Binding of FKBP23 to BiP in ER Shown by Gel Filtration Chromatography

Chen Chen[a], Hui Ma[a], Ying Wang[a], and Huaifeng Mi[a,b,*]

[a] Biochemical Section of Key Laboratory of Functional Polymer Materials, Ministry of Education of China, Institute of Polymer Chemistry, Nankai University, 300071 Tianjin, China. Fax: (+86) 22 23 50 27 49. E-mail: hfmi@nankai.edu.cn
[b] Medical School of Nankai University, 300071 Tianjin, China

* Author for correspondence and reprint requests

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FKBP23 was found in mouse endoplasmic reticulum (ER) in 1998. It consists of an *N*-terminal peptidyl-prolyl *cis/trans* isomerase (PPIase) domain and a *C*-terminal domain with Ca^{2+} binding sites. Previously, we reported that FKBP23 specifically binds to BiP, the main protein of the molecular chaperone Hsp70 in ER lumen, and the binding is interrelated with the Ca^{2+} concentration. In this work we have found the existence of the complex FKBP23/BiP by separation of an ER extract using gel filtration chromatography (GFC), and that the existence of this complex is Ca^{2+}-interrelated. This result further verified the Ca^{2+}-interrelated binding of these two proteins *in vivo*.

Key words: FKBP23, BiP, Gel Filtration Chromatography

Introduction

FK506 binding proteins (FKBPs) are known as immunophilin in cells. They are able to catalyze the *cis/trans*-isomerization of Xaa-Pro bonds in oligopeptides or proteins. FKBPs are ubiquitous and have been found in bacteria, fungi, plants and animals, and in all subcellular fractions. Up to now more than ten kinds of FKBPs of this protein family are found. All possess a functional region with PPIase (peptidyl-prolyl *cis/trans*-isomerase) activity. FKBP23 was found in mouse endoplasmic reticulum (ER) (Nakamura *et al.*, 1998). It consists of an *N*-terminal PPIase domain and a *C*-terminal domain, which possesses two Ca^{2+}-binding motifs. BiP, the Hsp70 in the ER lumen, is the first characterized member of the Hsp70 multigene family that was initially identified on the basis of its specific interaction with the immunoglobulin (Ig) heavy chain (Haas and Wabl, 1983; Munro and Pelham, 1986). BiP and all other Hsp70s are evolutionary conserved. BiP contains two functional domains, a 45 kDa amino-terminal ATPase domain and a 25 kDa carboxy-terminal substrate-binding domain. The *C*-terminal domain consists of two parts, eight β-sheets and five α-helices. The peptide binding site of Hsp70 or BiP is across the β-sheets in the *C*-terminal domain of BiP (Zhu *et al.*, 1996; Rüdiger *et al.*, 1997; Bukau and Horwich, 1998). The folded 8 β-sheets form a gap for the peptide bond, and the altered conformation caused by folding of 8 β-sheets and 5 α-helices can lead to open and close the peptide bond gap (Gething, 1999; Nishikawa *et al.*, 2001). Hsp70 is the most important protein of the molecular chaperones for transport and folding of nascent proteins (Rüdiger *et al.*, 1997; Zhu *et al.*, 1996).

Previously, we reported that FKBP23 binds to BiP specifically in ER and this bond is interrelated with the Ca^{2+} concentration (Zhang *et al.*, 2004a, b). Here we further confirm the bond *in vitro* and *in vivo* by using gel filtration chromatography (GFC).

Materials and Methods

Construction of plasmids expressing FKBP23 and BiP

For cloning of the full-length FKBP23 protein, mouse mRNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using a upstream primer (FKBP23f) including an *Eco*RI site (5′-ccggcc**gaattc**agcactgaggaagtgaaaat-3′) and a downstream primer (FKBP23r) including an *Xho*I site (5′-ccggc**ctcgag**ggaaaacaggtataacca-3′). The amplified PCR product was digested with *Eco*RI and *Xho*I restriction enzymes, and ligated into the bacterial expression vector pGEX5X1.

For cloning of the full-length BiP protein, mouse mRNA was amplified by RT-PCR using a upstream primer (BiPf) including a *Bam*HI site (5′-ccggcc**ggatcc**aggaggacaagaaggagga-3′) and a downstream primer (BiPr) including an *Xho*I site (5′-ggccgg**ctcgag**ctacaactcatctttttctgat-3′). The amplified PCR product was digested with *Bam*HI and *Xho*I restriction enzymes, and ligated into pGEX5X1.

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

Purification of GST fusion proteins and preparation of 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 by Smith and Johnson (1998). The amplified bacteria containing the recombinant plasmid with the target protein were induced with 0.1 mM isopropyl-L-D-thiogalactose. The target proteins of BiP and FKBP23 were expressed as GST fusion proteins and adsorbed with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as described by Heitman *et al.* (1991). The beads were washed three times with MTPBS (150 mM NaCl, 16 mM Na_2HPO_4 and 4 mM NaH_2PO_4, pH 7.3).

