Molecular characterization of FK-506 binding protein 38 and its potential regulatory role on the anti-apoptotic protein Bcl-2

Kang, Cong Bao; Feng, Lin; Chia, Joel; Yoon, Ho Sup

2005

Kang, C. B., Feng, L., Chia, J., & Yoon, H. S. (2005). Molecular characterization of FK-506 binding protein 38 and its potential regulatory role on the anti-apoptotic protein Bcl-2. *Biochemical and Biophysical Research Communications*, 337(1), 30-38.

<https://hdl.handle.net/10356/94816>

<https://doi.org/10.1016/j.bbrc.2005.09.023>

© 2005 Elsevier Inc. This is the author created version of a work that has been peer reviewed and accepted for publication by *Biochemical and Biophysical Research Communications*, Elsevier Inc. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [<http://dx.doi.org/10.1016/j.bbrc.2005.09.023>].

Downloaded on 26 Apr 2023 18:33:29 SGT

Molecular characterization of FK-506 binding protein 38 and its potential regulatory role on the anti-apoptotic protein Bcl-2

*Cong Bao Kang, Lin Feng, Joel Chia, Ho Sup Yoon **

Division of Structural and Computational Biology, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637511, Singapore

** Corresponding author. Fax: +65 6791 3856. E-mail address: hsyoon@ntu.edu.sg (H.S. Yoon).*

Abstract

The immunosuppressant FK-506 binding protein 38 (FKBP38) is localized at the mitochondrial membrane and appears to play an important role in apoptosis. Recent reports about the potential functions of FKBP38 in apoptosis appear to be controversial. To further understand the biological function of FKBP38, here, we studied its molecular characteristics and a potential regulatory role on the anti-apoptotic protein Bcl-2. Our results suggest that FKBP38 appears to show chaperone activities in the citrate synthase aggregation assays during thermal denaturation and affect solubility of Bcl-2 when they are co-expressed. The FKBP family proteins bind the immunosuppressive drug FK-506 through the FK-506 binding domain and consequently inhibit the activity of calcineurin. In this study, from our NMR studies and calcineurin assays in vitro, we demonstrate that the N-terminal fragment of FKBP38 which contains the FK-506 binding domain does not bind FK-506 at molecular level. Lastly, to investigate the effect of FKBP38 on Bcl-2, we suppressed FKBP38 by RNA interference (RNAi) of FKBP38. Our results suggest that the suppression of FKBP38 appears to make Bcl-2 unstable or unprotected from degradation in an unknown mechanism.

Keywords: FK-506; FKBP38; Bcl-2; Apoptosis; Chaperone; RNA interference

Apoptosis is programmed cell death, which is an essential regulatory process in multi-cellular organisms during development and homeostasis [1–5]. Bcl-2, which has an anti-apoptotic function, is a key regulator of apoptosis and is localized at the mitochondrial, endoplasmic reticulum, and nuclear membrane [6, 7]. Recently, it has been shown that FK-506 binding protein 38 (FKBP38), one of immunosuppressant FK-506 binding protein (FKBP) family, can interact with the anti-apoptotic proteins Bcl-2 and Bcl-Xl, and helps them to localize at the mitochondrial membrane [8].

From the analysis of sequence, FKBP38 contains FK-506 binding domain—a domain that is conserved among members of the FKBP family and is responsible both for binding to FK506

and for their peptidyl prolyl *cis-trans* isomerase (PPIase) activities, three tetracopeptide repeats (TPR), calmodulin-binding (CaM), and transmembrane (TM) domains [8, 9]. FKBP38 protein shows substantial sequence similarity to FKBP12 and FKBP52. The homology to FKBP12 and 52 is about 35% and 32%, respectively [9, 10]. FKBP38 has been shown to be unique among the FKBP family proteins [8]. The potential functions of FKBP38 have been previously described as an anti-apoptotic regulator, an important modulator in neuronal hedge-hog signaling and in controlling cell size [11–13]. Recently, it has also been shown that function of FKBP38 is pro-apoptotic in neuronal cells, and upon binding calmodulin, FKBP38 can be activated in the presence of calcium and shows interaction with Bcl-2 through its PPIase active site [14]. Three-dimensional structural studies revealed the presence of an unusually long unstructured loop in the anti-apoptotic proteins Bcl-2 and Bcl-Xl [15, 16]. Previously, we have shown that the unstructured loop of Bcl-2 is important for molecular interaction with FKBP38 [17].

