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The binding of FKBP23 to BiP modulates BiP's ATPase activity with its PPIase activity

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Abstract

Peptidyl-prolyl *cis–trans*-isomerases (PPIases) are enzymes that can *cis–trans*-isomerize a Xaa-Pro peptide bond. Three families of PPIases are known: cyclophilins, FKBPs, and parvulins. The physiological functions of the PPIases are only poorly understood. In previous work, we reported that the mouse FK506-binding protein 23 (mFKBP23), which comprises an N-terminal PPIase domain and a C-terminal domain with Ca^{2+}-binding sites, binds to mBiP in the endoplasmic reticulum (ER) and this binding is affected by the Ca^{2+} concentration. In this study, we demonstrate the ability of mFKBP23 to modulate the ATPase activity of BiP, and that the bound mFKBP23, but not the free mFKBP23, can suppress the ATPase activity of mBiP through its PPIase activity.

Keywords: FK506-binding protein; PPIase activity; BiP; ATPase activity; Ca^{2+}-binding relationship

Peptidyl-prolyl *cis–trans*-isomerases (PPIases; EC 5.2.1.8) are ubiquitous proteins that have been found in bacteria, fungi, plants, and animals. They are able to catalyze the *cis–trans* isomerization of Xaa-Pro bonds in oligopeptides and proteins [1,2]. The PPIases can be divided into three structurally unrelated families, namely the cyclophilins (CyPs), the FK506-binding proteins (FKBPs), and the parvulin-like proteins. They are characterized by their ability to bind to specific immunosuppressant drugs: cyclosporin A, FK506, and rapamycin, respectively. FKBP23 is a PPIase that is found in the endoplasmic reticulum (ER) and comprises an amino-terminal PPIase domain and a carboxy-terminal domain with two calcium-binding EF-hand motifs [3]. Sequence alignment of mouse FKBP23 (mFKBP23) and mFKBP12 showed that the N-terminus of mFKBP23 (amino acids 20–137) is part of a PPIase domain. It has also been reported that the C-terminus of mouse FKBP23 binds to BiP in the ER and that the binding is dependent on the Ca^{2+} concentration [4].

The molecular chaperone in ER, the immunoglobulin (Ig) heavy-chain-binding protein (BiP), is a member of the Hsp70 family and was the first member of the Hsp70 multigene family to be identified [5–7]. Many unfolded proteins are chaperoned by BiP in the ER. Through the maintenance of solubility by BiP, unfolded proteins are occasionally subjected to ER-associated degradation [8–10]. Furthermore, BiP is involved in many cellular processes, including the translocation of newly synthesized polypeptides across the ER membrane, participation in the folding and maturation of these polypeptides, assisting in refolding and renaturation, targeting misfolded proteins to the cytosol for degradation, maintaining selectivity of

Abbreviations: PPIase, peptidyl-prolyl *cis–trans*-isomerase; mFKBP, mouse FK506 binding protein; mBiP, mouse immunoglobulin heavy-chain-binding protein; ER, endoplasmic reticulum; GST, glutathione *S*–transferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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the ER membrane by closing the translocon pore, and regulating calcium homeostasis [11–14]. BiP and all other Hsp70s are evolutionarily conserved. They contain two functional domains: an N-terminal 45-kDa ATPase domain and a C-terminal 25-kDa substrate-binding domain. The C-terminal domain consists of eight β-sheets and five α-helices. The folded β-sheets form a site for peptide binding and conformation changes due to folding of the eight β-sheets and the five α-helices can open and close the peptide-binding gap [15–17]. Like all members of the Hsp70 family, BiP has weak ATPase activity to provide energy for protein translocation and protein folding [18]. ATP binding to the ATPase domain leads to rapid, but low-affinity, and subsequent ATP hydrolysis stabilizes substrate binding. Another molecular chaperone, Hsp90, has weak ATPase activity and this activity has been shown to be increased 200-fold by stimulation of protein binding [19]. The ATPase activity of BiP and other Hsp70s is stimulated by members of the J-domain family [20]. Recently, some regulatory proteins have also been reported, which bind to Hsp70/BiP and modulate its ATPase activity [21,22].

