The mouse FKBP23 binds to BiP in ER and the binding of C-terminal domain is interrelated with Ca^{2+} concentration

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Abstract FK506 binding protein 23 from mouse (mFKBP23) is a peptidyl-prolyl *cis-trans* isomerase (PPIase) from the endoplasmic reticulum (ER), which consists of an N-terminal PPIase domain and a C-terminal domain with Ca^{2+} binding sites. The assay of adsorption from ER extract with glutathione *S*-transferase-mFKBP23 attached to glutathione-Sepharose 4B shows that mFKBP23 binds to mouse immunoglobulin binding protein (mBiP). The same assay with the recombinant proteins of the N- and C-termini of mFKBP23 shows that the binding of the C-terminus is Ca^{2+}-dependent and the switch point is between 2 and 3 mM. By high concentration of Ca^{2+} this binding cannot be detected. Furthermore, the Ca^{2+}-regulated binding of mFKBP23 and mBiP in ER can be detected by means of co-immunoprecipitation.

Key words: Peptidyl-prolyl isomerase; FK506 binding protein 23; Immunoglobulin binding protein; Ca^{2+}-dependent protein interaction

1. Introduction

Peptidyl-prolyl *cis-trans* isomerases (PPIases; EC 5.2.1.8) are ubiquitous and abundant proteins and 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 belong to three structurally unrelated families, namely the cyclophilins (CyPs), the FK506 binding proteins (FKBPs) and the parvulin-like proteins. They are characterized by their binding to specific immunosuppressant drugs: cyclosporin A, FK506 and rapamycin.

CyPs and FKBPs are able to accelerate slow refolding steps in certain proteins in vitro and in vivo. Mitochondrial CyPs from *Neurospora crassa* and yeast have been shown to be a part of the protein folding machinery after the import involving molecular chaperones [3–5]. The immunophilin components of the inactivated estrogen receptor, cyclophilin 40 and FKBP52, bind to Hsp90 with their tetratricopeptide repeat motif [6–8] involving molecular chaperones [9]. FKBP23 from mouse heart [10] and FKBP22 from *N. crassa* [11] are PPIases from the endoplasmic reticulum (ER) and both consist of an N-terminal PPIase domain and a C-terminal domain of unknown function and FKBP23 is reported to possess Ca^{2+} binding ability in the C-terminal domain. The alignment of mouse (m) FKBP23 and mFKBP12 [12] showed that the N-terminus of mFKBP23 (amino acids 20–137) belongs to a PPIase domain.

Immunoglobulin binding protein (BiP), the Hsp70 in the ER lumen, was the first member of the Hsp70 multigene family that was initially identified on the basis of its specific interaction with the immunoglobulin (Ig) heavy chain [13–15]. BiP and all other Hsp70s are evolutionarily conserved. They contain two functional domains, an amino-terminal 45 kDa ATPase domain and a carboxy-terminal 25 kDa substrate binding domain. The C-terminal domain consists of two parts, eight β-sheets and five α-helices. The peptide binding site is across the β-sheets [16–18]. Like all members of the Hsp70 family, BiP has a weak ATPase activity [19] to provide energy for the protein translocation and the protein folding.

It was revealed in previous work that NcFKBP22, the FKBP from the ER of *N. crassa* [11], binds to BiP in the ER of *N. crassa* specifically by using the yeast two-hybrid system (Mi and Tropschug, unpublished). In view of the homology of mFKBP23 and NcFKBP22 in this work we study the binding specificity of mFKBP23 to mBiP and the results show that mFKBP23 binds specifically to mBiP both in vitro and in vivo. The binding of the C-terminus of mFKBP23 to mBiP is Ca^{2+}-dependent and the switch point is between 2 and 3 mM of $[Ca^{2+}]$. By high concentration of $[Ca^{2+}]$ this binding cannot be detected. The N-terminus of mFKBP23 can bind weakly to mBiP, but this binding is independent of the $[Ca^{2+}]$. These experiments show for the first time that a PPIase binds to a molecular chaperone of the Hsp70 family.

2. Materials and methods

2.1. Constructions of plasmids expressing mFKBP23, $mFKBP23_N$, $mFKBP23_C$ and mBiP

For cloning of the full-length mFKBP23 protein, mouse mRNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using an upstream primer (mFKBP23f) including an *Eco*RI site (5′-ccggccgaattcagcactgaggaagtgaaaat-3′) and a down-

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Abbreviations: mFKBP, mouse FK506 binding protein; mBiP, mouse immunoglobulin binding protein; ER, endoplasmic reticulum; PPIase, peptidyl-prolyl *cis-trans* isomerase; GST, glutathione *S*-transferase; IP, immunoprecipitation

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stream primer (mFKBP23r) including an *Xho*I site (5′-ccggcctcgag ggaaaacaggtataacca-3′). The amplified PCR product was digested with *Eco*RI and *Xho*I restriction enzymes, and ligated into a bacterial expression vector pGEX5X1.

