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Multimer formation by FKBP-12: roles for cysteine 23 and phenylalanine 36

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Abstract: FKBP-12 mediates the immunosuppressive actions of FK506 and rapamycin, and modulates the activities of the ryanodine, IP3 and type 1 TGF-ß receptors. Additionally, FKBP-12 possesses *cis-trans* peptidylprolyl isomerase (rotamase) activity. We have discovered that recombinant FKBP-12 readily forms a dimer and a small amount of trimer under nonreducing conditions. A mutant with substitution at the sole cysteine residue of FKBP-12 (C23S) did not form dimers or trimers. Using mutants with 5% or less rotamase activity, the formation of dimers was independent of enzymatic activity. The formation of trimers was abrogated by a F36Y substitution, even though dimer formation was preserved. Dimers were also observed with native FKBP-12 that was detached from rabbit skeletal muscle ryanodine receptors using FK590. The multimers of FKBP-12 could interact with molecular targets distinctly from the FKBP-12 monomer, for example, by facilitating the assembly of multimeric receptors or coordinating the activity of receptor subunits. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: FKBP-12; dimer; disulfide bonds; ryanodine receptor; IP3 receptor; TGF-β receptor; *cis-trans* peptidylprolyl isomerase

INTRODUCTION

FKBP-12 is a member of the immunophilin family of proteins that function as cytosolic receptors for immunosuppressant drugs such as rapamycin and FK506 [1,2]. The immunosuppressive functions of FK506 and rapamycin result from the ability of the drug–FKBP-12 complex to bind to and inhibit the phosphatase activity of calcineurin [3,4]. One of the substrates for calcineurin, nuclear factor of activated T cells (NFAT), is unable to translocate from the cytoplasm to the nucleus in the phosphorylated form that accumulates with calcineurin inhibition. Lack of NFAT in the nucleus leads to decreased interleukin-2 formation and to immunosuppressive effects [5,6].

FKBP-12 also functions as a *cis-trans* prolylisomerase [7], an activity with no known pathophysiological effects. FKBP-12 binds to all three forms of the ryanodine receptor (RyR1 -3) and is the predominant form bound to RyR1, the skeletal muscle isoforms [8]. FKBP-12 stabilizes a closed state of the channel and enhances cooperative interactions between the subunits of the RyR1 tetramer [9], including regulating the phenomenon of coupled gating in which neighboring channels open simultaneously [10]. FKBP-12 binds to the inositol 1,4,5-triphosphate receptor and to all four subtypes of the type 1 TGF-β receptor, recognizing the conserved 'SGSGSGLP' motif on the cytoplasmic tail [11]. The interaction of FKBP-12 with the type I receptor inhibits signaling pathways of the TGF-β family ligands [12].

During the course of purifying recombinant FKBP-12, we have observed that the protein readily forms disulfide-linked dimers. The formation of dimers was abolished in a C23S mutant. Furthermore, a small amount of trimer was observed, which was dependent on the presence of phenylalamine 36, being abolished in an F36Y mutant. We postulate that the dimer and trimer formation of FKBP-12 could have unique functions in interactions with multimeric macromolecules including receptors.

MATERIALS AND METHODS

Materials

The FKBP-12 wild type cDNA cloned in the pET3d vector (Novagen, Madison, WI) has been previously described [13]. Two mutants of FKBP-12, F36Y and F99Y, which have 5 and 0.1% PPIase activity, respectively, of wild type FKBP-12, have also been cloned into pET3d [14]. Oligonucleotide primers were synthesized on an ABI 394 DNA/RNA synthesizer using standard cyanoethyl phosphoramide chemistry in the DNA Synthesis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University School of Medicine:

wt-Nco: 5′-CATGCCATGGGAGTGCAGGTGG-3′
wt-Bam: 5′-CGGGATCCTCATTCCAGTTTTAGAAGC-3′
cys-forwards: 5′-GCGCGGCCAGACC***A***GCGTGGTGCAC-3′
cys-backwards: 5′-GTGCACCACGC***T***GGTCTGGCCGCGC-3′

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Cloning of FKBP-12 C23S

To examine the role of disulfide bonds in FKBP-12 dimer and trimer formation, the only cysteine (residue 23) in FKBP-12 was changed to serine, using the process of splicing by overlap extension. To introduce the cysteine to serine substitution at residue 23, three separate PCR reactions were performed:

PCR Ia: The *N*-terminal part (1–67) of FKBP-12 wt in pET3d was amplified with primers wt-Nco and cys-backwards. This primer has a T for A substitution (shown underlined).