In this study, we investigated the effect of the binding of FKBP23 on the ATPase activity of BiP. The results show that bound FKBP23, but not the free FKBP23, can suppress the ATPase activity of BiP through its PPIase activity.

Materials and methods

Construction of plasmids expressing mFKBP23, mBiP, mFKBP23$_C$, mBiP$_N$, and mBiP$_C$. The plasmids expressing mFKBP23, mBiP, mFKBP23$_C$ were constructed as described previously [4]. To subclone the N-terminal and C-terminal domains of mBiP (mBiP$_N$ and mBiP$_C$, respectively) the previously obtained pGEX5X1-mBiP was used as a template, the primers used were: mBiPf as an upstream primer and a downstream primer including an *Xho*I site and a TAG stop codon (5′-GGCCGGCTCGAGCTAAAGCAGTACCAGATCACCTG-3′) for mBiP$_N$; and an upstream primer including an *Bam*HI site (5′-CCGGCCGGATCCTCTCTGGTGATCAGGA-3′) and mBiPr as a downstream primer for mBiP$_C$, respectively. After digestion with *Bam*HI and *Xho*I restriction enzymes, the PCR fragments were ligated into pGEX5X1.

Purification of GST fusion proteins and recombinant proteins without tag of GST. In this paper, the glutathione *S*-transferase (GST) fusion protein system was used. The expression and isolation of the recombinant proteins were performed as described previously [4,23]. The purified GST-mBiP and GST-mFKBP23 on the beads were restriction-digested with factor Xa (Amersham Pharmacia Biotech) in a digestion buffer (50 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$ and 100 mM NaCl) overnight at 4 °C.

Preparation of ER extract from mouse liver. This was carried out as described previously [4,24]. About 150 g mouse livers were suspended with 50 mL Buffer E (1 M sorbitol, 10 mM Hepes, pH 7.4 and 1 mM EDTA) and applied to homogenate. Then 1/10 volume of NP-40 (3% solution) was added and shaken on ice for 5 min to break the cells. The suspension was centrifuged for 20 min at 1000*g* and the supernatant was collected. The pellet was suspended with 10 mL buffer E and centrifuged for 20 min at 1000*g* once more. The two supernatants were collected together and then centrifuged sequentially at 12,000*g* and 40,000*g* for 1 h, respectively. The pellet was washed cautiously with 5 mL buffer E and centrifuged for 30 min at 40,000*g*. Then the pellet was suspended with 2 mL buffer E, sheared using a syringe and centrifuged for 2 h at 100,000*g*. The supernatant is ER lumen extract and which was tested by Western blot to ensure there was no contamination with cytosol.

Protein ATPase assays. A spectrophotometric assay was used to measure the decrease of [NADH] in absorbance at 338 nm coupled with ATP hydrolysis, involving pyruvate kinase and lactate dehydrogenase as described previously [25,26].

Tested components (mBiP + mFKBP23, mBiP + mFKBP23 + FK506, mBiP + mFKBP23 + GST-mFKBP23$_C$) were added to 350 μL reaction mixtures in buffer A (20 mM Tris–HCl pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 300 μM NADH, 2 mM phosphoenolpyruvate, 1 mM ATP, 3 U/mL pyruvate kinase and 3 U/mL lactate dehydrogenase). The reduction of NADH was monitored by measuring at 37 °C the absorbance at 338 nm. The results were linear from 0 to 20 min. In these coupled reactions, the hydrolysis of one molecule ATP can lead to oxidation of one molecule NADH. The ATPase activity of BiP, which is shown as relationship of hydrolysis rates of ATP (nmol/min) to amount of BiP, can be calculated with the reducing rates of extinction of NADH at 338 nm.