Accumulating reports on potential function of FKBP38 suggest that FKBP38 appears to be multi-functional protein in many regulatory processes. In our study, to better understand the biological function of FKBP38 in the apoptotic regulatory pathway, we investigated the biochemical characteristics of FKBP38 including chaperone activity, FK-506 binding activity, the effect on the phosphatase activity of calcineurin *in vitro*, and the regulation of FKBP38 on the anti-apoptotic protein Bcl-2.

Materials and methods

Materials. Antibodies against Bcl-2 (Δ C21: sc-783), Bcl-Xl (S-18: sc-634), Bax (N-29: sc-493), and PARP (H-250: sc-7150) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibody against GAPDH was purchased from Ambion (Austin, TX, USA). Antibody against human FKBP38 was a kind gift from Prof. Keiichi I. Nakayama. C-Jun-N-terminal Kinase (JNK) was from Upstate (Lake Placid, NY, USA). [γ - 32 P]ATP (3000 Ci/mmol) was from Amersham BioScience (Uppsala, Sweden), ethylene glycol-bis-(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), RPMI 1640 Medium, and other chemicals were purchased from Sigma–Aldrich (St. Louis, Mo, USA). Isopropyl-thio- β -Dgalactopyranoside (IPTG) was from Promega (Madison, WI, USA). MitoTracker Deep Red 633 and Prolong Antifade reagent, Alexa Fluor 546 goat anti-rabbit IgG were from Molecular Probes (Eugene, OR, USA). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen (Carlsbad, CA, USA). Protease inhibitors complete-mini protease tablets were from Roche Applied Science (Indianapolis, IN, USA).

Cell cultures. HeLa, MDA-MB-231, MCF-7, A549, and Jurkat cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37 °C in a humidified CO₂ incubator. Cells, except Jurkat cells, were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Jurkat cells were grown in RPMI 1640 medium.

Protein purification. Recombinant human FKBP38 lacking the trans-membrane domain and the N-terminal fragment of FKBP38 (FKBP38NTD) were expressed and purified as described

before [8,17]. And purifications of kinase ERK2 and JNK were carried out as described before [20]. Uniformly ^{15}N -labeled FKBP38NTD and FKBP12 were expressed in *Escherichia coli* BL21(DE3) cells grown on M9 media containing $^{15}\text{NH}_4\text{Cl}$ and purified using Ni^{2+} -NTA affinity chromatography and Sephacryl S-200 gel filtration column chromatography. Concentration of purified proteins was determined by a protein assay kit from Bio-Rad Laboratories (Hercules, CA, USA). Proteins were stored at 4 °C before use.

Citrate synthase aggregation assay. To measure the chaperone activity, the thermal denaturation of citrate synthase was performed as previously described [18,19]. Briefly, prior to using the citrate synthase in the assay, the citrate synthase was changed from the buffer (2.2 M $(\text{NH}_4)_2\text{SO}_4$) into TE buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA) and concentrated to 1 mg/ml. The citrate synthase or citrate synthase mixed with 1 mg/ml FKBP38 or other proteins was incubated at 42 °C for 20 min, and the level of aggregation during denaturation was measured by monitoring the increase in absorbance at 360 nm.

