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Mouse FKBP23 mediates conformer-specific functions of BiP by catalyzing Pro^{117} *cis*/*trans* isomerization

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ABSTRACT

FK506-binding proteins (FKBPs) are cellular receptors for the immunosuppressant FK506 and rapamycin. They belong to the ubiquitous peptidyl-prolyl *cis*/*trans* isomerases (PPIases) family, which can catalyze the *cis*/*trans* isomerization of peptidyl-prolyl bond in peptides and proteins. In previous work, we revealed that mouse FKBP23 binds immunoglobulin binding protein (BiP), the major heat shock protein (Hsp) 70 chaperone in the ER, and the binding is interrelated with [Ca^{2+}]. Furthermore, the binding can suppress the ATPase activity of BiP through the PPIase activity of FKBP23. In this work, FKBP23 is demonstrated to mediate functions of BiP by catalyzing the Pro^{117} *cis*/*trans* conformational interconversion in the ATPase domain of BiP. This result may provide new understanding to the novel role of PPIase as a molecular switch.

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1. Introduction

Peptidyl-prolyl *cis*/*trans* isomerases (PPIases) are ubiquitous proteins that have been found in both prokaryotes and eukaryotes. They are able to catalyze the slow *cis*/*trans* isomerization of proline-peptide bonds in peptides and proteins [1,2]. So far, three subfamilies of PPIases have been identified: cyclophilins (CyPs), FK506 binding proteins (FKBPs) and parvulins. They are known as cellular receptors for immunosuppressive drugs cyclosporine A, FK506 and rapamycin, respectively. Mouse FKBP23 (mFKBP23) is an ER-resident PPIase comprising an N-terminal PPIase domain and a C-terminal domain with two calcium-binding EF-hand motifs (Fig. 1A). In previous studies, we reported that mFKBP23 can bind mouse immunoglobulin binding protein (mBiP) in the ER and that the binding is regulated by [Ca^{2+}] [3]. Besides, only the bound, full-length mFKBP23 can suppress the ATPase activity of mBiP through its N-terminal PPIase activity [4]. As the major Hsp70 molecular chaperone in the ER, BiP is involved in many cellular processes, including protein biogenesis, signal transduction and calcium homeostasis [5–7]. Except for binding Ca^{2+}, all of these functions require the ATPase activity of BiP to provide energy.

In this work, we aimed to find the substrate site of the PPIase activity of mFKBP23 on the ATPase domain of BiP. We compared mFKBP23 with its homolog, FKBP22, which was reported to cooperate with BiP as a chaperone in the ER of *Neurospora crassa*. It is reported that FKBPs have a narrow specificity and are sensitive to the specific residue preceding Pro in the substrate [8]. FKBP22 shows the highest PPIase activity against the peptide substrate succinyl-Pro-Phe-*p*-nitroanilide [9]. And a unique Phe is found at position 125, preceding the Pro residue, in the N-terminus of *N. crassa* BiP, while a unique Leu^{116} occurs at the corresponding position in mBiP, as shown in Fig. 1C. The findings above strongly imply that the Pro following Leu^{116} in $mBiP_N$ could likely be the target site for $mFKBP23_N$.

2. Materials and methods

2.1. Mutagenesis and protein expression

Mutations were created by PCR using the MutanBest kit (Takara) with pUC18-BiP as the template DNA. This plasmid was constructed by subcloning the coding sequence of mBiP into the pUC18 plasmid from pGEX5X1-mBiP plasmid. The primers used for mutagenesis were shown in Supplementary Table 1. After sequencing (Supplementary Fig. 1), each mutant was transferred into expression vector pGEX-5X1 between *Xho* I and *Bam*H I. WT and mutant proteins were all expressed and purified as previously described [3]. The purified proteins were analyzed by SDS–PAGE. The concentration was determined by the Bradford method with BSA as the standard.

Abbreviations: mBiP, mouse immunoglobulin binding protein; ER, endoplasmic reticulum; CyPs, cyclophilins; mFKBP, mouse FK506-binding protein; PPIase, peptidyl-prolyl *cis*/*trans* isomerase; Hsp, heat shock protein; $mFKBP23_N$, N-terminus of mFKBP23; $mBiP_N$, N-terminus of mBiP; Suc, succinyl; *p*NA, *p*-nitroanilide; WT, wild-type.

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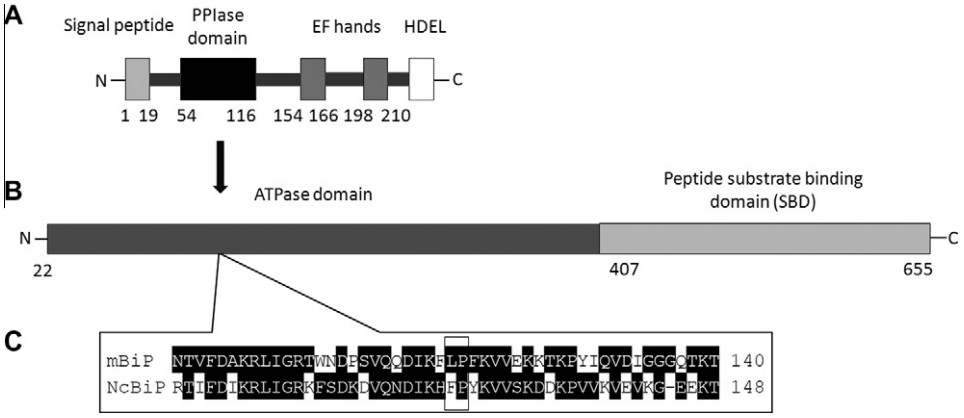

Fig. 1. Putative substrate site for the PPIase activity of mFKBP23 in the ATPase domain of mBiP. (A) Schematic diagram of the structural domains of mFKBP23. (B) Schematic diagram of the structural domains of mBiP. (C) Alignment of NcBiP and mBiP shows the unique potential substrate site for the PPIase activity of mFKBP23.