Gel filtration chromatography for binding test in vivo. A Sephadex G-75 column (1 × 30 cm) was used for GFC analysis. The column was equilibrated with binding buffer (20 mM Tris–HCl pH 8.0, 50 mM KCl, 1 mM $MgCl_2$), without or with 5 mM Ca^{2+}. One milliliter sample containing 4 μg recombinant BiP, 1 μg recombinant FKBP23 or 1 mL mouse ER extract without or with 5 mM Ca^{2+} was loaded onto the column. The column was eluted with the same buffer with a flow rate of 0.2 mL/min and fraction volume of 1 mL/tube. Eighty microliter of each tube was precipitated by TCA/DOC as probe for Western blot.

Binding assay in vitro. 0.025 nmol mFKBP23 in 400 μL binding buffer (20 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM $MgCl_2$), without or with 5 mM Ca^{2+}, was incubated with GST-mBiP$_C$ or GST-mBiP$_N$ (0.2 nmol each) bound to glutathione–Sepharose 4B beads at room temperature for 40 min. The beads were washed five times with 1 mL binding buffer containing 0.1% Tween and once with 1 mL binding buffer without Tween. The bound mFKBP23 was detected by using Western blot as described previously [4].

Results

The binding of mFKBP23 to mBiP suppresses the ATPase activity of mBiP

To investigate if the binding of mFKBP23 to mBiP affected mBiP's ATPase activity, a coupled spectrophotometric assay was used [25,26]. As shown in Fig. 1A, the ATPase activity of mBiP was reduced with increasing concentration of mFKBP23. At an mFKBP23/mBiP molar ratio equal to 1:1, the ATPase activity of mBiP was reduced from 4.31 nmol ATP/mg BiP·min (without mFKBP23) to 3.09 nmol ATP/mg BiP·min, which is a reduction to 71.6%. At an mFKBP23/mBiP molar ratio of 4:1, the ATPase activity of mBiP was reduced to 1.38 nmol ATP/mg BiP·min, which is a reduction to only 32.1%. The same assay using mFKBP12 instead of mFKBP23 was performed, and no effect on mBiP's ATPase activity could be detected (data not shown).

There are two EF-hand motifs in the C-terminal domain of mFKBP23 (mFKBP23$_C$). Each EF-hand motif has Ca^{2+}-binding ability according to Kretsinger's rule. In previous work, we have reported that the concentration of Ca^{2+} can affect the binding of mFKBP23 to mBiP [4]. At low Ca^{2+} concentrations (<2 mM), FKBP23 can bind to

