De-Novo Cloning of FKBP23 cDNA from Pig ER Using Nested PCR[§]

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FK506 binding proteins (FKBPs) in cells are known as immunophilins. We have identified and characterized a cDNA encoding an endoplasmic reticulum (ER) immunophilin, FKBP23, from pig liver by nested PCR. The predicted amino acid sequence of pig FKBP23 shows high identity to those of human FKBP23 and mouse FKBP23. It possesses a conserved FKBP-type peptidylprolyl *cis-trans* isomerase (PPIase) domain and EF-hand domain. We constructed a plasmid to express pFKBP23. Furthermore, we proved that the recombinant pFKBP23 can specifically bind to natural BiP, the main protein of the molecular chaperone Hsp70 in ER lumen; the binding is interrelated with the Ca^{2+} concentration just as the FKBP23 from mice.

Key words: FKBP23, Nested PCR, Peptidylprolyl *cis-trans* Isomerase

Introduction

FK506 binding proteins (FKBPs) in cells are known as immunophilins and highly conserved proteins widely distributed from *Escherichia coli* to mammals. They are able to catalyze the *cis-trans* isomerization of Xaa-Pro bonds in oligopeptides and proteins (Schreiber, 1992; Fischer, 1994). Up to now more than ten kinds of FKBPs of this protein family were found. All FKBPs possess a functional region with PPIase (peptidylprolyl *cis-trans* isomerase) activity (Barth *et al.*, 2007; Lima *et al.*, 2006; Solscheid and Tropschug, 2000; Suzuki *et al.*, 2004). FKBP23 was first found in mouse heart endoplasmic reticulum (ER) (Nakamura *et al.*, 1998). It consists of an *N*-terminal PPIase domain and a *C*-terminal domain with Ca^{2+} binding sites.

FKBP23 is a glycoprotein retained in the ER. The ER is a compartment specialized for the maturation of proteins, which are destined for secretion, membranes, and endocytotic or exocytotic organelles. It provides unique conditions for processing and folding of these proteins such as cleavage of the signal sequence, disulfide bond formation and protein glycosylation (Ellgaard and Helenius, 2003; Trombetta and Parodi, 2003; Kleizen and Braakman, 2004; Helenius and Aebi, 2004; Bukau *et al.*, 2006). We are interested in purifying natural FKBP23 and researching the function of glycosyl in FKBP23. A new method of preparing a protein-imprinted polymer (PIP) with a cloned bacterial protein template is reported, which recognizes/adsorbs authentic target protein present at a relatively low level in a cell extract (Zhao *et al.*, 2006). Pig liver is a handy and abundant source to acquire the ER. If cloned pig FKBP23 as template protein can be prepared, natural pig FKBP23 in the ER can be acquired by the same above-mentioned method.

The nucleotide sequences of FKBP23 from humans (Patterson *et al.*, 2002), mice (Nakamura *et al.*, 1998), lizards (Klein *et al.*, 2002), fishs (Lo *et al.*, 2003), etc. have been detected. According to two highly conservative regions of nucleotide sequences of these species, we designed primers and used the polymerase chain reaction (PCR) to amplify the middle segment of the pFKBP23 gene. From this sequence we used the nested PCR (Frohman, 1993; Bertling *et al.*, 1993; Martin *et al.*, 2005) to extend the nucleotide sequence towards the 5′ and 3′ ends for detecting the full length cDNA encoding pig FKBP23. Our result is available from GenBank with accession No.

§ The nucleotide sequence of pig FKBP23 is available from GenBank accession No. EU545235.

EU545235 and is contributed for elucidating the mRNA library of pig.

Material and Methods

Materials

Glutathione-Sepharose 4B, factor Xa and Hybond-C nitrocellulose membranes were from Amersham Biosciences UK Limited (Little Chalfont, UK). Restriction endonuclease, T4 DNA ligase, DNA polymerase I and ribonuclease H were purchased from TaKaRa (Dalian, China). Alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (IgG) was from Sigma-Aldrich (USA), and Trizol reagent from BBI (Markham, Canada), Revertaid™ first strand cDNA synthesis kit from Fermentas (Hanover, USA). All PCR primers were synthesized by Sangon (Shanghai, China). All other chemical reagents were of analytical grade.

cDNA synthesis

Total RNA was prepared using the Trizol reagent. The first strand cDNA was prepared using the Revertaid™ first strand cDNA synthesis kit according to the manufacturer's instructions. The second strand cDNA was synthesized, which was performed with the hybrid of the first strand cDNA and mRNA, DNA polymerase I, T4 DNA ligase and ribonuclease H. The ends of double strands cDNA were filled and ligated with adaptor 1 (5′-ccatcctaatacgactcactatagggctcgagcggc-3′) or adaptor 2 (5′-gccgctcgagccctata-3′).