For cloning of the full-length mBiP protein, mouse mRNA was amplified by RT-PCR using an upstream primer (mBiPf) including a *Bam*HI site (5′-ccggccggatccaggaggacaagaaggagga-3′) and a downstream primer (mBiPr) including an *Xho*I site (5′-ggccggctcgagctaca actcatcttttctgat-3′). The amplified PCR product was digested with *Bam*HI and *Xho*I restriction enzymes, and ligated into pGEX5X1.

For subcloning of the partial mFKBP23 proteins $mFKBP23_N$ and $mFKBP23_C$, the previously obtained pGEX5X1-mFKBP23 was used as a template, and the primers used for PCR were: mFKBP23f as an upstream primer and a downstream primer including an *Xho*I site and a TAG stop codon, whose complementary sequence in primer is labeled in bold (5′-ggccggctcgag**cta**ggtcacagcataaagttcaat-3′), for $mFKBP23_N$, an upstream primer including an *Eco*RI site (5′-ggccg gaattctatgctgtgaccaaaggacc-3′) and mFKBP23r as a downstream primer for $mFKBP23_C$, respectively. After digestion with restriction enzymes, the PCR fragments were ligated into pGEX5X1.

The recombinant plasmid DNAs was transformed into competent cells of *Escherichia coli* strain DH5α and the positive clones were amplified. The purified recombinant plasmids DNAs were identified with sequencing.

2.2. Purification of GST fusion protein and pure protein

The amplified bacteria containing the recombinant plasmid with the target protein were induced with 0.1 mM isopropyl-β-D-thiogalactose. The target proteins were expressed as glutathione *S*-transferase (GST) fusion proteins and purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as described [20]. The purified fusion proteins could be restriction-digested on the beads 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 25°C. The purified mFKBP23 was then confirmed by determining its PPIase activity as described [21].

2.3. Preparation of ER extract from mouse liver

This was carried out as described by Borgeson and Bowman [22]. All steps of this procedure were performed at 4°C and all buffers and the equipment were precooled at 4°C. About 30 g mouse livers were suspended with 10 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 $1000\times g$ and the supernatant was collected. The pellet was suspended with 10 ml buffer E and centrifuged for 20 min at $1000\times g$ once more. The two supernatants were collected together and then centrifuged sequentially at $12\,000\times g$ and $40\,000\times g$ for 1 h, respectively, to get the fractions 12P (pellet after centrifugation at $12\,000\times g$, nuclear and mitochondrial), 40P (pellet after centrifugation at $40\,000\times g$, microsome/endoplasmic reticulum) and 40S (supernatant after centrifugation at $40\,000\times g$, cytoplasm). Fraction 40P was washed cautiously with 5 ml buffer E and centrifuged for 30 min at $40\,000\times g$. The pellet 40P was suspended with 2 ml buffer E, sheared using a syringe and centrifuged for 2 h at $100\,000\times g$. The supernatant is ER lumen extract and which was tested by Western blot to ensure there was no contamination with cytosol.

2.4. In vitro binding assays

0.025 nmol mBiP in 400 μl binding buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$) or 500 μl ER extract was incubated with glutathione-Sepharose 4B beads-attached GST, GST-mFKBP23, $GST\text{-}mFKBP23_N$ or $GST\text{-}mFKBP23_C$ (0.2 nmol each) at room temperature for 30 min. The beads were washed five times with 1 ml binding buffer containing 0.1% Tween and once with 1 ml binding buffer. The assay for reverse adsorption was conducted with 0.025 nmol mFKBP23 in 400 μl binding buffer and 0.2 nmol GST-mBiP attached to glutathione-Sepharose 4B beads, too. For the Ca^{2+}-regulated binding assay 1 mM $CaCl_2$ in the binding buffer was replaced by 2 mM, 3 mM, 4 mM, 5 mM $CaCl_2$ or 5 mM EDTA. For the peptide competition assay, free or bead-attached mBiP was preincubated with different doses of peptide V7 (FYQLAKT) (50 μM, 100 μM or 200 μM per assay) for 2 h prior to the procedure of binding with mFKBP23.