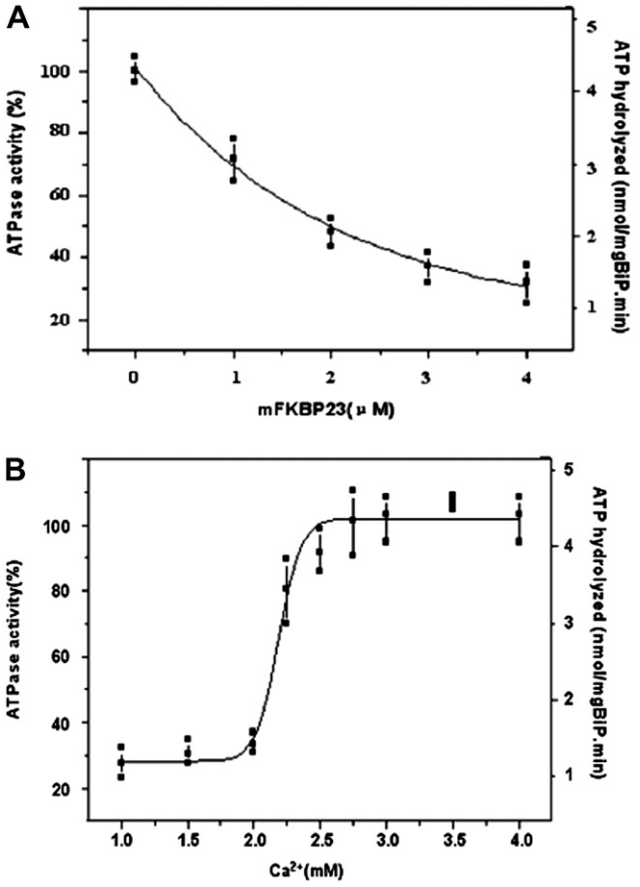

Fig. 1. Binding of mFKBP23 to mBiP suppresses the ATPase activity of mBiP. (A) After preincubation of 0.35 nmol mBiP with different amounts of mFKBP23 at room temperature for 20 min, the reaction mixtures were filled to 350 μL in buffer A (final concentration of mBiP: 1 μM; mFKBP23: 0, 1, 2, 3, and 4 μM, respectively). The mixtures were incubated at 37 °C and the decrease in NADH absorbance at 338 nm was monitored for calculating the ATPase activities. (B) One micromolar of mBiP and 4 μM mFKBP23 were pre-incubated at room temperature for 20 min, the reaction mixtures were filled to 350 μL in buffer A and increasing concentrations of $CaCl^{2+}$ (final concentration 1.0, 1.5, 2.0, 2.25, 2.5, 2.75, 3.0, 3.5, and 4.0 mM).

BiP, but dissociates when the $[Ca^{2+}]$ increases to 3 mM. Therefore, we investigated whether the $[Ca^{2+}]$ could also affect the suppression by mFKBP23 on the ATPase-activity of mBiP by means of its influence to the binding of mFKBP23 to mBiP. We used an mFKBP23/mBiP molar ratio of 4:1 for this test. As shown in Fig. 1B, when the $[Ca^{2+}]$ was less than 2 mM, the ATPase activity of mBiP was inhibited. By contrast, when the $[Ca^{2+}]$ increased to 2.5 mM, the ATPase activity of mBiP was restored almost completely. As a control, the $[Ca^{2+}]$ was shown to have no effect on the ATPase activity of BiP in the absence of mFKBP23 (see Supplementary data). The results indicate that only the binding mFKBP23 can suppress the ATPase activity of BiP and the free mFKBP23 have no influence to this activity. These results also show that the $[Ca^{2+}]$ limit at which mFKBP23 can bind to mBiP is between 2 and 2.5 mM.

The ATPase activity of mBiP can be recovered by suppression of the PPIase activity of mFKBP23 with FK506 or by competition between the PPIase activity free C-terminal domain of mFKBP23 and full-length mFKBP23 for the binding to mBiP

FK506 can inhibit the PPIase activity of the FKBPs [27]. To prove if the inhibition of mBiP's ATPase activity due to the PPIase activity of mFKBP23, we used FK506 to inhibit the PPIase activity of mFKBP23. At a FK506 concentration of up to 20 μM, which is five times the concentration of mFKBP23, the ATPase activity of mBiP could be restored by 96.7% (Fig. 2A). This showed that when the PPIase activity of mFKBP23 was inhibited with FK506, the ATPase activity of mBiP was restored. As a control, it was shown that FK506 alone does not affect the ATPase activity of mBiP (see Supplementary information).

It is reported that binding of mFKBP23 to mBiP is mainly due to the binding of its C-terminal domain ($mFKBP23_C$), which lacks the PPIase domain [4]. We know that the C-terminal domain of mFKBP23 cannot