Kinase assay. Purified FKBP38 was incubated with the c-Jun-N-terminal kinase (JNK) for 2 h at 30 °C in a buffer containing 10 mM Tris-HCl, pH 7.5, 25 mM MgCl_2 1 mM EGTA, 1 mM ATP, 1 μCi [γ - ^{32}P], and 250 μM PMSF. For the phosphorylation of FKBP38 by ERK2, a different buffer was used. ERK2 was incubated with purified FKBP38 in the buffer containing 50 mM Tris-HCl, 10 mM MgCl_2 , 2 mM dithiothreitol, 1 mM EGTA, and 0.01% Brij 35, pH 7.5. The samples were loaded on SDS-PAGE followed by autoradiography for visualization.

Phosphatase assay. The phosphatase activity of calcineurin was carried out with an assay kit from Calbiochem (San Diego, CA, USA). Briefly, the synthetic phosphopeptide derived from the RII subunit of protein kinase A was mixed with calcineurin and incubated at 30 °C for 1 h. After the reaction, malachite green reagent was added, and the absorbance at 620 nm was measured. The release of phosphate was calculated according to the standard curve [18].

NMR spectroscopy. All NMR data were acquired at 300 K on a Bruker Avance AV700 equipped with a cryoprobe accessory. NMR sample contained 0.1–0.5 mM protein in 90% H_2O /10% D_2O in 20 mM phosphate, pH 7.0, 20 mM NaCl, 1 mM DTT, and 0.01% NaN_3 . Chemical shift perturbations to ^{15}N -labeled FKBP38NTD and FKBP12 were monitored with ^{15}N -heteronuclear single quantum correlation spectroscopy (HSQC) upon addition of FK-506. NMR spectra were processed on Linux work-stations using Bruker Topspin software.

RNA interference. Three siRNAs were designed for RNA interference experiments. The mRNA sequence for Bcl-2-specific small interfering RNA (siRNA) was: 5'-AAGUACAUCCAUAUAAGCUG-3'. The mRNA sequence for FKBP38-specific siRNA was: 5'-AAGAGUGGCUGGA CAUUCUGG-3'. The sequence for the scramble siRNA was: 5'-AAGU CUCCAAGCGGAUCUCGU-3'. All the siRNAs were purchased from Dharmacon (Lafayette, CO, USA). The siRNAs (20 μM) were dissolved in a buffer containing 100 mM potassium acetate, 30 mM Hepes-KOH, and 2 mM magnesium acetate, pH 7.4, and stored at -80 °C before use. Prior to experimentation, the siRNA solution was heated at 90 °C for 1 min, and incubated at 37 °C for 60 min, to disrupt higher aggregation and ensure siRNA silencing efficiency [21,22].

Western blotting. Cells were washed with ice-cold 1x PBS and then lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors before being freeze-thawed to prepare the cell lysate. The lysate was collected and centrifuged at 14,100g for 15 min. The supernatant was collected and the total protein amount was determined using the Bio-Rad Protein Assay kit, followed by SDS-PAGE. Samples of equal protein amount (100 µg) were separated on 12% SDS-polyacrylamide gel, transferred to PVDF membrane, and probed with specific antibodies followed by detection with the Immun-Star Chemiluminescent protein detection system (Bio-Rad Laboratories, Hercules, CA, USA).

Semi-quantitative RT-PCR. Cells were harvested and total RNA was isolated by using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration of total RNA was measured at UV 260 nm. For cDNA synthesis, 1 µg of total RNAs was used for reverse transcription reaction and PCR amplification. The RT-PCR product was detected on a 1% agarose gel.

Immunofluorescence and confocal microscopy. 1×10^4 cells/well were seeded on 22 mm² coverslips within a 6-well plate. Cells were stained with 200 nM of MitoTracker Deep Red 633 after 24 h culture for 30 min under growth conditions. After washing with 1x PBS for several times, cells were fixed with 3.7% paraformaldehyde in 1x PBS at 37 °C for 20min. Then cells were soaked with 0.1% Triton X-100 at 25 °C for 3–5 min. After blocking with 5% non-fat milk, cells were incubated at 4 °C overnight with corresponding primary antibodies. Primary antibodies were detected with Alexa Fluor 546 goat anti-rabbit IgG. After washing with 1x PBS for six times, the coverslips were mounted with Prolong Antifade reagent and the slides were observed using the Zeiss LSM 510 META confocal microscope at excitation wavelengths of 543 and 633 nm simultaneously to capture images with a 60x oil immersion lens.

