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Expression, purification, and molecular characterization of *Plasmodium falciparum* FK506-binding protein 35 (PfFKBP35)

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Abstract

The immunosuppressive drug FK506 binds its targets FK506-binding protein (FKBP) family and modulates cellular processes. Recent studies demonstrated that FK506 shows anti-malaria effects. Newly identified FK506-binding protein 35 from *Plasmodium falciparum* (PfFKBP35) is assumed to be the molecular target of FK506 in the parasite. Currently, molecular and structural basis of growth inhibition of the parasite by FK506 remains unclear. In this study, to examine characteristics of PfFKBP35 and also understand its molecular mechanism of the inhibition by FK506, we have cloned, expressed, and purified the full-length PfFKBP35 and its FK506-binding domain (FKBD). We demonstrate that the full-length PfFKBP35 and the FKBD were properly folded, and suitable for biochemical and biophysical studies. PfFKBP35 showed a basal activity in inhibiting the phosphatase activity of calcineurin in the absence of FK506, but the presence of FK506 greatly enhanced its calcineurin-inhibitory activity. Our NMR data indicate that the FKBD binds FK506 with a high affinity.

Keywords: FK506; FKBP; Calcineurin; Malaria; *Plasmodium falciparum*; NMR

Human malaria still remains a major threat to the public health of countries in the tropical and subtropical regions of the world [1]. About 40% of the world's population

lives in areas where malaria is transmitted [1–3]. Human malaria is caused by infection with intracellular parasites *Plasmodium* that are transmitted by Anopheles mosquitoes. *Plasmodium falciparum* is the most lethal pathogen among the four species of *Plasmodium* that infect human beings. In recent years, extensive efforts have been made for the development of various tools and drugs to prevent the infection by *P. falciparum* [4]. The variation of parasite antigens hinders the development of vaccine against the parasite. In the midst of efforts to develop potential vaccines against the parasite, the identification of molecular targets, and attempts to develop inhibitors against authentic targets may serve as an alternative option in combating malaria [3].

Previous studies demonstrated that the immunosuppressive drug FK506 shows an anti-malarial effect [5], suggesting that the parasite may contain a potential FK506-binding protein as the molecular target of the drug. Recent efforts, mainly through a genomic analysis, resulted in the identification of a FKBP family protein (PfFKBP35) in *P. falciparum* [3,6]. PfFKBP35 shows a high similarity to FKBP12 in the catalytic core domain, whereas the overall structural architecture resembles the multiple tetratricopeptide repeat (TPR)¹-containing FKBP family including FKBP38, FKBP51, and FKBP52 [7]. The canonical FKBP family proteins possess peptidylprolyl *cis–trans* isomerase activity, FK506-binding activity, and chaperon activity [6]. PfFKBP35 as one of the multiple TPR-containing FKBP family members contains a FKBD (FK506-binding domain), a tripartite TPR domain, and one putative calmodulin-binding domain (CBD) (Fig. 1). It was demonstrated that FKBP38 and FKBP52, which show similar structural characteristics to that of PfFKBP35, interact with proteins in the cell cycle or apoptosis, and regulate their activities [8–10]. This suggests that PfFKBP35 may play an important role in the pathogenesis of *P. falciparum* in humans. Currently, molecular basis of the growth inhibition of the parasite by FK506 remains unclear. Towards a better understanding on the biological function of PfFKBP35, in this study, we have cloned, expressed, purified PfFKBP35, and subsequently performed biochemical and biophysical characterizations. Our results demonstrate that the purified proteins were properly folded and showed a basal level of inhibitory activity on calcineurin. NMR analysis showed that the FKBD of PfFKBP35 binds FK506 with a high affinity.

Materials and methods

Materials

Antibody against His-tag was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Ni^{2+} -nitriloacetic acid (NTA) resin was purchased from Qiagen (Hilden, Germany). Protein molecular weight marker was from Bio-Rad Laboratories (Hercules, CA, USA). HiPrep 26/ 60 Sephacryl S-200 column was from Amersham Biosciences (Buckinghamshire, UK). Chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). *Escherichia coli* BL21 (DE3) cells and kanamycin were from Invitrogen (Carlsbad, CA, USA). The vector pET29b was from Novagen (Madison, WI, USA). Isopropyl-thio- β -D-galactopyranoside (IPTG) was from Promega (Madison, WI, USA). C₄ and C₁₈ ZipTips were from Millipore (Billerica, MA, USA).