The purified fusion proteins GST-BiP and GST-FKBP23 on the beads could be restriction-digested with factor Xa (Amersham Pharmacia Biotech) in a digestion buffer [50 mM tris(hydroxymethyl)aminomethane-HCl, pH 8.0, 1 mM $CaCl_2$ and 100 mM NaCl] overnight at 4 °C.

After gentle centrifugation, the supernatant containing the recombinant protein without tag of GST was removed to a clean tube. The beads were washed with factor Xa digestion buffer for 5 times and the supernatant fractions were collected. The obtained proteins of BiP and FKBP23 in every fraction were quantitatively checked by 12% SDS-polyacrylamide gel electrophoresis (PAGE) with bovine serum albumin (BSA-V) of different concentrations as reference of the protein amount.

Preparation of polyclonal antiserum

Anti-FKBP23 and anti-BiP antisera were prepared by immunization of rabbits with purified recombinant FKBP23 and BiP. Two rabbits for each antiserum were immunized at the same time. Before immunization, 1.5 mL blood were collected by bleeding the rabbits from the marginal vein of the ear for preparing the preimmunized sera as negative controls. Afterwards, 1.2 mL of sample A containing 250 μg antigen and the same volume of Freund's complete adjuvant was injected intradermally in the back and proximal limbs of the rabbit using 30–50 μL per each site. On the 3rd day, the above injection was repeated to strengthen the first immunization stimulation. On the 28th day, 1.2 mL of sample B containing 150 μg antigen and the same volume of Freund's incomplete adjuvant was injected using the same way. On the 35th day, 0.5 mL blood was collected by the same way used before for checking the titer of the antisera by Western blot analysis. The antisera were withdrawn from the carotid. The separated antisera were incubated at 56 °C for 60 min to inactivate the protease activity of the complements.

Preparation of ER extract from mouse liver

This was carried out as described by Borgeson and Bowman (Miller and Moon, 1997). All steps of this procedure were performed at 4 °C and all buffers and the equipment were precooled at 4 °C. About 200 g pig liver were suspended in 200 mL 2 × buffer E (2 M sorbitol, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4, and 2 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 in 10 mL buffer E and centrifuged for 20 min at $1000 \times g$. 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 in 2 mL buffer E, sheared using a syringe, and centrifuged for 2 h at $100{,}000 \times g$. The supernatant was ER lumen extract which was tested by Western blot

analysis to ensure that there was no contamination with cytosol.

Gel filtration chromatography

A Sephadex G-75 column (1 cm × 30 cm) was used for GFC analysis. The standard compounds including rabbit phosphorylase b (97,400), bovine serum albumin (66,200), rabbit actin (43,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (20,100), and hen egg white lysozyme (14,400) were used for demarcating the column. The standard molecular weight curve was drawn by the logarithmic values of molecular weights as ordinate versus elution volumes of the corresponding protein peaks as abscissa. In the test range it is a straight line. For the investigation the column was equilibrated with buffer B [binding buffer: 10 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$ and 5 mM EDTA] or buffer B with 5 mM Ca^{2+} overnight at room temperature. 1 mL sample containing 4 μg recombinant BiP, 1 μg recombinant FKBP23, 4 μg BiP + 1 μg FKBP23 that was preincubated at room temperature for 40 min in the binding buffer or 1 mL 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. The proteins in each fraction were precipitated by TCA/DOC and detected by Western blot analysis.

Western blot analysis

The samples were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto a 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(hydroxymethyl)aminomethane-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(hydroxymethyl)aminomethane-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$].

Results

Chromatographic behavior of recombinant FKBP23 and BiP

4 μg recombinant BiP (78 kDa) and 1 μg recombinant FKBP23 (23 kDa) in 1 mL buffer B were loaded onto the GFC column, respectively, and the column was eluted with buffer B. Fig. 1 shows that BiP was eluted from 7 mL to 11 mL and the bands of FKBP23 appeared at the elution volume from 12 mL to 15 mL. It is in consistence with the demarcating curve.

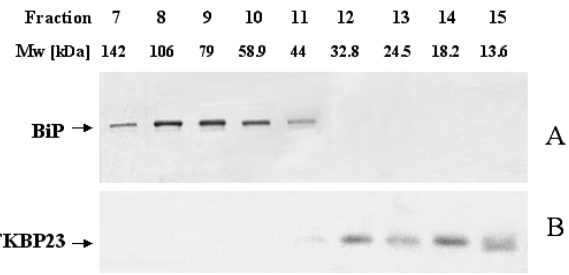

Fig. 1. Western blot of the assay of recombinant BiP and FKBP23 by GFC. 4 μg BiP and 1 μg FKBP23 in 1 mL buffer B were loaded onto the column, respectively. The column was eluted with buffer B. The eluent was collected as 1 mL/fraction and 80 μL of eluted solution were prepared as a probe for SDS-gel electrophoresis and Western blot analysis. The upper line of the figure represents the corresponding molecular weight in the standard molecular weight curve at the related elution volume of the corresponding fraction.

