found to be 8.2 Å away from the nearest FK506 atom [Fig. 1(D)]. In this inhibitor free form, it seems that residues Ile57 and Phe100 may hinder the interaction of the inhibitor [Fig. 1(C,D)] with the sulfur atom of Cys77. The nearest atoms of these two residues, namely Ile57 CD1 and Phe100 CE1, were found to be separated by 3.7 Å but can act like a split-bridge

Figure 2. Stereo view of the superposition of *AaFKBP12* (light orange) on *HsFKBP12* (pale pink), in ribbon representation, indicating a similar fold with subtle variations in loop regions (blue). The orientation of the protein is the same as in Figure 1(B) and residues have been labeled to trace the chain. This figure is generated using the program PyMOL.²⁰ [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

triggered by inhibitor binding. This split-bridge feature can be validated from the NMR structure of the protein, solved by our group recently (unpublished), where this distance has increased to 8.25 (57) Å. The distance between these two atoms in the X-ray and NMR structures of *HsFKBP12* is 4.0 Å and 4.63 (32) Å, respectively, indicating lesser fluctuation. Interestingly, in a similar situation, His87 in *HsFKBP12* is found to be replaced by Cys105/106 in *P. falciparum/vivax* FKBP35 [Fig. 1(A)] as described previously,^{5,6,24,25} and recently, these residues are being targeted with new inhibitors (unpublished data). Further binding studies show that the immunosuppressant FK506 binds tightly with *AaFKBP12* (data not shown), and its inhibitory effect on the dengue-transmitting mosquito is yet to be studied. In addition, from this structure, a MOPS buffer molecule sitting in the active site [Fig. 1(C)] could be a good nucleus for designing inhibitors for *AaFKBP12*. The morpholine ring in MOPS buffer is also present in mycophenolate mofetil, an immunosuppressant and pro-drug of mycophenolic acid, a known inhibitor of DEN-2 replication.^{26,27} Molecular interaction between mycophenolic acid and *AaFKBP12* and inhibition of PPIase activity of *AaFKBP12* in the presence of mycophenolic acid remain to be examined.

Materials and Methods

Cloning, expression, and purification

The gene sequences encoding *AaFKBP12* were synthesized from GenScript (Piscataway, NJ). The PCR-amplified DNA fragment was inserted into pETSUMO (Novagen, Madison, WI) to generate pETSUMO-*AaFKBP12* with a hexahistidine tag at the N-terminus. The protein was expressed in *Escherichia coli* BL21(DE3) cells and purified by consecutive cycles of Ni-NTA metal affinity chromatography, before and after cleaving the SUMO-tag.

Table I. Crystallographic Data Reduction and Refinement Statistics of *Aedes aegypti* FKBP12

Data reduction	
Wavelength (Å)	1.0000
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell parameters	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	29.82, 38.67, 79.58
Resolution range	30–1.30 (1.35–1.30) ^a
<i>R</i> _{merge}	0.030 (0.32)
No. of unique reflections	23,141 (2228)
Mean[<i>I</i> /σ(<i>I</i>)]	55.1 (4.7)
Completeness (%)	99.5 (98.2)
Multiplicity	7.3 (5.5)
Refinement	
No. of reflections	23,012
Resolution range (Å)	24–1.30
No. of atoms	
Protein/hetero atoms/water molecules	814/18/133
<i>R</i> -value	0.1631
<i>R</i> -free	0.1903
Average B-factor (Å ²)	
Overall/protein/hetero atoms/water molecules	23.83/21.05/33.09/39.92
R.m.s.d. from ideal values	
Bond lengths (Å)	0.013
Bond angles (°)	1.556
Ramachandran plot statistics (%)	
Most favored regions	94.2
Additionally allowed regions	5.8
Generously allowed regions	0.0
Disallowed regions	0.0

^a Values in parentheses refer to the corresponding values of the highest-resolution shell.

Crystallization, data collection, and structure determination

Crystal screening was performed at 15 mg/mL protein concentration, using hanging drop vapor diffusion method, with an ammonium sulfate-buffer grid. The reservoir contained 500 μL of the screening solution, and the drop constituted of 4 μL with equal volume of protein and reservoir solution. Crystals of 0.5 × 0.5 × 0.1 mm³ size appeared in 3.0M ammonium sulphate and 0.1M MOPS buffer pH 8.0, in 5 days. The crystals were cryo protected with 20% glycerol added to the reservoir solution and data, up to 1.3 Å resolution, was collected at 100 K on beamline 13B1 at the National Synchrotron Radiation Research Center (Hsinchu, Taiwan) using an ADSC-Quantum 315 detector. Two datasets (a low and high resolution) were collected from a single crystal and merged to improve the completeness in low-resolution shell. The diffraction data was indexed, integrated, merged, and scaled using the HKL2000 suite of programs.²⁸ The structure was solved by molecular replacement method using the program PHASER,²⁹ with initial phases from the *Human* (*Hs*)FKBP12 (PDB ID 2PPN) as search model. Refinement was performed using the program PHE-NIX³⁰ and map fitting with COOT.³¹ The sequence of AaFKBP12 (UNIPROT ID Q1HR83_AEDAE) was

used for mutating the residues using the program COOT. Further cycles of refinement, including the anisotropic parameters, ended up at the final model with good geometry and stereochemistry (Table I). Water molecules were added manually from the 2Fo-Fc and Fo-Fc electron density maps contoured at 1σ and 3σ cutoffs, respectively. In addition, we could observe a MOPS molecule, from the crystallization solution, occupying a portion of the active site pocket. The program PROCHECK³² showed none of the residues outside Ramachandran plot.³³ The data collection and refinement statistics are given in Table I. The coordinates and structure factors of AaFKBP12 have been deposited in the Protein Data Bank (PDB ID 3UQI).

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