Construction of bacterial expression vectors for PfFKBP35 and its FKBD

The coding sequence for PfFKBP35 was amplified using the genomic DNA of *P. falciparum* 3D7 (A kind gift from Dr. Peter Preiser) as a template. The following primers were used for the cDNA amplification: forward primer contains *Nde*I site (5'-gctatctcatatgactaccgaacaagaattt-3'); reverse primer contains *Xho*I site (5'-agctagactcgagattgcactatttttttttc-3'). The amplified DNA fragment was digested with *Nde*I and *Xho*I and the resulting product was inserted into pET29b to generate pET29-FKBP35 with a hexahistidine tag at the C-terminus. The FKBD (M1-E127) of PfFKBP35 was also sub-cloned into pET29b using the same restriction enzymes and using pET29-FKBP35 as a template. The primers used were as follows: forward primer (5'-gctatctcatatgactaccgaacaagaattt-3'); reverse primer (5'-agctagactcgagttctctaaagcttaataattc-3') [11].

Expression and purification of recombinant PfFKBP35

The plasmid pET29-FKBP35 was transformed into *E. coli* BL21(DE3) cells, and transformed cells were grown in LB medium containing 30 $\mu\text{g/ml}$ of Kanamycin at 37 °C. The protein was induced by adding IPTG to 1 mM final concentration when the cell culture reached $\text{Abs}_{600} = 0.7\text{--}1.0$, and the culture was further incubated for additional 3 h

at 25 °C. The cells were harvested by centrifugation at 8000g for 10 min, re-suspended in the re-suspension buffer (20 mM NaPO₄, 500 mM NaCl), and broken by sonication for 20–30 min on ice. The cell lysate was cleared by centrifugation at 20,000g for 20 min and purified by Ni²⁺–NTA resin as described before [11]. The elution fractions from Ni²⁺–NTA were loaded onto a Sepharyl S-200 filtration column for a further purification. The purification of uniformly ¹⁵N-labeled protein was done using the same procedure as previously described [9,11]. The purified proteins were analyzed on a 12% SDS–polyacrylamide gel electrophoresis. The protein concentration was determined by Bradford dye assay kit (Bio-Rad Laboratories).

Matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF/TOF) analysis

Molecular weight and protein identification were confirmed by mass spectrometry (MS). Briefly, the purified PffKBP35 (20 µM) was desalted by using C₄ ZipTips. Impurities were then washed away with water prior to MS analysis. Ten picomoles of protein sample (0.3 µl) was mixed with 0.3 µl of Sinapinic acid matrix solution, spotted on the MALDI plate and subjected to air-dry. For protein ID determination, protein sample (5 µl) was digested overnight with trypsin followed by desalting with C₁₈ ZipTips. An aliquot (0.3 µl) of protein was mixed with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamic acid (αCHCA) matrix solution and was spotted on the MALDI sample plate and allowed to air-dry. The protein sample was analyzed on a MALDI-TOF/TOF (Applied Bio-systems MDS SCIEX 4800 MALDI-TOF/TOF). For the molecular weight determination, MS experiment was run in a linear TOF mode. For protein ID, the digested peptide was run in reflection mode first for peptide mass determination followed by MS/MS mode for peptide sequencing. All spectra acquired consisted of averaged signal from 600 laser shots, and the data were processed using accompanying Bio-systems mass spectrometry software.