Binding of recombinant FKBP23 to BiP

4 μg BiP and 1 μg FKBP23 were incubated at 37 °C with buffer B for 40 min and the mixture was subjected to GFC by elution with buffer B. The result showed that a part of FKBP23 appeared in the high molecular weight region where BiP was detected (Fig. 2A). This result indicates that the protein complex of BiP/FKBP23 existed. The peak of the protein complex appeared at the elution volume of about 8 mL, which is related to the apparent molecular weight of 106 kDa. It corresponds to the sum of molecular weight of BiP (78 kDa) and FKBP23 (23 kDa), 101 kDa. This result testifies the conclusion of the binding of BiP to FKBP23.

In a system using buffer B with 5 mM Ca^{2+}, the protein bands of FKBP23 that appeared in the high molecular weight region in Fig. 2A disap-

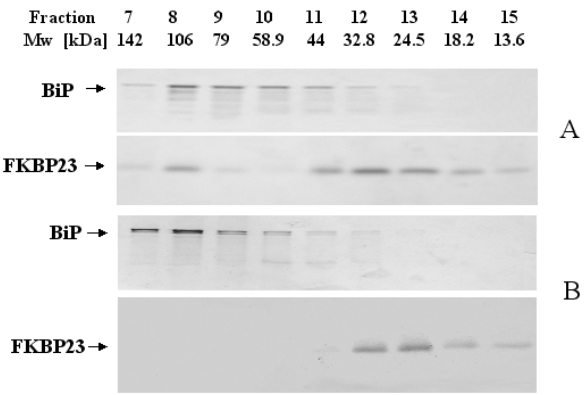

Fig. 2. Western blot of the binding assay of recombinant FKBP23 to BiP by GFC. (A) 4 μg BiP and 1 μg FKBP23 were preincubated at room temperature for 40 min in buffer B, then loaded onto the column and eluted with buffer B. (B) 4 μg BiP and 1 μg FKBP23 were preincubated at room temperature for 40 min in binding buffer B with 5 mM Ca^{2+}, then loaded onto the column and eluted with buffer B with 5 mM Ca^{2+}. The eluent was collected as 1 mL/fraction and 80 μL of eluted solution were prepared as a probe for SDS-gel electrophoresis and Western blot analysis.

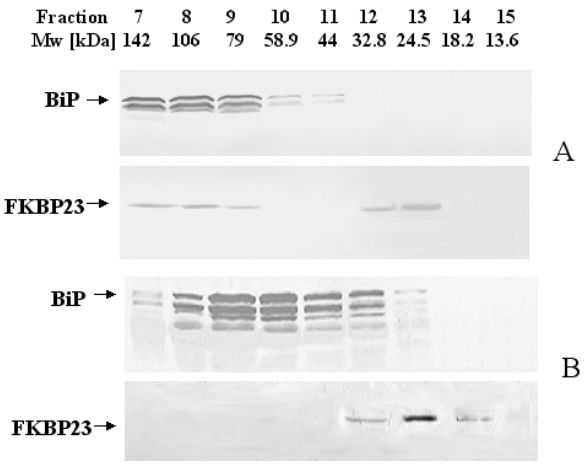

Fig. 3. Western blot of the binding assay of FKBP23 to BiP *in vivo* by GFC. (A) 1 mL ER lumen extract was loaded onto the column and eluted with buffer B. (B) 1 mL ER lumen extract with 5 mM Ca^{2+} was loaded onto the column and eluted with buffer B with 5 mM Ca^{2+}. The eluent was collected as 1 mL/fraction and 80 μL of eluted solution were prepared as a probe for SDS-gel electrophoresis and Western blot analysis.

peared (Fig. 2B). This means that the protein complex was disassociated. This result verifies our former outcome that BiP binds to FKBP23 and the binding is interrelated with the Ca^{2+} concentration (Zhang *et al.*, 2004a, b).

Binding of BiP and FKBP23 in vivo

1 mL ER extract was loaded onto the column and eluted with buffer B. The result showed that the bands of FKBP23 appeared in the high molecular weight region (Fig. 3A). When 1 mL ER extract was added to 5 mM Ca^{2+} and the study was carried out by the same procedure, but using buffer B + 5 mM Ca^{2+} for elution, the protein bands of FKBP23, which appeared in the high molecular weight region in Fig. 3A, disappeared (Fig. 3B). This result shows that the complex of BiP/FKBP23 exists in ER extract and the binding of these two proteins is Ca^{2+}-interrelated.

Discussion

In our previous work we proved that FKBP23 binds to BiP specifically and the bond is interrelated with the Ca^{2+} concentration. In this work we have testified it using a different method and verified the existence of the complex of these two proteins in ER. Furthermore we can estimate that the 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 according to the molecular weight curve.

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