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 as described by Heitman *et al.* (1991). The purified fusion proteins were restriction-digested on the beads with factor Xa 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.

Preparation of ER extract from pig liver

This preparations was carried out as described by Borgeson and Bowman (cited in 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 the 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 in 200 mL 2×buffer E and centrifuged for 20 min at 1000 × *g*. The two supernatants were collected and centrifuged sequentially at 12,000 × *g* and 40,000 × *g* for 1 h, respectively, to get the fractions 12P (pellet after centrifugation at 12,000 × *g*, nuclear and mitochondrial), 40P (pellet after centrifugation at 40,000 × *g*, microsome/endoplasmic reticulum) and 40S (supernatant after centrifugation at 40,000 × *g*, cytoplasm). Fraction 40P was washed cautiously with 5 mL buffer E and centrifuged for 30 min at 40,000 × *g*. The pellet 40P was suspended in 2 mL buffer E, sheared using a syringe, and centrifuged for 2 h at 100,000 × *g*. The supernatant was ER lumen extract which was tested by Western blot to ensure that there was no contamination with cytosol.

Western blot analysis

The samples were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto a Hybond-C nitrocellulose membrane 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-BiP antiserum (1:500 diluted in TBS) for 1 h. The blots were washed with TBS/Tween (0.1%) and then developed with alkaline phosphatase-labeled goat anti-rabbit IgG (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, 5 mM $MgCl_2$].

Results and Discussion

Amplification of the middle segment of pFKBP23 gene

To detect the middle segment of the pig FKBP23 gene, the first strand pig cDNA was amplified by PCR using a upstream primer (pFKBP23mf) including an *Eco*RI site (5′-ccggccgaattccccccaaatggtttgttcttgg-3′) and a downstream primer (pFKBP23mr) including a *Xho*I site (5′-ggccggctcgagacattgtattcctt-3′) according to the conservation of homologous sequences of human FKBP23, mouse FKBP23, lizard FKBP23, fish FKBP23 in these two regions (Fig. 1A). The PCR product of the middle segment gene of pFKBP23 was identified as shown in Fig. 1B.

Detection of the pFKBP23 cDNA sequence by nested PCR

The pFKBP23 cDNA encoding the 3′ partial sequence and 5′ partial sequence, respectively, were detected by nested PCR. According to the middle segment gene of pFKBP23, we designed the 3′ partial outer primer (P1, 5′-ccccaaatggtttgttcttg-3′) and the inner primer (P2, 5′-ggccgcggccgcgacattgccatgatggatat-3′) including a *Not*I site, and the 5′ partial outer primer (P4, 5′-ttaggtggaatcttgctt-3′) and the inner primer (P3, 5′-ggccgcggccgctttccatatgcaaaggaagg-3′) including a *Not*I site. Adaptor primer 1 (AP1, 5′-ccatcctaatacgactcac-3′) and adaptor primer 2 (AP2) including an *Eco*RI site (5′-ggccgaattctatagggctcgagcggc-3′) were also designed according to the adaptors ligating with two strands cDNA. The first PCR was performed using AP1/P1 as the primer pair for 3′ partial sequence, AP1/P4 as the primer pair for 5′ partial sequence and the double strands cDNA as template. The second PCR was performed using AP2/P2 as the primer pair for 3′ partial sequence, AP2/P3 as the primer pair for 5′ partial sequence and the diluted first PCR product as template (Fig. 2). The positive clones were identified by sequencing. The 5′ and 3′ partial sequences were ligated by Lasergene 7.0 software and the 632 bp cDNA sequence was acquired with the GenBank accession No. EU545235 (Fig. 3A).