Gel filtration analysis

Gel filtration analysis was performed on HiPrep 26/60 Sephacryl S-200 column (Amersham BioScience) with the buffer containing 20 mM NaPO₄, pH 6.8, 150mM NaCl, 1 mM DTT, 0.01 % NaN₃ at a flow rate of 1 ml/min. The theoretical molecular

weight of FKBP35 including LEHHHHH tag is 35.9 kDa. The molecular weight of pfFKBP35 was determined based upon the K_{av} values [$K_{av} = (V_e - V_0)/(V_t - V_0)$, $V_0 = 73.2$ ml, $V_t = 332.8$ ml], and standard curve drawn using the standard proteins: bovine thyroglobulin, 670.0 kDa; bovine γ -globulin, 158.0 kDa; chicken ovalbumin, 44 kDa; horse myoglobin, 17 kDa; vitamin B12, 1.35 kDa. The protein molecular weight was calculated according to its V_e and the standard curve.

NMR experiments

The unlabeled and uniformly ^{15}N -labeled FKBP35 samples in 20mM NaPO_4 , pH 6.8, 20mM NaCl, 1mM DTT, 0.01% NaN_3 were used for NMR studies. One-dimensional ^1H NMR and 2D ^1H - ^{15}N heteronuclear single quantum correlation spectroscopy (HSQC) and TROSY-HSQC spectra were acquired at 298K on a Bruker Avance AV 700 equipped with a cryoprobe accessory and processed with Topspin version 1.3 (Bruker, Switzerland). For NMR titration between FK506 and FKBD, 2D ^1H - ^{15}N HSQC spectrum of a uniformly ^{15}N -labeled FKBD (0.1mM) was recorded on a 700MHz NMR, and then an increasing amount of FK506 was added and the spectra of free FKBD and FKBD complexed with FK506 were superimposed. The chemical shift perturbations were examined as described [9].

Calcineurin assay

The calcineurin assay using commercial substrate **R11** (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-ValpSer-Val-Ala-Ala- Glu, MW. 2192.0) was performed according to the protocol provided by the manufacturer (CalBiochem, USA). The reaction buffer for the assays contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6 mM MgCl_2 , 0.5 mM CaCl_2 , 0.5 mM DTT, 0.025% NP-40, and 250 nM calmodulin. The reaction was performed in 50 μl at 37 °C for 45 min, and stopped by adding 100 μl Malachite-green dye followed by measuring the absorbance at 620 nm. The release of phosphate was calculated according to the standard curve as described [12].

Results

Expression and purification of PfFKBP35

For biochemical and structural characterization, the full-length PfFKBP35 and its FKBD (Fig. 1) were cloned into *E. coli* expression vector pET29b. The proteins were overexpressed and purified by a Ni²⁺-NTA affinity and gel filtration column chromatography. Our expression system allowed us to obtain the recombinant PfFKBP35 and its FKBD to a near homogeneity (Fig. 2a and b, lane 6). The purified proteins were stable after storage for one month at 4°C.

Characterization of PfFKBP35 and its FKBD

MS was used to characterize the purified full-length PfFKBP35. For the molecular weight determination, the sample was prepared, and subjected to MALDI-TOF/TOF experiment. The resulting spectrum (Fig. 3) showed the peak at 35,886Da which is in a good agreement with the theoretical mass of PfFKBP35 including the C-terminal LEHHHHHH calculated. For protein ID, the MS results were submitted to database search using MASCOT software which retrieved the protein ID of PfFKBP35 from *P. falciparum* 3D7 (AAN36539). The Mascot search result confirmed the purified protein was correct. Based on the MS data, the reduced molecular weight of PfFKBP35 is 35.9kDa. However, our gel filtration results showed that the recombinant full-length pfFKBP35 eluted at the elution volume of 147ml. The calculated molecular weights of the PfFKBP35 and FKBD from the elution profile were 74 and 15kDa, respectively (Fig. 4). This suggests that the full-length PfFKBP35 appear to exist as a dimer while the FKBD exists as a monomer in solution.

The analysis of the dispersion of NMR resonance signals is a good indicator of folded globular proteins [13]. One-dimensional ¹H NMR spectra of both full-length PfFKBP35 and its FKBD exhibited characteristic of a well-folded protein featuring good resonance dispersions in the regions of the methyl protons, α -protons, and amide protons (Fig. 5a and b). Two-dimensional ¹H-¹⁵N TROSY-HSQC and HSQC spectra showed a good dispersion of backbone amides, indicating that the PfFKBP35 and the FKBD are correctly folded (Fig. 5c and d) and ready for biochemical and structural studies.