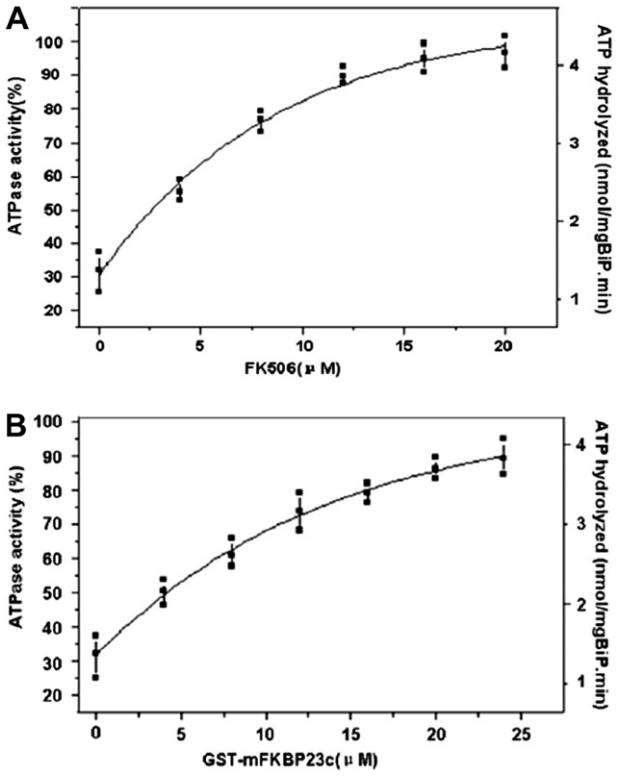

Fig. 2. The ATPase activity of mBiP can be recovered. (A) After preincubation of 0.35 nmol mBiP and 1.4 nmol mFKBP23 at room temperature for 20 min, different amounts of FK506 were added to the reaction mixtures for a further incubation at room temperature for 10 min. The reaction mixtures were filled to 350 μL in buffer A (final concentration of mBiP: 1 μM; mFKBP23: 4 μM, FK506: 4, 8, 12, 16, and 20, respectively). (B) After preincubation of 0.35 nmol mBiP, 1.4 nmol mFKBP23 with different amounts of GST-$mFKBP23_C$ at room temperature for 20 min, the reaction mixtures were filled to 350 μL in buffer A (final concentration of mBiP: 1 μM; mFKBP23: 4 μM, GST-$mFKBP23_C$: 4, 8, 12, 16, 20, and 24 μM, respectively).

suppress the ATPase activity of mBiP (see Supplementary data). Then, we performed an experiment by using GST-mFKBP23$_C$ for competing the binding of mFKBP23. As shown in Fig. 2B, the ATPase activity was restored with increasing concentration of GST-mFKBP23$_C$. As a control, the same test with GST alone was performed and the ATPase activity of mBiP could not be restored (see Supplementary data). This result shows once more that only the bound mFKBP23, but not the free mFKBP23, inhibits the ATPase activity of mBiP. Furthermore, we can also infer that mFKBP23 inhibition of the mBiP ATPase activity is due to the N-terminal PPIase domain of mFKBP23.

Therefore, we can conclude that the binding of mFKBP23 to mBiP can suppress the ATPase activity of mBiP; only the bound mFKBP23, but not the free mFKBP23, inhibits the ATPase activity of mBiP; and this suppression is due to the PPIase activity of mFKBP23. The determination of the PPIase activity of mFKBP23 is shown in Supplementary information.