Analysis of nucleotide and predicted amino acid sequences of pFKBP23

Comparing the nucleotide sequence of pFKBP23 with those of human and mouse FKBP23, we found that the sequence of pFKBP23 lost 25 bp nucleotide in the 5′ partial sequence. So the cDNA sequence was not full. The homologous analysis of amino acid sequences of human FKBP23 and mouse FKBP23 together with pig FKBP23 showed 91.4% and 87.6% identity, respectively. The first 19 amino acids of human and mouse FKBP23 in the *N*-terminal domain were signal sequence analyzed with signal P3.0; so we could construct plasmid to express pFKBP23 in *Escherichia coli* with the acquired sequence. The predicted amino acid sequence was analyzed with the conserved domain architecture retrieval tool in NCBI, and we found the conserved FKBP-type peptidylprolyl *cis-trans* isomerase domain and EF-hand domain (Fig. 3).

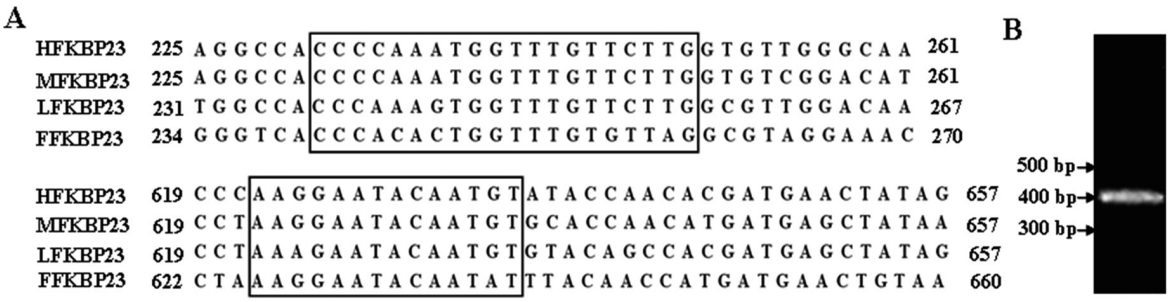

Fig. 1. Part sequence comparisons of four FKBP23 species and the PCR product of the middle segment of the pFKBP23 gene. (A) Alignment of the part nucleotide sequence of four FKBP23 species: human (HFKBP23), mouse (MFKBP23), lizard (LFKBP23), and fish (FFKBP23); the regions used for the initial design of the PCR primers to identify the middle segment of the pFKBP23 gene are boxed. (B) PCR amplified product of the middle segment of the pFKBP23 gene analyzed by agarose gel electrophoresis at about 400 bp.

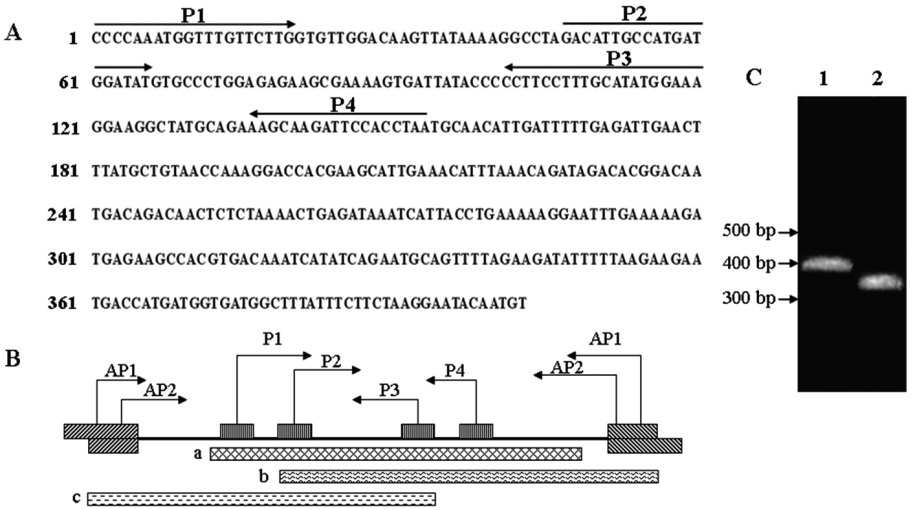

Fig. 2. Molecular cloning strategy of pig FKBP23 cDNA. (A) The nucleotide sequence of the middle segment of pFKBP23. P1 and P2 are the nested PCR primers used for the 3′ partial sequence of pFKBP23; P3 and P4 are the nested PCR primers used for the 5′ partial sequence of pFKBP23. (B) A schematic diagram of the cloning strategy for pFKBP23 cDNA. The full-length cDNA of pFKBP23 was amplified by nested PCR, the primers were AP1/P1, AP2/P2 and AP1/P4, AP2/P3. a, The middle segment of pFKBP23; b, the 3′ partial pFKBP23 by nested PCR; c, the 5′ partial pFKBP23 by nested PCR. (C) PCR-amplified products of 3′ and 5′ partial pFKBP23 analyzed by agarose gel electrophoresis; lane 1, PCR-amplified products of 3′ partial pFKBP23 at about 400 bp; lane 2, PCR-amplified products of 5′ partial pFKBP23 at about 350 bp.