Calcineurin-inhibitory activity of PfFKBP35 and effect of FK506

To examine whether the recombinant PfFKBP35 is active in its molecular function, we tested its calcineurin-inhibitory activity. As shown in Fig. 6, our results demonstrated that PfFKBP35 showed the calcineurin-inhibitory at 20 μ M independent of FK506 while the calcineurin inhibition by FKBP12 was FK506-dependent and FKBP38 showed little effect on calcineurin. To our surprise in this study we observed that the presence of FK506 significantly enhanced the ability of PfFKBP35 to inhibit the phosphatase activity of calcineurin. Our results showed that the degree of inhibition on the phosphatase activity of calcineurin by PfFKBP35 complexed with FK506 was comparable to that of FKBP12. This finding appears to be different from the earlier observations demonstrating FK506-independent inhibition of calcineurin [3,6] and remains to be further studied.

Identification of molecular interaction between FK506 and PfFKBP35

The fact that the calcineurin-inhibitory activity of PfFKBP35 was enhanced in the presence of FK506 prompted us to directly confirm the molecular interaction between PfFKBP35 and FK506. Since FK506 binds the FK506-binding domain of the canonical FKBP family proteins, in this study, we prepared uniformly ^{15}N -labeled FKBD of PfFKBP35, and we examined the molecular interaction by employing NMR-based binding assays. To this end, the perturbations of chemical shifts on a 2D ^1H - ^{15}N HSQC spectra were monitored before and after the addition of FK506. We demonstrated that several residues undergoing significant chemical shifts were detected when the spectra recorded in the absence and presence of FK506 were superimposed (Fig. 7). With resonance assignments available, we were able to locate the residues affected upon binding the drug. Our NMR data showed that the chemical shifts of aromatic residues (Y44, W78, and F118) conserved in FK506 binding were perturbed upon the addition of FK506. This suggests that the mode of the drug binding in the active site appears to resemble that of the canonical FKBP family. In addition, we noticed that the perturbed cross-peaks appear to exist in two different forms, without having step-wise chemical shift changes between the initial and final stage (data not shown), suggesting the drug binds tightly to the FKBD. Taken together, our NMR data suggest that FK506 clearly interacts with the FK506-binding domain of PfFKBP35 with a high affinity.

Discussion

The PfFKBP35 is a newly identified chaperone in the *P. falciparum*, and belongs to a multiple TPR-containing FKBP family. The multiple TPR-containing FKBP family deserves attention, because they show noncanonical catalytic and molecular characteristics and also they were shown to be associated with the anti-apoptotic proteins [10,11]. In this study, to examine the characteristics of PfFKBP35 we obtained the full-length PfFKBP35 and its FKBD. From our study we demonstrated that the full-length PfFKBP35 exists as a dimer while the FKBD shows monomer state in solution (Fig. 4). These data suggest that the TPR domain of PfFKBP35 might be important for the dimer formation. In our calcineurin assay, we showed that the purified recombinant FKBP35 could inhibit calcineurin at a relatively high concentration (20 μ M) in the absence of FK506 (Fig. 6). But, we observed that the presence of FK506 greatly influence PfFKBP35's calcineurin-inhibitory activity. Our observation is inconsistent with the previous studies showing the FK506-independent calcineurin-inhibitory activity of PfFKBP35 [3,6]. The sequence comparison shows that most of the residues involved in the ligand-binding for the canonical FKBP family are well conserved in PfFKBP35 (Fig. 1b). Indeed, our NMR data clearly demonstrated that the FKBD of PfFKBP35 binds FK506 with a high affinity (Fig. 7). The FK506-binding site on PfFKBP35 is localized in the conserved ligand-binding site. We speculate that the complex formation between PfFKBP35 and FK506 is important for the inhibitory effect on calcineurin. However, we cannot exclude the possibility that the discrepancy between this study and earlier studies [3,6] in PfFKBP35's ability to inhibit the phosphatase activity of calcineurin could be due to the different constructs used or the assay conditions employed in both studies. A future study would be necessary to answer the discrepancy.