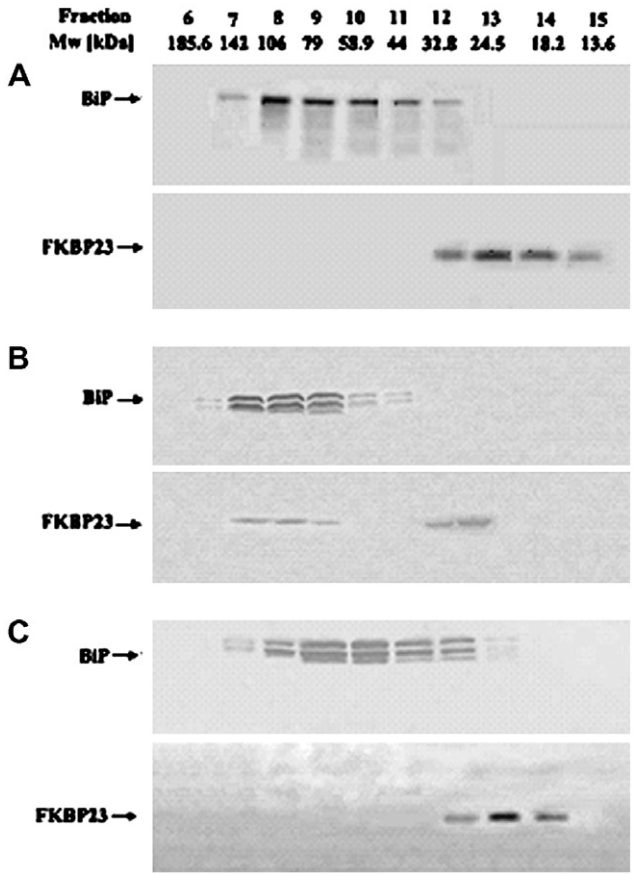

Fig. 3. Binding of mFKBP23 and mBiP *in vivo*. (A) Four micrograms of BiP or 1 μg FKBP23 in the binding buffer was loaded onto the column, respectively. The upper line of Mw represents the corresponding molecular weight in the standard molecular weight curve at the related elution volume of the corresponding fraction. (B) One milliliter of ER extract was loaded onto the column and eluted with the binding buffer of 0 mM [Ca^{2+}]. (C) one milliliter of ER extract was loaded onto the column and eluted with the binding buffer of 5 mM [Ca^{2+}]. The elution was prepared as probe for Western blot.

Existence of the [Ca^{2+}]-dependent complex of mFKBP23/mBiP in ER

The gel filtration chromatography (GFC) is used to confirm the existence of the [Ca^{2+}]-dependent complex of mFKBP23/mBiP *in vivo*. Recombinant mBiP (78 kDa), mFKBP23 (23 kDa) or ER extract at [Ca^{2+}] of 0 or 5 mM was loaded onto the GFC column, respectively. As shown in Fig. 3A, mBiP alone was eluted from 7 to 12 mL and the bands of FKBP23 alone appeared at the elution volume from 12 to 15 mL. Whereas, the bands of FKBP23 in ER extract, appeared also in the high molecular weight region at 0 mM [Ca^{2+}] (Fig. 3B). The protein bands of FKBP23 in the high molecular weight region disappeared when the [Ca^{2+}] was enhanced to 5 mM (Fig. 3C). This result shows that the complex of BiP/FKBP23 exists in ER extract and the binding of these two proteins is Ca^{2+}-interrelated. Furthermore, we can estimate that complex consists of one molecule BiP and one molecule FKBP23 according to the chromatographic behavior of the complex, which was eluted in the volume region of 106 kDa corresponding to the molecular weight curve.

The binding of mBiP$_C$ to mFKBP23 is also dependent on Ca^{2+} concentration

The C-terminal domain of mFKBP23 binds to mBiP in the ER interrelated with the [Ca^{2+}], and that this domain does not bind to the common peptide-binding site of mBiP [4]. To determine the site in mBiP to which mFKBP23