Cell survival assay. Cells in 96-well plates were used 24 h later after initial seeding. The cells were treated with 30 nM siRNA by double transfection in a 24 h interval. Cell viability was determined using the Promega CellTiter 96 AQueous One Solution Reagent kit according to the manufacturer's instructions 48 h after the second transfection. Absorbance of the colored product was read at 490 nm using Bio-Rad Benchmark plus micro-plate [23,24].

Caspase-3 activity assay. Caspase-3 activity was determined by using the Promega CaspACE assay kit. Briefly, cells in 6-well plates were transfected with siRNAs as described above. For inhibited apoptosis samples, Z-VAD-FMK was added into the wells to a final concentration of 100 µM together with siRNAs. Cells were incubated at 37 °C in a humidified incubator with 5% CO₂, and cell extracts were obtained by freezing and thawing. One hundred micrograms of the total protein from each sample was added to each well of a 96-well plate containing the CaspACE assay buffer containing the colorimetric substrate, Ac-DEVD-pNA, according to the manufacturer's protocol. The contents were incubated for 24 h and the absorbance of the developed color was read at 405 nm by the micro-plate reader.

Results

Expression of FKBP38

FKBP38 has been shown to be abundantly expressed in the brain tissue [11]. In this study, to first evaluate the expression of FKBP38 in cancer cells, we analyzed FKBP38 in several cancer cells including HeLa, MDA-MB-231, MCF-7, A549, and Jurkat cells. Our results showed that FKBP38 is widely expressed in different cancer cell lines (Fig. 1A). Also, to confirm the distribution of FKBP38 in cancer cells, we demonstrated that immunofluorescence images of FKBP38 coincide with those of the mitochondria-specific dye MitoTracker in HeLa cells (Fig. 1B), indicating that FKBP38 is primarily localized at the mitochondria, which is consistent with previous observation [8].

FKBP38 exhibits chaperone activity

FKBP38 is a unique member among the FKBP family proteins. It is different from typical FKBP family proteins; it contains a FKBP domain—a domain which is conserved among FKBP family proteins, a calmodulin-binding domain, and three tetratricopeptide repeats (TPR), but it appears to lack conserved residues for FK-506 binding and peptidyl prolyl *cis-trans* isomerase (PPIase) activity conserved in other FKBP family proteins [9]. It has been shown that the FKBP family proteins assist protein folding along with other biological functions in cells [25]. Recently, it has been reported that FKBP38 exhibited PPIase activity after the formation of the complex with Ca^{2+} and calmodulin [14]. This observation led us to further characterize and test potential chaperone activities from FKBP38. Here, we employed citrate synthase as a model substrate for the chaperone activity assay. It has been shown that the thermal denaturation and subsequent refolding of citrate synthase is a good system to monitor the effect of potential chaperone activity, since it is characterized by low recovery of active enzyme and high degree of forming aggregates during the assay procedure [18,19]. First, for the study of potential chaperone activity of FKBP38, we expressed and purified human recombinant FKBP38 to near homogeneity (Fig. 2A). The purified FKBP38 proteins were confirmed by Western blotting using anti-FKBP38 antisera (Fig. 2B). Molecular chaperones exert their function by forming complexes with their substrates. Previously, we showed the binding between FKBP38 and Bcl-2 *in vitro* as well as in cells [17]. In the present study, we first confirmed binding between FKBP38 and Bcl-2 by co-expressing them in bacterial cells. Our data showed that FKBP38 and Bcl-2 formed a complex when they were co-expressed, while Bcl-Xl, a close member of the Bcl-2 family, showed a considerably reduced interaction with FKBP38 (Fig. 3A). We then evaluated the effect of FKBP38 on the solubility of Bcl-2. When Bcl-2 was expressed in the absence of FKBP38, a majority of Bcl-2 protein stayed in an insoluble form, but in the presence of FKBP38, we observed a significant increase in soluble fraction, suggesting that FKBP38 affected the solubility of Bcl-2 during protein expression or folding in bacterial cells (Fig. 3B). After we checked the effect of FKBP38 on the solubility of Bcl-2, we then used the citrate synthase model for further evaluation of the potential chaperone activity. Our data showed that FKBP38 significantly reduced the level of aggregates of citrate synthase, while FKBP12, which belongs to the FKBP family, and bovine serum albumin (BSA) had no effect on the aggregate formation of citrate synthase (Fig. 3C).