PCR Ib: The *C*-terminal part (67–327) of FKBP-12 wt in pET3d was amplified with primers cys-forwards and wt-bam. The primer cys-forward has an A for T substitution.

PCR II: The purified fragments of PCR Ia and Ib were amplified with the outside primers, wt-Nco and wt-Bam. The restriction sites in the primers are underlined.

Ligation and Transformation

The FKBP-12 C23S cDNA fragment was first TA-cloned into pCR2.1 (Invitrogen, San Diego, CA) and the construct transformed into *E. coli* INVα' (Invitrogen). Both strands were sequenced to verify the construction and to eliminate PCR-induced errors. For the ligation into pET3d, the vector and insert were digested with restriction enzymes NcoI and BamHI. The vector pET3d containing FKBP-12 C23S was then transformed into *E. coli* BL21(DE3) from Novagen (Madison, WI).

Expression of Wild Type and Mutant Forms of FKBP-12 in *E. coli*

E. coli BL21(DE3) transformed with recombinant pET3d (Novagen, Madison, WI) containing the inserted FKBP-12 wt, FKBP-12 F36Y, FKBP-12 F99Y or FKBP-12 C23S cDNAs cloned into the NcoI-BamHI site were grown at 37 °C in LB medium containing 100 μg/ml ampicillin. When the OD_{600} reached 0.5–1.0, isopropyl-β-D-thiogalactopyranoside was added (0.5 mM), and incubation was continued for another 3–5 h. Bacteria were then harvested by centrifugation (5000*g* for 10 min at 4 °C) and resuspended in 20 ml of 20 mM Tris pH 7.4 with protease inhibitors (leupeptin, pepstatin, bestatin and aprotinin, each at 1 μg/ml). The sample was frozen overnight at −70 °C, afterwards thawed at RT and vortexed to release the recombinant protein from the periplasmic region. The bacteria were pelleted (12 000*g* for 30 min at 4 °C) and the FKBP-12-containing supernatant was used for analysis and purification.

Protein Purification

Purification of FKBP-12 wt and mutants was done with fast performance liquid chromatography (FPLC), using gel filtration with a HiPrep 26/60 Sephacryl S-100 high resolution column (Pharmacia) and the following buffer: 100 mM KCl, 20 mM potassium phosphate (pH 7.5), 0.02% NaN_3, and 2 mM β-mercaptoethanol. This buffer composition, including the presence of β-mercaptoethanol has been used for previous purifications of recombinant FKBP-12 [15]. The fractions containing FKBP-12 were pooled, concentrated with Centriprep 3 (Amicon, Beverly, MA) and then dialyzed with Slide-A-Lyzer 2K (2000 MWCO; Pierce, Rockford, IL) in 150 mM KCl pH 7.0. The protein concentration was measured at $\lambda = 280$ nm ($\varepsilon_{280\ FKBP-12} = 9530\ M^{-1}cm^{-1}$).

SDS-PAGE and Immunoblotting

SDS-PAGE was performed in 15% SDS gels under both reducing (5% β-mercaptoethanol) and nonreducing conditions. Samples were boiled for 5 min prior to SDS-PAGE, and then transferred to nitrocellulose. The membrane was blocked with 3% nonfat dry milk in TBS. The monoclonal anti-FKBP-12 antibody (clone 2C1-87, Pharmingen, San Diego, CA) was used at 1 : 500 dilution in 3% milk/Tris-buffered saline. The secondary antibody was a monoclonal anti-mouse IgG/peroxidase conjugate (Amersham Life Sciences, Arlington Heights, IL) at 1 : 2000 dilution in 3% milk/Tris-buffered saline. Bound antibodies were detected using chemiluminescence with the ECL kit (Amersham Life Sciences, Arlington Heights, IL).