2.5. Immunoprecipitation (IP) for in vivo binding assays

Bound mFKBP23 or mBiP in 500 μl ER extract was co-immunoprecipitated by addition of anti-mFKBP23 or anti-mBiP antibodies and incubated overnight. Then 40 μl protein A-Sepharose (Pharmacia Biotech) was added to the ER extract to absorb the antigen–antibody complex. The beads were washed five times with 500 μl washing buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40 and 10% glycerol). For the Ca^{2+}-regulated binding assay, $CaCl_2$ was added to the ER extract to a final concentration of 1 mM, 2 mM, 3 mM or 4 mM and incubated for 40 min, the extracts were then subjected to IP as before.

2.6. Western blot

Proteins bound, described in Sections 2.4 and 2.5, were eluted by the addition of sodium dodecyl sulfate (SDS) sample buffer. The samples were analyzed by SDS–polyacrylamide gel electrophoresis and transferred onto Hybond-C nitrocellulose membrane (Amersham Life Science) at 0.8 mA/cm^2 for 2 h. The membrane was blocked with 5% non-fat milk powder in TBS (50 mM Tris–HCl, pH 7.5 and 150 mM NaCl) for 1 h and incubated with rabbit anti-mFKBP23 or anti-mBiP antiserum (1:500 diluted in TBS) overnight. The blots were washed with TBS/Tween (0.1%) and then developed with alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma, 1:2000 diluted in TBS). After washing with TBS/Tween the blots were developed by the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate system in alkaline phosphatase buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$).

3. Results

3.1. Recombinant mFKBP23 binds specifically to recombinant mBiP

To investigate the binding of recombinant mFKBP23 and mBiP to each other in vitro, we used purified mFKBP23 and mBiP to carry out the binding assay. The result in Fig. 1A shows that mBiP binds to GST-mFKBP23 in vitro, but does not bind to GST alone. Fig. 1B demonstrates that mFKBP23 binds to GST-mBiP in vitro, but does not bind to GST alone. Furthermore, there is no effect of FK506 on this binding.

BiP is a member of the Hsp70 multigene family [23]. We wanted to know if the mFKBP23 binds specifically to mBiP or if it binds also to Hsp70 or to other heat shock proteins.

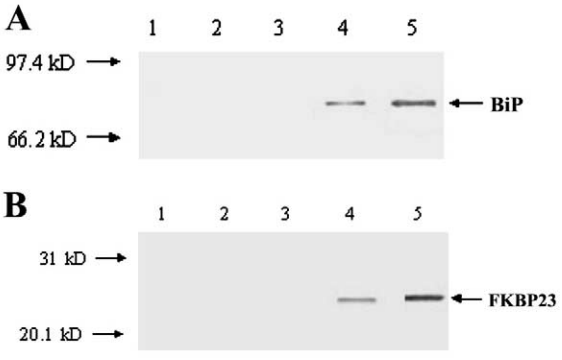

Fig. 1. Evidence of binding of mFKBP23 to mBiP in vitro. A: Western blot result of binding of GST-mFKBP23 to mBiP specifically. Lane 1: 0.2 nmol GST alone; lane 2: 0.2 nmol GST incubated with 0.025 nmol mBiP as control; lane 3: 0.2 nmol GST-mFKBP23 alone; lane 4: 0.2 nmol GST-mFKBP23 incubated with 0.025 nmol mBiP; lane 5: 0.005 nmol recombinant mBiP. B: Western blot result of binding of GST-mBiP to mFKBP23 specifically. Lane 1: 0.2 nmol GST alone; lane 2: 0.2 nmol GST incubated with 0.025 nmol mFKBP23 as control; lane 3: 0.2 nmol GST-mBiP alone; lane 4: 0.2 nmol GST-mBiP incubated with 0.025 nmol mFKBP23; lane 5: 0.005 nmol recombinant mFKBP23.

Binding tests with mFKBP23 to human Hsp60, Hsp70, Hsp90 or hamster BiP (StressGen) were performed. The result shows that the binding of mFKBP23 to mBiP is specific (data not shown).

To test if the binding of mFKBP23 to mBiP also occurs in the common peptide binding site of mBiP, the test peptide [24] V7 (FYQLAKT, a peptide with high affinity for all Hsp70s) was used for competition binding assay and the competitor peptide cannot influence the binding of mFKBP23 to mBiP at different doses of peptide V7. It means that the mFKBP23 does not non-specifically bind to the common peptide binding site of mBiP.