FKBP38 is phosphorylated by ERK2

From the analysis of the amino acid sequence of FKBP38, there appears to be phosphorylation sites in FKBP38 [26], suggesting that FKBP38 may be regulated by some kinases. To check potential phosphorylation of FKBP38, in this study, in vitro phosphorylation reactions were performed using two kinases, JNK and ERK2. Our data showed that FKBP38 was phosphorylated by ERK2 but not by JNK (Fig. 4A). Provided the biological function of FKBP38 is still unclear and somewhat controversial [8,14], these in vitro phosphorylation data on the potential phosphorylation of FKBP38 might provide information about the role of FKBP38 during apoptosis in cells.

Effect of FKBP38 on the activity of calcineurin

The phosphatase activity of calcineurin is inhibited by forming a ternary complex with FK-506 and FKBP12 [27]. Given that contradictory observations about the inhibitory effect of human FKBP38 on calcineurin have been reported [8,28], we attempted to investigate the effect of FKBP38 on calcineurin. Our results showed unlike FKBP12/FK-506, FKBP38 had no inhibitory effect on the activity of calcineurin in the absence or presence of FK-506 (Fig. 4B). To further probe this observation, we employed a NMR-based binding study using ¹⁵N-labeled FKBP domain of human FKBP38 (FKBP38NTD). We added FK-506 to the ¹⁵N-labeled FKBP38NTD. Upon addition of FK-506, no apparent spectral changes were detected in the ¹⁵N-HSQC spectrum (Fig. 5A) while chemical shift perturbations were observed in the ¹⁵N-HSQC spectrum of FKBP12 upon addition of FK-506 (Fig. 5B). Taken together, our data suggest FKBP38 did not appear to bind FK-506 and apparently it showed no inhibitory effect on calcineurin.

FKBP38 regulates Bcl-2

FKBP38 and Bcl-2 have been shown to be co-localized at the mitochondrial membrane, and the depletion of FKBP38 by RNA interference resulted in a change of Bcl-2 localization in cells [8]. In this study, we addressed a question of how the suppression of FKBP38 would affect the mislocalized Bcl-2. First, our data showed that the mRNA and protein levels of FKBP38 after the treatment of the siRNA targeting FKBP38 were extensively reduced as predicted (Fig. 6A). To our surprise, unexpectedly, the level of Bcl-2 protein after the treatment of FKBP38-specific siRNA was also significantly reduced, in which the effect was similar to that of Bcl-2 siRNA treatment. In contrast, the same anti-apoptotic family member Bcl-Xl was relatively unaffected and pro-apoptotic member Bax was slightly increased (Fig. 6A). To further check whether the observed effect is at the protein level or RNA level, we performed a semi-quantitative RT-PCR analysis. Our data showed that the treatment of FKBP38-specific siRNA resulted in silencing most of its mRNA, but no apparent change in the level of Bcl-2 mRNA was observed (Fig. 6B). Taken together, our results suggest that after the depletion of FKBP38 by RNA interference of

FKBP38, Bcl-2 protein, without the presence of FKBP38, becomes unstable and appears to be degraded in an unknown mechanism