Table 1
Kinetic constants for PPIase activity of mFKBP23 for different peptide substrates. All assays consisted of 50 mM Hepes buffer (pH 7.5), 100 mM NaCl, 32 μM chymotrypsin, 60 nM mFKBP23, 80 μM peptide substrates. The decrease in absorbance at 390 nm was recorded at 12 °C. The blank was assayed with all the ingredients except for mFKBP23. The data represent at least three independent experiments.

Compound[a]	k_{obs}[b] ($\times 10^{-3}$ s^{-1})	k_{nonenz} ($\times 10^{-3}$ s^{-1})	k_{enz} ($\times 10^{-3}$ s^{-1})
Suc-Ala-*Leu*-Pro-Phe-*p*NA	8.18 ± 0.13	6.07 ± 0.20	2.11 ± 0.07
Suc-Ala-*Lys*-Pro-Phe-*p*NA	8.29 ± 0.11	7.90 ± 0.21	0.39 ± 0.10
Suc-Ala-*Phe*-Pro-Phe-*p*NA	5.28 ± 0.09	4.91 ± 0.13	0.37 ± 0.04
Suc-Ala-*Val*-Pro-Phe-*p*NA	4.57 ± 0.15	4.31 ± 0.11	0.26 ± 0.04
Suc-Ala-*Glu*-Pro-Phe-*p*NA	3.47 ± 0.12	3.40 ± 0.10	0.07 ± 0.02

[a] Changed residues are italicised.
[b] k_{obs} is the observed rate constant in the presence of mFKBP23. k_{enz} and k_{nonenz} represent the rate constant of enzymetic and nonenzymetic isomerization, respectively. $k_{enz} = k_{obs} - k_{nonenz}$.

2.2. PPIase assay

The PPIase activity of mFKBP23 was measured in a chymotrypsin-coupled spectrophotometric assay based on previously described methods [10]. Substrate specificity was determined with the Suc-Ala-Xaa-Pro-Phe-*p*NA (Xaa = Glu, Leu, Lys, Phe, or Val) substrate series (GL Biochem). The peptide substrates were dissolved in tetrahydrofuran containing 470 mM LiCl at a stock concentration of 2.8 mM. A 0.7-ml reaction mixture, containing 32 μM chymotrypsin (Worthington) and 60 nM FKBP23 in a buffer (50 mM HEPES and 100 mM NaCl, pH 7.5), was prechilled to 12 °C. The reaction was initiated by addition of the peptide substrate at a final concentration of 80 μM. The absorbance change at 390 nm due to release of *p*-nitroaniline was recorded at 1-s intervals until the reaction was complete. The blank was assayed with all the ingredients except for mFKBP23. The rate constant of the reaction (k_{obs}) was calculated from the slope of the plot of ln $(A_{390}(\infty) - A_{390}(t))$ versus t (s) for 200 data points.

2.3. ATPase assay

The spectrophotometric assay used to measure ATP hydrolysis was previously described [4]. The regeneration of ATP from ADP and phosphoenolpyruvate coupled with the oxidation of NADH can be monitored by the decrease in absorbance at 338 nm. Various mBiP proteins (1 μM each) were each preincubated with different amounts of mFKBP23 at room temperature for 20 min. The protein mixtures were added to a buffer containing 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 reactions were monitored at 37 °C for 30 min. ATPase activities were calculated from the slope of the linear part of the curves.

3. Results and discussion

3.1. Substrate specificity of FKBP23 towards standard peptides

We initially measured the PPIase activity of mFKBP23 towards a series of peptide substrates containing amino acid substitutions in the standard test peptide Suc-Ala-Xaa-Pro-Phe-*p*NA (Xaa = Glu, Leu, Lys, Phe or Val). As expected, mFKBP23 showed the highest activity against the substrate Suc-Ala-Leu-Pro-Phe-*p*NA (Table 1 and Fig. 4).

3.2. Mutation at Pro^{117} made BiP insensitive to the catalyzing activity of cis/trans isomerization by mFKBP23

Based on the substrate preference of FKBP23, we then mutated the Pro^{117} to Leu in the ATPase domain of mBiP, which is also neutral and hydrophobic. We compared the ATPase activity of WT and P117L BiP using a coupled spectrophotometric assay in the absence or presence of mFKBP23. In these reactions, the hydrolysis of one molecule of ATP leads to the oxidation of one molecule of NADH. The ATPase activity of BiP, which is shown by relating the hydrolysis rate of ATP (nmol/min) to the amount of BiP, was calculated from the reduction rate of NADH.

As Fig. 2 shown, the distinction in the ATPase activity curves between WT and Pro^{117} mutant suggested that the mutation in BiP_N did affect the inhibition activity of FKBP23 on BiP, which was reasonablely attributed to the catalyzing activity of FKBP23 as our prediction described. However, the ATPase activity of P117L did not maintain its basal value in the presence of FKBP23. Since mFKBP23 has only one PPIase motif, it was supposed to have unique substrate site on BiP_N. Besides, several earlier reports described that PPIase binds its substrate peptide through several hydrogen bonds around its target residue proline, but the precise mechanisms of action at work in PPIases are still not entirely understood [11–13]. Thus we speculated that the remaining inhibitory action of FKBP23 is probably due to the steric hindrance introduced by $FKBP23_N$ which weakly binds the residues around

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