Protein Microsequencing

Protein sequencing was performed on proteins that were separated by SDS-PAGE, and then electroblotted in 10 mM CAPS, 10% methanol (pH 11) onto PVDF membrane. The monomeric, dimeric and trimeric protein bands were excised from the membrane and subjected to automated Edman degradation using an Applied Biosystems 477A protein sequencer in the Protein Core Laboratory of the Comprehensive Cancer Center of Wake Forest University School of Medicine. The PTH derivatives of the amino acids were analyzed by an on-line PTH analyzer. The first 12 residues were sequenced for the monomer, dimer and trimer. The resulting sequences were compared with protein libraries (BLAST, SWISS-PROT).

The Dissociation of Native FKBP-12 from Rabbit Sarcoplasmic Reticulum vesicles

To examine the formation of dimers in native FKBP-12, a crude heavy fraction of sarcoplasmic reticulum was isolated from New Zealand White rabbit white skeletal muscle as previously described [16] and suspended at a concentration of 25 mg/ml in 20 mM histidine, 150 mM KCl, 0.3 M trehalose and protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 10 μg/ml soybean trypsin inhibitor). To remove FKBP-12, 600 μl of crude heavy SR preparation was diluted to 1.5 ml total volume in the same buffer and FK-506 or FK-590 in 95% ethanol was added to give a final concentration of 50 μM. The SR vesicles were incubated for 16 h at 4 °C. As a control for the effect of the solvent, some preparations were incubated in an equivalent volume of 95% ethanol without FK-506 or FK-590. The vesicles were centrifuged at 25 000 rpm in a Beckman TLA 100.3 rotor at 4 °C for 30 min. The supernatant was concentrated two-fold and used for SDS-PAGE and immunoblotting to verify that FKBP-12 had been removed by immunophilin treatment and to determine whether dimers formed under these conditions. The rabbit FKBP-12 amino acid sequence is identical to that of the human one [17].

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RESULTS

Wild type FKBP-12 and Mutants Form Dimers

The recombinant wild type FKBP-12 and the mutants were expressed in *E. coli* BL21(DE3) and isolated from the periplasmic region by a freeze/thaw cycle. The resulting extracts were analyzed in reducing and nonreducing SDS gels. The gels showed ~80% pure FKBP-12 in a background of several bacterial proteins (Figure 1(A)). One prominent protein at ~26 kDa, present on reducing and nonreducing gels, was a protein of bacterial origin. To prove the identity of FKBP-12 we also carried out immunoblots of reducing and nonreducing SDS gels. In immunoblots derived from reducing gels, the monoclonal anti-FKBP-12 antibody recognized only the expected FKBP-12 band at 12 kDa (not shown). The immunoblots of nonreducing gels, on the other hand, showed two bands for wild type and the F36Y and F99Y mutants, and the second band with an apparent size around 24 kDa (Figure 1(B)).

FKBP-12 C23S

To examine whether the presumed dimers seen in immunoblots of nonreducing SDS gels were formed by disulfide bonds, we substituted a serine for the only cysteine in FKBP-12 (residue 23) to eliminate any possibility of forming disulfide bonds. On reducing gels and their immunoblots, the new mutant C23S showed no difference to wild type FKBP-12 or mutants F36Y and F99Y (not shown). However, for the C23S mutant there was only one recognized band with the size of FKBP-12 monomer (Figure 1(B), lane 4).