Suppression of FKBP38 induces apoptosis

Given the contradictory reports about the potential function of FKBP38 [8,14,28], we studied whether the suppression of FKBP38 might cause a change in cellular homeostasis. We treated HeLa cells with the siRNAs targeting FKBP38 and Bcl-2, respectively. Our data demonstrated that the suppression of FKBP38 by the siRNA resulted in a decrease in cell viability (Fig. 7). And we then checked the induction of apoptotic cell death after the treatment of the FKBP38 siRNA. Our data showed that the silencing of FKBP38 by the siRNA induced the activation of DEVDase activity (Fig. 8A), and the cleavage of PARP, which is a manifestation of the activation of caspase-3 (Fig. 8B), and also nuclear condensation (Fig. 8C), suggesting that caspase-3 dependent apoptosis was induced after the downregulation of FKBP38. In our study, together, the effect of silencing of FKBP38 on apoptotic cell death appeared to be similar to that of Bcl-2. From our results, we speculate that FKBP38 exerts an anti-apoptotic activity in conjunction with Bcl-2 in HeLa cells.

Discussion

FKBP38, unlike other FKBP family proteins, has been shown to lack the FK-506 binding activity and inhibitory activity on calcineurin [8]. To further understand the biological function of FKBP38, we, in the present study, attempted to investigate the molecular characteristics of human FKBP38 and its regulatory role on the anti-apoptotic protein Bcl-2. Recently, it has been shown that PPIase activity of FKBP38 was activated by complex formation with Ca^{2+} /calmodulin [14]. This led us to further investigate potential chaperone activities of FKBP38. From our results we demonstrated that FKBP38 exerted molecular chaperone activities by reducing the degree of aggregation in the citrate synthase model system and also increasing the solubility of Bcl-2, while FKBP12, which was used as a control in this study, did not appear to show similar chaperone activities with those substrates (Figs. 3B and C), suggesting that FKBP38 appears to work differently in chaperoning other proteins as compared to the typical FKBP family proteins. To further test whether or not FKBP38 binds FK-506 at molecular level and shows any inhibitory activity on calcineurin, we performed NMR-based binding studies of FKBP38 and phosphatase assays of calcineurin. Our data showed that FKBP38 did not show any apparent chemical shift perturbations in the 2D ^1H , ^{15}N -HSQC spectrum of FKBP38NTD upon addition of FK-506 (Fig. 5A), suggesting no molecular interaction with FK-506. In contrast, FKBP12 demonstrated significant chemical shift perturbations in the presence of FK-506, indicating apparent binding to FK-506. Sequence comparison study among FKBP family proteins reveals that FKBP38 lacks the well-conserved aromatic residues involved in binding to FK-506 in the active site pocket [29,34]. FKBP38 contains Leu instead at those positions. The Trp in FKBP12 is important for the interaction with FK506 and forms the base of the

hydrophobic drug-binding cavity. Those sequence variations found in FKBP38 might be one of the potential reasons contributing to the difference between FKBP38 and other FKBP family members in recognizing FK-506 as a ligand. On the other hand, FKBP38 contains an extra N-terminal tail, not found in other FKBP family proteins. The significance of this tail of FKBP38 on FK-506 binding remains to be explored. Three-dimensional structural determination of FKBP38 would provide important clues for explaining the unique properties of FKBP38. Previously, it was shown that FKBP38 is an endogenous inhibitor of calcineurin [8]. Our results demonstrate that FKBP38 did not appear to influence the calcineurin activity *in vitro*. Whether the binding of FK-506 is prerequisite for the inhibition on the phosphatase activity of calcineurin remains to be further explored.