Construction of plasmid expressing pFKBP23

For cloning of the pFKBP23 protein, the first strand pig cDNA was amplified by PCR using a upstream primer (pFKBP23f) including a *Bam*I site (5′-ccggccggatccaaaagacagaggagagca-3′) and a downstream primer (pFKBP23r) including a *Xho*I site (5′-ccggcctcgagctatagttcatcatgttgatat-3′). The amplified PCR product was digested with *Bam*I and *Xho*I restriction enzymes and ligated into the bacterial expression vector pGEX5X2. 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.

Recombinant pFKBP23 can bind to pBiP from the ER extract and the binding is interrelated with the Ca^{2+} concentration

Previously, we reported that FKBP23 binds to BiP specifically in the ER and this bond is interrelated with the Ca^{2+} concentration (Zhang *et al.*, 2004a, b; Wang *et al.*, 2007). BiP is a member of the Hsp70 multigene family and one of the best-studied folding helpers in the ER. It binds to stretches of hydrophobic residues exposed by incompletely folded proteins and prevents their aggregation (Kleizen and Braakman, 2004; Bukau *et al.*, 2006; Flynn *et al.*, 1991; Blond-Elguindi *et al.*, 1993). To investigate the binding of recombinant pFKBP23 to natural pBiP in the ER, GST-pFKBP23 attached to glutathione-Sepharose 4B beads was used for the adsorption of pBiP from the ER extract. GST alone attached to glutathione-Sepharose 4B beads was used as control. As shown in Fig. 4A, pBiP in the ER binds to GST-pFKBP23, but does not bind to GST alone. We also investigated the binding capacity of pFKBP23 to pBiP in the binding buffer with different Ca^{2+} concentration. As shown in Fig. 4B, the binding of pFKBP23 and pBiP could be hardly detected when the Ca^{2+} concentration increased to 3 mM, whereas it can be observed when the Ca^{2+} concentration was below 2 mM, suggesting that this binding is regulated by the Ca^{2+} concentration.

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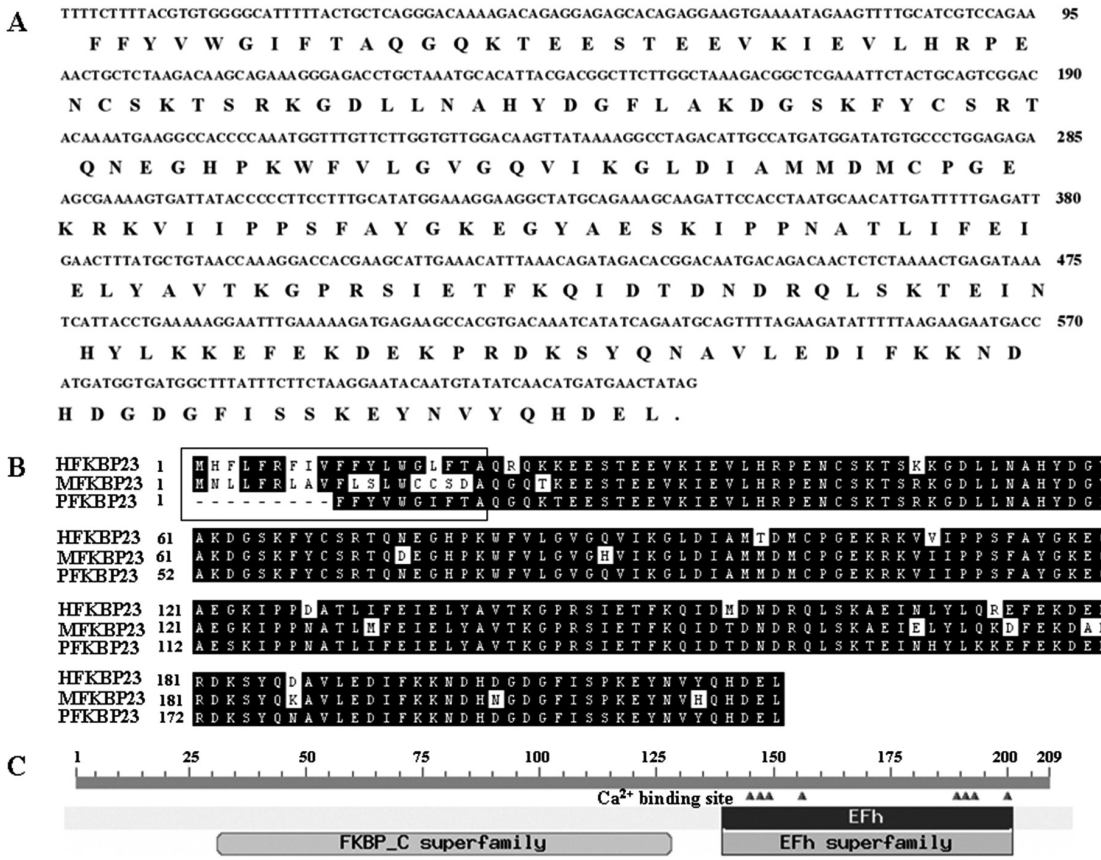