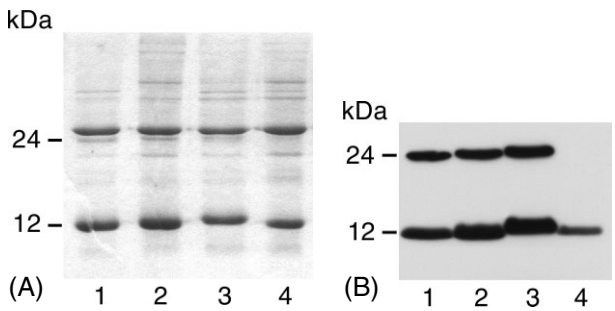

Figure 1 FKBP-12 wt and mutants from periplasmic extract. Wild type and mutant forms of FKBP-12 were expressed in *E.coli* BL21(DE3), then extracted from the periplasmic space as described in 'Materials and Methods'. Replicate 15% SDS-PAGE gels were run under nonreducing conditions and either Coomassie-stained (Figure 1(A)) or immunoblotted with anti-FKBP-12 (Figure 1(B)). Lanes 1: wild type FKBP-12; Lanes 2: F36Y FKBP-12; Lanes 3: F99Y FKBP-12; Lanes 4: C23S FKBP-12. The major band at ~26 kDa seen on the Coomassie-stained gels represents a bacterial protein, not the FKBP-12 dimer (see Figure 2, lanes 2).

Purification of FKBP-12 Wild Type and Mutants

To further purify wild type FKBP-12 and mutants, the periplasmic extract was loaded on an FPLC column using a reducing buffer. Using this method we could purify the proteins to more then 95% purity. When analyzed on SDS gel directly after purification, almost pure monomer was shown in case of wild type FKBP-12 (Figure 2(A)) and the mutants F36Y, F99Y and C23S (not shown). But after leaving the monomer-containing fractions open at 4 °C for hours to days (Figure 2(B)), increasing amounts of dimer were formed (probably because of evaporating β-mercaptoethanol), except for FKBP-12 C23S, which stayed completely monomeric (not shown). We also purified the ~26 kDa bacterial protein to show that it was distinct in size to the FKBP-12 dimer (Figure 2(B)) and did not cross-react with the antibody in immunoblots (not shown).

Trimer Formation

After FPLC purification, the FKBP-12-containing fractions of each mutant and the wild type protein were individually pooled, concentrated and dialyzed into KCl. They were again analyzed on gels and immunoblots. Now the gels (Figure 3(A)) and immunoblots (Figure 3(B)) showed another additional band in case of wild type FKBP-12 and the F99Y mutant, which was recognized by antibodies and therefore could be an FKBP-12 trimer. The C23S mutant had neither a dimer nor a trimer (Figure 3(B), lane 4) and

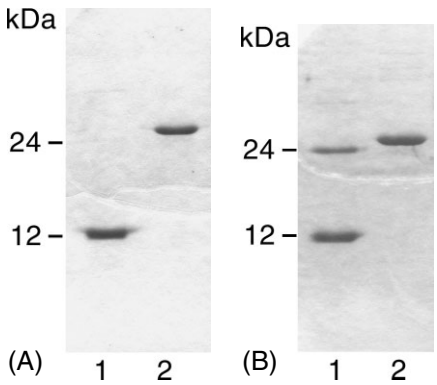

Figure 2 Purified FKBP-12 and 26 kDa bacterial protein. Wild type FKBP12 was expressed and purified as described in 'Materials and Methods'. A bacterial protein of ~26 kDa was purified under reducing conditions. Purified recombinant wild type FKBP-12 was loaded immediately after FPLC purification (Figure 2(A), Lane 1) or after 2 days storage at 4 °C (Figure 2(B), Lane 1). Both gels are nonreducing 15% SDS-PAGE with Coomassie-staining. Lanes 1: purified recombinant FKBP-12. Note that the FKBP-12 dimer that forms after 2 days is slightly smaller than the bacterial protein (Figures 2(A) and (B), lanes 2). Lanes 2: ~26 kDa bacterial protein that migrates near the FKBP-12 dimer. This protein is not detected by anti-FKBP-12 in immunoblots (not shown).