Previously potential posttranslational modification sites in the murine FKBP38 were predicted [26]. Since the human FKBP38 has very high homology with the murine FKBP38, to check potential posttranslational modifications such as phosphorylations on the human FKBP38, in this study, we performed *in vitro* phosphorylation reaction using purified protein kinases. Our results showed that FKBP38 was phosphorylated by ERK2, but not JNK (Fig. 4A), suggesting that FKBP38 potentially could be phosphorylated by a specific kinase such as ERK2. More studies are needed to further characterize the phosphorylation of FKBP38 in cells.

Apoptosis is a well-organized process, and Bcl-2 plays an important role in the regulation of apoptosis. FKBP38 is a docking molecular which can help Bcl-2 to localize at the mitochondria [8]. To check the effect on Bcl-2 after the depletion of FKBP38, we attempted to employ RNA interference to suppress FKBP38 function. From our studies, we demonstrated that the knocking down of FKBP38 unexpectedly resulted in a reduction of anti-apoptotic protein Bcl-2 without much influence on the expression of pro-apoptotic protein Bax, while the similar anti-apoptotic protein Bcl-Xl was slightly affected (Fig. 5A), suggesting that the ratio between pro- and anti-apoptotic proteins was changed, and consequently led to induction of apoptosis. The treatment of FKBP38 siRNA caused to reduce the level of Bcl-2 protein, but it did not affect Bcl-2 mRNA. This suggests that the down-regulation of Bcl-2 after suppression of FKBP38 takes place at protein level. Three-dimensional structural studies revealed that the anti-apoptotic proteins Bcl-2 and Bcl-Xl contain an unusually long disordered loop [15,16]. It was shown that the loop is regulated by kinases, in response to diverse external stimuli [30,31], and also, proteasomes are involved in the posttranslational regulation of Bcl-2 [32]. Recently, we have shown that the disordered loop of Bcl-2 is involved in molecular interaction with FKBP38 [17]. Taken together, we speculate that Bcl-2 might be protected when complexed with FKBP38. But when Bcl-2 loses FKBP38, Bcl-2 might become unstable or left unprotected from degradation, and eventually contributing to apoptotic cell death. There have been controversial reports about the function of FKBP38 in apoptosis; Shirane and Nakayama [8] demonstrated the anti-apoptotic activity of FKBP38 by showing that FKBP38 inhibited Bcl-2 dependent apoptosis. On the other hand, Edlich et al. [16] demonstrated the pro-apoptotic activity of FKBP by showing that the activated FKBP38 with Ca^{2+} /calmodulin resulted in induction of apoptosis by interacting with Bcl-2 in an analogous manner to pro-apoptotic proteins. Recently, it was shown that presenilins 1

and 2 (PS1/2), which have shown to be associated with familial Alzheimer's disease (FAD) and involved in apoptotic neuronal cell death, increased the susceptibility of cells to apoptosis by antagonizing anti-apoptotic function of FKBP38 [33]. Our results showed that the effect of RNA interference of FKBP38 was similar to that of Bcl-2 in inducing apoptosis (Figs. 6–8). Therefore, based on our data, we speculate that FKBP38 exerts an anti-apoptotic activity by chaperoning the antiapoptotic protein Bcl-2 at the mitochondrial membrane. It, however, cannot be excluded that the dual function— anti-apoptotic and pro-apoptotic—of FKBP38 could be due to differences in cells used in those studies [14]. Different cells originated from different tissues may operate different apoptotic regulatory pathways. Further studies remain to be conducted for better understanding of function and role of FKBP38 in apoptosis.

Acknowledgements

This work was generously supported by A*STAR Bio-medical Research Council of Singapore Grant 04/1/22/ 12/362. C.B. Kang is a recipient of the Singapore Millennium Foundation PhD scholarship.