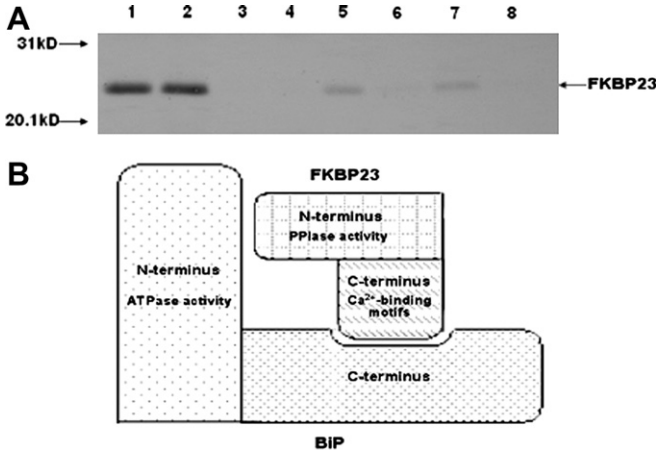

Fig. 4. (A) Evidence of the binding of mBiP$_C$ and mBiP$_N$ to mFKBP23. 0.025 nmol mFKBP23 was adsorbed with 0.2 nmol GST-mBiP$_C$ on the glutathione–Sepharose 4B beads in the binding buffer (lane 1, 0 mM [Ca^{2+}] without FK506; lane 2, 0 mM [Ca^{2+}] and 0.5 μM FK506; lane 3, 5 mM [Ca^{2+}] without FK506; lane 4, 5 mM [Ca^{2+}] and 0.5 μM FK506). The same assays were performed using mFKBP23 and GST-mBiP$_N$ on the glutathione–Sepharose 4B beads (lane 5, 0 mM [Ca^{2+}] without FK506; lane 6, 0 mM [Ca^{2+}] and 0.5 μM FK506; lane 7, 5 mM [Ca^{2+}] without FK506; lane 8, 5 mM [Ca^{2+}] and 0.5 μM FK506). The adsorbed mFKBP23 was detected by Western blot analysis. (B) Model of the interaction of mBiP and mFKBP23.

binds, we assessed the binding of mFKBP23 at different [Ca^{2+}] to GST-$mBiP_C$ and GST-$mBiP_N$. The effect of FK506 to FKBP23 was used as a control. As shown in Fig. 4A, the binding of the C-terminal of mBiP ($mBiP_C$) to mFKBP23 cannot be detected at [Ca^{2+}] of 5 mM, whereas binding was observed when the [Ca^{2+}] was 0 mM and this binding is not affected by FK506. The weak binding of the N-terminal of mBiP ($mBiP_N$) to mFKBP23 can be detected and is unrelated to the [Ca^{2+}]. In the presence of FK506 the weak binding could not be detected. So we suppose that the C-terminal domain of mFKBP23 binds to the C-terminal domain of mBiP and the weak binding of $mFKBP23_N$ to $mBiP_N$ is the binding of enzyme (PPIase) and its substrate in BiP_N, where the ATPase activity of BiP is located as illustration of Fig. 4B.

Discussion

FKBP23 has two EF-hand motifs downstream of the FKBP-type PPIase motif. FKBP23 binds Ca^{2+}. Like many other EF-hand proteins, they are suggested to be involved in the two possible functions, regulation of Ca^{2+} storage within the ER or Ca^{2+}-dependent ER functions such as protein folding and trafficking. Considering that FKBP23 has only two EF-hand motifs and has a PPIase motif, it is unlikely that FKBP23 functions as a Ca^{2+}-storage molecule [3,28–30]. Considered together with the fact that FKBP23 has a PPIase motif, an intriguing possibility is that FKBP23 may act as a Ca^{2+}-dependent molecular chaperone. In this study, we found that the binding of mFKBP23 to mBiP can have a negative effect on the ATPase activity of mBiP. This negative effect is due to the binding of C-terminal domains of the two proteins and the PPIase activity of the mFKBP23 N-terminus on the ATPase region of mBiP (Fig. 4B). The result that one molecule BiP binds one molecule FKBP23 (Fig. 3B) also supports our inference. It is important to note that only the FKBP23, which is bound to BiP, but not the free FKBP23, can suppress the ATPase activity of BiP. In the ER extract, the molar concentration of FKBP23 is much lower than that of BiP. According to our assessment of the prepared ER extract, the concentration of BiP is about 20 nM, whereas the concentration of FKBP23 is only about 2 nM. Thus, the FKBP23 is not able to inhibit all the BiP's ATPase activity. Therefore, we suppose that the binding of FKBP23 to BiP, and suppression of the BiP's ATPase activity, allows BiP to have another certain function. In addition to its classical role of chaperone, BiP has a second role in the storage of Ca^{2+} within the ER lumen and in control of the ER lumenal Ca^{2+} homeostasis [13]. The ER [Ca^{2+}] is between 2 and 3 mM [31]. Therefore, we presume that binding of FKBP23 to BiP is interlinked with its role in Ca^{2+} import in the ER lumen. When the [Ca^{2+}] increases, FKBP23 can not bind to BiP, and consequently, the Ca^{2+} in the ER lumen can be maintained at a constant concentration. Further studies are in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.209.

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