3.2. *The full-length and C-terminal domain of mFKBP23 can bind to mBiP from the ER extract*

To further investigate the binding of the recombinant mFKBP23 to the natural mBiP in the ER, the GST-mFKBP23 attached to glutathione-Sepharose 4B beads was used for adsorption of mBiP from ER extract. As shown in Fig. 2A, mBiP in the ER binds to GST-mFKBP23, but does not bind to GST alone.

mFKBP23 consists of two domains. The N-terminus is a PPIase domain with an FK506 binding site. The function of the C-terminal domain is not clear so far apart from the fact that it possesses Ca^{2+} binding ability on the two EF-hand motifs. To examine which of the two domains is required for the binding, GST fusion protein of the N-terminal domain (amino acids 20–137) and the C-terminal domains of mFKBP23, GST-mFKBP23$_N$ and GST-mFKBP23$_C$, were prepared for binding tests with ER extract. The result shows that mFKBP23$_C$ can bind to mBiP strongly. For the mFKBP23$_N$ a very weak binding to mBiP can be detected, too (Fig. 2B).

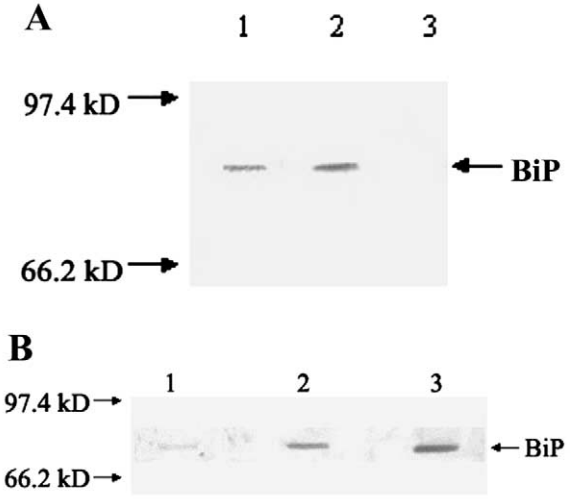

Fig. 2. Evidence of the binding of the C-terminal domain of mFKBP23 to mBiP in the ER. A: Western blot result of adsorbed mBiP from ER extract. Lane 1: 100 μl ER extract only; lane 2: adsorption of 500 μl ER extract with 0.2 nmol GST-mFKBP23 bound on glutathione-Sepharose 4B beads; lane 3: adsorption of 500 μl ER extract with 0.2 nmol GST only bound on glutathione-Sepharose 4B beads. B: The C-terminal domain of mFKBP23 binds to mBiP in the ER. Lane 1: 0.2 nmol GST-mFKBP23$_N$ incubated with 500 μl ER extract; lane 2: 0.2 nmol full-length mFKBP23 incubated with 500 μl ER extract; lane 3: 0.2 nmol GST-mFKBP23$_C$ incubated with 500 μl ER extract.

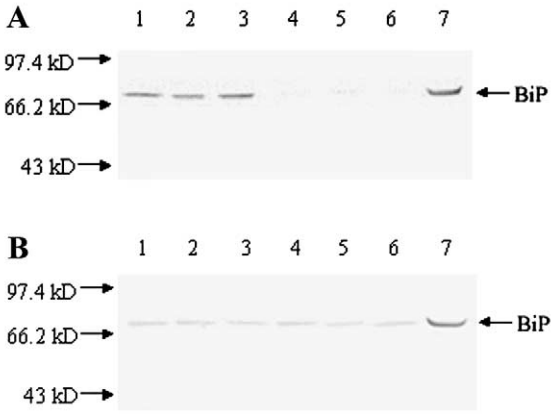

Fig. 3. The binding of mFKBP23 with mBiP is interrelated with Ca^{2+} concentration. A: 0.025 nmol mBiP was adsorbed with 0.2 nmol GST-mFKBP23$_C$ in the binding buffer of different Ca^{2+} concentrations: 0, 1, 2, 3, 4 or 5 mM (lanes 1–6). The recombinant mBiP was loaded to act as control (lane 7). The adsorbed mBiP was detected with Western blot. B: The same assay using mBiP and GST-mFKBP23$_N$ showed that the weak binding is unrelated to [Ca^{2+}]. Lane 7 is the recombinant mBiP as control.

3.3. *The binding of mFKBP23$_C$ to mBiP is interrelated with Ca^{2+} concentration*

There are two EF-hand motifs on the C-terminal domain of mFKBP23 protein [10]. Each EF hand possesses Ca^{2+} binding ability according to Kretsinger's rule [25]. Therefore, we asked if the Ca^{2+} concentration could influence the binding of mFKBP23 to mBiP. We investigated the binding capacity of mFKBP23 to mBiP through different Ca^{2+} concentrations in the binding buffer. As shown in Fig. 3A, the binding of mFKBP23$_C$ and mBiP could be hardly detected when the [Ca^{2+}] increased to 3 mM, whereas binding was observed when [Ca^{2+}] was below 2 mM, suggesting that this binding is regulated by the Ca^{2+} concentration in the solution, and the switch point is between 2 and 3 mM. Compared with the above observation, weak binding of mFKBP23$_N$ can be detected in vitro, too. But this binding is unrelated to the concentration of Ca^{2+} (Fig. 3B).