Acknowledgement

We thank Dr. Peter Preiser for providing the genomic DNA of *Plasmodium falciparum* 3D7.

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List of Figures

- Fig. 1 Comparison of PfFKBP35 with other FKBP family proteins. (a) The diagram of PfFKBP35, its FKBD, and human FKBP38. The diagram shows domain layout of PfFKBP35. Compared to human FKBP38 (AAB00102), PfFKBP35 contains no transmembrane domain: FKBD, FK506-binding domain; TPR, tetratricopeptide repeat; CBD, calmodulin-binding domain; TM, transmembrane domain. (b) The amino acid alignment of human FKBP12 and the FKBD of PfFKBD35, human FKBP38, and human FKBP52 (A46437) is shown. ∇, represent aromatic residues in the FK506-binding pocket. •, represent residues involved in interaction with FK506 through hydrogen bonds. The protein sequences were aligned by Vector NTI (InforMax).
- Fig. 2. Purification of PfFKBP35 and its FKBD. PfFKBP35 was expressed and induced as described in Material and methods. The overexpressed proteins were first purified by Ni^{2+} -NTA affinity purification and followed by the gel filtration analysis. Samples of PfFKBP35 (a) and its FKBD (b) from various steps were analyzed by 12.5% SDS-PAGE. 1, molecular weight; 2, *E. coli* cell lysate without IPTG induction; 3, IPTG-induced lysate; 4, sample loaded onto the Ni^{2+} -NTA column; 5, elution fraction of affinity column; 6, purified PfFKBP35 from gel filtration.
- Fig. 3. MS analysis of PfFKBP35. The purified protein sample was subjected to MALDI-TOF/TOF analysis on Applied Bio-systems MDS SCIEX 4800 MALDI-TOF/TOF analyzer as described in Materials and methods.
- Fig. 4. Gel filtration analysis of PfFKBP35 and its FKBD. The purified PfFKBP35 and FKBD from affinity purification were loaded onto Sephacryl S-200 and molecular weights of the proteins were calculated according to the standard curve using the molecular weight markers. The

calculated molecular weight of FKBD and PFKBP35 was indicated.

Fig. 5. 1D ^1H NMR and 2D- ^1H - ^{15}N HSQC NMR spectra of PFKBP35 and its FKBD. The full-length PFKBP35 (a) and its FKBD (b) were examined by 1D ^1H NMR. The purified proteins (0.1 mM) were in the NMR buffer containing 20 mM NaPO_4 , pH 6.8, 20 mM NaCl, 1 mM DTT, and 0.01% NaN_3 . The spectrum was recorded on 700 MHz at 298 K with water suppression. Two-dimensional ^1H - ^{15}N TROSY-HSQC spectra of PFKBP35 (c) and its FKBD (d) were recorded on a 700 MHz at 298 K using ^{15}N uniformly labeled PFKBP35 as described in Materials and methods.

Fig. 6. Inhibitory effect of PFKBP35 on calcineurin. Calcineurin-inhibitory activity of the recombinant PFKBP35 was examined. For comparison, the activity of human calcineurin was measured by observing the release of phosphate with the addition of malachite green. The human FKBP38 and human FKBP12 were also used in the assay. In the assay, the concentration of FK506 is two times of the protein concentration. The phosphatase activities are averages of three independent experiments.

Fig. 7. Molecular interaction of FK506 with PFKBP35. FK506 interacts with the FKBD of PFKBP35 in a NMR-based assay. (a) ^{15}N -labeled FKBD was monitored on a ^{15}N -heteronuclear single quantum correlation spectroscopy (HSQC) upon the addition of FK506. Spectrum of FKBD alone is presented in black. Spectrum of FKBD with FK506 is presented in red. Concentration of ^{15}N -labeled FKBD was 0.1 and 0.25mM of FK506 was added for binding studies. (b), (c) Sections of a ^{15}N -HSQC spectrum and chemical shift changes of the residues involved in ligand-binding (Y44, W78, and F118) upon the addition of FK506 were indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Fig. 1

Fig. 2

Fig. 3

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Fig. 5

Fig. 6

Fig. 7