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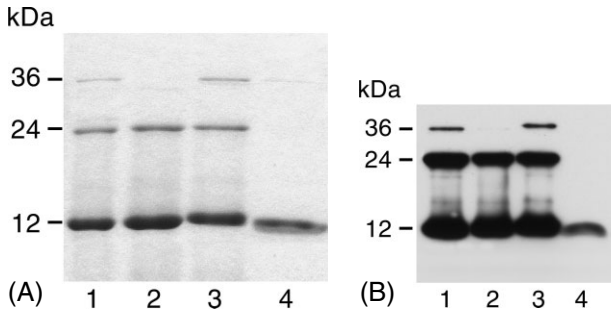

Figure 3 Formation of dimers and trimers by wild type and mutant forms of purified recombinant FKBP-12. Wild type and mutant forms of FKBP-12 were expressed, purified, concentrated and dialyzed into 150 mM KCl, pH 7.0 as described in 'Materials and Methods'. The purified proteins were separated by 15% nonreducing SDS-PAGE on replicate gels which were Coomassie-stained (Figure 3(A)) or transferred to nitrocellulose and immunoblotted using anti-FKBP-12 (Figure 3(B)). Lanes 1: wild type FKBP-12, Lanes 2: the F36Y mutant, Lanes 3: the F99Y mutant and Lanes 4: the C23S mutant.

the F36Y mutant had only one dimer band (Figure 3(B), lane 2).

Protein Sequencing

To exclude the possibility that the assumed FKBP-12 dimer could be a heterodimer of one FKBP-12 molecule binding to a bacterial protein of similar size, we performed protein sequencing on purified wild type FKBP-12 in 150 mM KCl. The Coomassie-stained PVDF membrane blot from a nonreducing SDS-PAGE gel showed four discrete bands, two dominant ones at 12 kDa and 24 kDa and two light ones at 36 kDa and around 40 kDa (not shown). Protein sequencing proved three of them to be pure FKBP-12: one at 12 kDa, one at 24 kDa and the last one at 36 kDa. This proves the existence of FKBP-12 monomers, dimers and trimers. Protein sequencing also identified the fourth band slightly bigger than the FKBP-12 trimer as the bacterial maltose-binding periplasmic protein (43 kDa). We do not have an explanation for why this protein co-purified with FKBP-12.

Dimer formation in sarcoplasmic reticulum (SR) vesicles. In order to determine whether native FKBP-12 formed dimers or trimers, vesicles were treated with FK590 or with 95% ethanol as a control. In the supernatant of the vesicles treated with FK590, the FKBP-12 specific antibody recognized three bands at 12, 24 and 61 kDa (Figure 4). Only the upper band, which remained unidentified, also appeared in the control supernatant. This immunoblot clearly shows that intrinsic rabbit FKBP-12 can also form dimers.

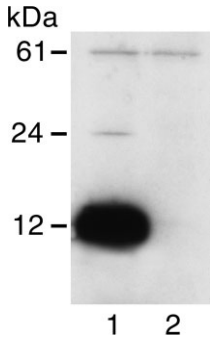

Figure 4 Formation of dimer by native FKBP-12 released from rabbit skeletal muscle sarcoplasmic reticulum with FK-509. Rabbit skeletal muscle sarcoplasmic reticulum vesicles were treated with FK590 or with vehicle (95% ethanol). After centrifugation and concentration, the supernatant was separated on 15% nonreducing SDS-PAGE replicate gels and immunoblotted. Lane 1: FK590-treated vesicles show a 12 kDa band and also shows the presence of a 24 kDa dimer. Lane 2: ethanol treated vesicles (control). The FKBP-12 monomer and dimer are not detected in these lanes. A 61 kDa band of unknown origin was detected by immunoblotting in both the control- and FK590-treated lanes.

DISCUSSION

In this paper, we demonstrate the formation of disulfide-linked dimers of the immunophilin, FK 506 binding protein-12 (FKBP-12). The formation of a dimer of FKBP-12 was previously reported using a recombinant protein which was electrophoresed under nonreducing conditions [18]. In that study, immunoblotting was performed using plasma from patients with a variety of autoimmune diseases, demonstrating that a significant percentage of them had circulating autoantibodies to FKBP-12 [18]. The autoantibodies recognized both the monomeric and dimeric forms of FBBP-12 [18]. However, the molecular mechanisms for dimer formation were not investigated. In the present work, we have shown that the formation of dimers occurs via intermolecular disulfide bond formation utilizing the single cysteine residue in FKBP-12 (residue 23). Dimer formation was abolished under reducing conditions and failed to occur under nonreducing conditions with the C23S mutant.