3.4. *mFKBP23 and mBiP exist in a bound complex form in mouse ER and the binding is also influenced by Ca^{2+} concentration*

To determine if the binding between mFKBP23 and mBiP also occurs in vivo, we subjected isolated mouse ER extract to co-immunoprecipitation. As shown in Fig. 4A, mBiP was co-precipitated from the ER extract in the presence of anti-mFKBP23. To investigate the Ca^{2+} influence on the binding in vivo, we then adjusted the buffer of ER extract to different Ca^{2+} concentrations and carried out IP as mentioned above. Fig. 4B shows that mBiP can be detected when the [Ca^{2+}] was lower than 2 mM, which accorded with the results in vitro.

We also wanted to find out whether the mFKBP23 can be detected when anti-mBiP was used. As expected, the mFKBP23 could be co-precipitated when the [Ca^{2+}] was lower than 2 mM (Fig. 4C). These results also provide support for the investigation in vitro: the binding between mFKBP23 and mBiP exists in vivo, the binding is Ca^{2+}-regulated and the switch point is between 2 and 3 mM [Ca^{2+}].

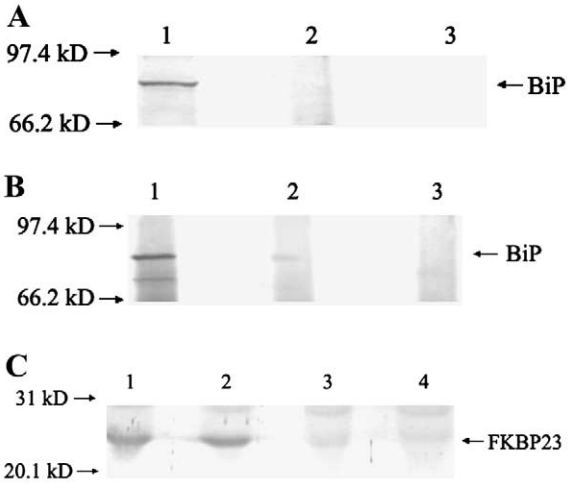

Fig. 4. mFKBP23 and mBiP in the ER are bound and the binding is Ca^{2+}-regulated. A: Western blot result of mBiP co-precipitated in the presence of anti-mFKBP23 by IP. Lane 1: mFKBP23 antibody (1 μl) adsorbed from 500 μl ER extract and the complex was tested using anti-mBiP; lane 2: unrelated antibody (anti-cyclophilin 18) was used as a control instead of anti-mFKBP23; lane 3: no antibody was used. B: The binding complex in ER was regulated by $[Ca^{2+}]$. The $[Ca^{2+}]$ in the ER extract was adjusted to 2 mM, 3 mM or 4 mM (lanes 1–3) and was subjected to IP as before. C: mFKBP23 was co-precipitated by adding 5 μl mBiP antibody to the ER extract. Furthermore, the $[Ca^{2+}]$ was 1 mM, 2 mM, 3 mM or 4 mM (lanes 1–4).

4. Discussion

In this study we revealed binding of mFKBP23 to mBiP in ER. This is evidence for the first time that a PPIase binds to a molecular chaperone of the Hsp70 family. We also indicated that the binding of the full-length (in vivo) and the C-terminus of mFKBP23 with mBiP are interrelated with Ca^{2+} concentration and the switch point of this binding is between 2 and 3 mM $[Ca^{2+}]$. The concentration of Ca^{2+} in the ER is between 2 and 3 mM [26]. Therefore, this $[Ca^{2+}]$-interrelated binding is probably physiologically significant.

In the ER BiP is involved in polypeptide translocation, protein folding or digestion of misfolded proteins and assembly in the formation of multichain protein molecules, such as antibody molecules [23,27]. Recent studies showed that BiP maintains the permeability barrier of the ER membrane by sealing the luminal end of the translocon pore before and early in translocation [28]. The $mFKBP23_N$ can bind weakly to mBiP. It could be a binding between PPIase and its substrate site in BiP. We can presume that the complex of BiP and FKBP23 represents one of BiP's functions that will be modulated by FKBP23's PPIase activity. It will be a subject of our further studies.

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