Fig. 3. Nucleotide and predicted amino acid sequences of pFKBP23 cDNA. (A) The nucleotide sequence is shown in the upper lines and the amino acid sequence is shown in the lower lines using the single-letter amino acid code. cDNA sequence data are avaible from GenBank accession No. EU545235. (B) Alignment of the amino acid sequence of three FKBP23 species: human (HFKBP23), mouse (MFKBP23), and pig (PFKBP23). The first 19 amino acids in *N*-terminal predicted signal sequences are boxed. (C) Analysis of the conserved domain architecture. The PFKBP23 protein possesses a conserved FKBP-type peptidylprolyl *cis-trans* isomerase domain and an EF-hand domain.

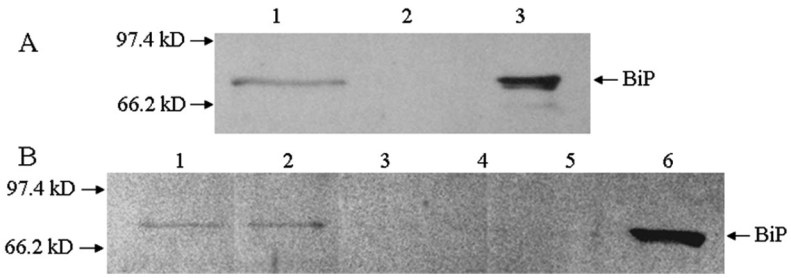

Fig. 4. Evidence of the binding of pFKBP23 to pBiP. (A) Western blot of adsorbed pBiP from an ER extract. Lane 1, adsorption of 100 μL ER extract with 0.2 nmol GST-pFKBP23 bound on glutathione-Sepharose 4B beads; lane 2, adsorption of 100 μL ER extract with 0.2 nmol GST alone bound on glutathione-Sepharose 4B beads; lane 3, recombinant mBiP loaded to act as control. (B) Western blot of the binding assay of pFKBP23 to pBiP at different Ca^{2+} concentrations. Lanes 1–5, adsorption of 100 μL ER extract with 0.2 nmol GST-pFKBP23 bound on glutathione-Sepharose 4B beads in the binding buffer of different Ca^{2+} concentrations, adjusted to 1, 2, 3, 4, or 5 mM; lane 6, recombinant mBiP loaded to act as control.

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Barth S., Nesper J., Hasgall P. A., Wirthner R., Nytko K. J., Edlich F., Katschinski D. M., Stiehl D. P., Wenger R. H., and Camenisch G. (2007), The peptidyl prolyl *cis/trans* isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. Mol. Cell. Biol. **27**, 3758–3768.

Bertling W. M., Beier F., and Reichenberger E. (1993), Determination of 5′ ends of specific mRNAs by DNA ligase-dependent amplification. PCR Methods Appl. **3**, 95–99.

Blond-Elguindi S., Cwirla S. E., Dower W. J., Lipshutz R. J., Sprang S. R., Sambrook J. F., and Gething M. J. (1993), Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell **75**, 717–728.

Bukau B., Weissman J., and Horwich A. (2006), Molecular chaperones and protein quality control. Cell **125**, 443–451.

Ellgaard L. and Helenius A. (2003), Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell. Biol. **4**, 181–191.