Studies with another mutant of FKBP-12 may provide mechanistic insights into the dimerization process [19]. Mutant FKBP-12 with a substitution at phenylalanine 36 (F36M) was found to undergo rapid dimerization. Dimer formation occurred with relatively weak affinity and was reversible with the ligand FK506, suggesting a noncovalent interaction rather than disulfide bonding [19]. The investigators proposed that the F36M substitution removes a steric hindrance that is induced by Phe-36, allowing a latent property of self-dimerization to become expressed [19]. This tendency may align two FKBP-12 molecules in a manner that encourages the ultimate formation

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of disulfide bonds, despite the steric hindrance of phenylalanine-36.

Using concentrated protein preparations, we also observed a small amount of trimer with wild type FKBP-12 and with the F99Y mutant. However, interestingly, the F36Y mutant failed to form a trimer, even though dimer formation was preserved. The mechanism for trimer formation with wild type FKBP-12 and the failure to detect trimers with F36Y are currently not explained. It is possible that the dimeric form of FKBP-12 has increased affinity for monomeric FKBP-12 which serves as its substrate. FKBP-12 is known to catalyze its own folding [20]. There is a previous example of a dimer of a member of the FKBP family having increased rotamase activity. The FK506 binding protein from *Legionella pneumophila* (FKBP25mem or Mip) exists as a homodimer in solution and on the bacterial cell surface rotamase [21]. The dimerization of FKBP25mem resulted in a significant enhancement of activity compared to the monomer [21]. The F36Y mutant lacks significant rotamase activity and also may have a distorted structure that prevents it from being a rotamase substrate, since phenylalanine 36 clearly plays an important role in regulating the quarternary structure of FKBP-12 [19].

The formation of disulfide-linked dimers of FKBP-12 could have functional significance. Two of the receptors to which FKBP-12 binds, including the ryanodine receptors [8] and the TGF-β1 receptor [12], are multimeric proteins. It is possible that dimers of FKBP-12 could facilitate simultaneous interactions with two subunits of these receptors. Coupled gating in the skeletal muscle ryanodine receptor is dependent on FKBP-12 [10], suggesting the interesting possibility that FKBP-12 dimers could facilitate this process by binding simultaneously to neighboring calcium release channels.

The cytoplasm of the cell is usually a strongly reducing environment, rendering the formation of disulfide bonds less likely. Under certain conditions, however, the oxidant stress to the cytoplasm increases and these conditions could favor the formation of disulfide-linked FKBP-12 dimers. For example, strenuous exercise leads to the production of oxygen free radicals including hydroxyl radicals, superoxide anions and H_2O_2 [22,23]. The rabbit skeletal muscle type 1 ryanodine receptor contains 100 cysteine residues [24]. Modifications of certain hyper-reactive cysteine residues [25] has marked effects on RyR1 channel open probability. Furthermore, the redox state of ryanodine receptors influences their binding affinity for FKBP proteins [26]. It is possible that the cysteine residue of FKBP-12 protects the RYR1 sulfhydryl residues from these modifications, an effect that would be lost by FKBP-12 disulfide dimer formation.

These considerations stress the importance of knowing the molecular form of FKBP-12, which is used in studying its interactions with the ryanodine receptor and other receptors. Under conditions that are commonly used for studying the interaction of FKBP-12 with RYR1, we have found that approximately one-third of the molecule exists as a dimer. It is possible that the monomeric and dimeric forms of FKBP-12 have distinct functional effects on the RYR1 receptor and other binding proteins. The C23S mutant may be a useful molecular tool for differentiating the functional effects of monomeric and multimeric FKBP-12, and for a better understanding of the mechanism by which FKBP-12 multimers form.

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