Fischer G. (1994), Peptidyl-prolyl *cis/trans* isomerases and their effectors. Angew. Chem. Int. Ed. Engl. **33**, 1415–1436.

Flynn G. C., Pohl J., Flocco M. T., and Rothman J. E. (1991), Peptide-binding specificity of the molecular chaperone BiP. Nature **353**, 726–730.

Frohman M. A. (1993), Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE. Methods Enzymol. **218**, 340–356.

Heitman J., Movva N. R., Hiestand P. C., and Hall M. N. (1991), FK506-binding protein proline rotamase is a target for the immunosuppressive agent FK506 in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **88**, 1948–1952.

Helenius A. and Aebi M. (2004), Roles of *N*-linked glycans in the endoplasmic reticulum. Annu. Rev. Biochem. **73**, 1019–1049.

Klein S. L., Strausberg R. L., Wagner L., Pontius J., Clifton S. W., and Richardson P. (2002), Genetic and genomic tools for *Xenopus* research: The NIH *Xenopus* initiative. Dev. Dyn. **225**, 384–391.

Kleizen B. and Braakman I. (2004), Protein folding and quality control in the endoplasmic reticulum. Curr. Opin. Cell Biol. **16**, 343–349.

Lima A., Lima S., Wong J. H., Phillips R. S., Buchanan B. B., and Luan S. (2006), A redox-active FKBP-type immunophilin functions in accumulation of the photosystem II supercomplex in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **103**, 12631–12636.

Lo J., Lee S., Xu M., Liu F., Ruan H., Eun A., He Y., Ma W., Wang W., Wen Z., and Peng J. (2003), 15000 Unique zebrafish EST clusters and their future use in microarray for profiling gene expression patterns during embryogenesis. Genome Res. **13**, 455–466.

Martin A. R., Brown G. K., Dunstan R. H., and Roberts T. K. (2005), Anaplasma platys: an improved PCR for its detection in dogs. Exp. Parasitol. **109**, 176–180.

Miller J. R. and Moon R. T. (1997), Analysis of the signaling activities of localization mutants of beta-catenin during axis specification in *Xenopus*. J. Cell Biol. **139**, 229–243.

Nakamura T., Yabe D., Kanazawa N., Tashiro K., Sasayama S., and Honjo T. (1998), Molecular cloing, characterization, and chromosomal localization of FKBP23, a novel FK506-binding protein with Ca^{2+}-binding ability. Genomics **54**, 89–98.

Patterson C. E., Gao J., Rooney A. P., and Davis E. C. (2002), Genomic organization of mouse and human 65 kDa FK506-binding protein genes and evolution of the FKBP multigene family. Genomics **79**, 881–889.

Schreiber S. L. (1992), Immunophilin-sensitive protein phosphatase action in cell signaling pathways. Cell **70**, 365–368.

Solscheid B. and Tropschug M. (2000), A novel type of FKBP in the secretory pathway of *Neurospora crassa*. FEBS Lett. **480**, 118–122.

Suzuki Y., Haruki M., Takano K., Morikawa M., and Kanaya S. (2004), Possible involvement of an FKBP family member protein from a psychrotrophic bacterium *Shewanella sp*. SIB1 in cold-adaptation. Eur. J. Biochem. **271**, 1372–1381.

Trombetta E. S. and Parodi A. J. (2003), Quality control and protein folding in the secretory pathway. Annu. Rev. Cell. Dev. Biol. **19**, 649–676.

Wang Y., Han R., Wu D., Jie L., Chen C., Ma H., and Mi H.(2007), The binding of FKBP23 to BiP modulates BiP's ATPase activity with its PPIase activity. Biochem. Biophys. Res. Commun. **354**, 315–320.

Zhang W., Li H., Zhang X., Shen Y., Wang Y., and Mi H. (2004a), FKBP23 in ER binds BiP specifically. Sci. China B **47**, 17–23.

Zhang X., Wang Y., Li H., Zhang W., Wu D., and Mi H. (2004b), The mouse FKBP23 binds to BiP in ER and the binding of *C*-terminal domain is interrelated with Ca^{2+} concentration. FEBS Lett. **559**, 57–60.

Zhao Z., Wang C., Guo M., Shi L., Fan Y., Long Y., and Mi H. (2006), Molecular imprinted polymer with cloned bacterial protein template enriches authentic target in cell extract. FEBS Lett. **580**, 2750–2754.