References

- [1] M.G.V. Heiden, C.B. Thompson, Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nat. Cell Biol.* 1 (1999) 209–214.
- [2] S. Cory, J.M. Adams, The Bcl2 family: regulators of the cellular life or-death switch, *Nat. Rev.* 2 (2002) 647–656.
- [3] S.J. Riedl, Y. Shi, Molecular mechanisms of caspase regulation during apoptosis, *Nat. Rev.* 5 (2004) 897–907.
- [4] S.W. Fesik, Insights into programmed cell death through structural biology, *Cell* 103 (2000) 273–282.
- [5] R.M. Kluck, E. Bossy-Wetzel, D.R. Green, D.D. Newmeyer, The release of cytochrome c from mitochondrial: a primary site for Bcl-2 regulation of apoptosis, *Science* 275 (1997) 1132–1136.
- [6] C.M. Sorenson, Bcl-2 family members and disease, *Biochimica. Biophysica. Acta* 1664 (2004) 169–177.
- [7] G. Kroemer, The proto-oncogene Bcl-2 and its role in regulating apoptosis, *Nat. Med.* 3 (1997) 614–620.
- [8] M. Shirane, K.I. Nakayama, Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis, *Nat. Cell Biol.* 5 (2003) 28–37.
- [9] E. Lam, M. Martin, G. Wiederrecht, Isolation of a cDNA encoding a novel human FK506 binding protein homlog containing leucine zipper and tetratricopeptide repeat motif, *Gene* 160 (1995) 297–302.
- [10] B. Wu, P. Li, Y. Liu, Z. lou, Y. Ding, C. Shu, S. Ye, M. Bartlam, B. Shen, Z. Rao, 3D structure of human FK506-binding protein⁵²; implications for the assembly of the glucocorticoid receptor/Hsp90/ immunophilin heterocomplex, *Proc. Natl. Acad. Sci. USA* 101 (2004) 8348–8353.
- [11] O.V. Bulgakov, J.T. Eggenschwiler, D.H. Hong, V. Anderson, T. Li, FKBP8 is a negative regulator of mouse sonic hedgehog signaling in neural tissues, *Development* 131 (2004) 2149–2159.
- [12] M. Rosner, K. Hofer, M. Kubista, M. Hengstschlager, Cell size regulation by the human TSC tumor suppressor proteins depends on PI3K and FKBP38, *Oncogene* 22 (2003) 4786–4798.

- [13] J.V. Nielsen, C. Mitchelmore, K.M. Pedersen, K.M. Kjaerulff, B. Finsen, N.A. Jensen, FKPB8: novel isoforms, genomic organization and characterization of a forebrain promoter in transgenic mice, *Genomics* 86 (2004) 181–192.
- [14] F. Edlich, M. Weiward, E. Frank, J. Fanghanel, F. Jarczowski, J.U. Rahfeld, G. Fische, r Bcl-2 regulator FKBP38 is activated by Ca^{2+} / calmodulin, *EMBO J.* 24 (2005) 2688–2699.
- [15] A.M. Petros, A. Medek, D.G. Nettesheim, D.H. Kim, H.S. Yoon, K. Swift, E.D. Matayoshi, T. Itersdorf, S.W. Fesik, Solution structure of the antiapoptotic protein Bcl-2, *Proc. Natl. Acad. Sci. USA* 98 (2001) 3012–3017.
- [16] S.W. Muchmore, M. Sattler, H. Liang, R.P. Meadows, J.E. Harlan, H.S. Yoon, D. Nettesheim, B.S. Chang, C.B. Thompson, S.L. Wong, S.C. Ng, S.W. Fesik, X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death, *Nature* 381 (1996) 335–341.
- [17] C.B. Kang, J. Tai, J. Chia, H.S. Yoon, The flexible loop of Bcl-2 is required for molecular interaction with immunosuppressant FK-506 binding protein 38 (FKBP38), *FEBS Lett.* 579 (2005) 1469–1476.
- [18] R. Kumar, B. Adams, A. Musiyenko, O. Shulyayeva, S. Barik, The FK506-binding protein of the malaria parasite, *Plasmodium falciparum*, is a FK506-sensitive chaperone with FK506-independent calcineurin-inhibitory activity, *Mol. Biochem. Parasitol.* 141 (2005) 163–173.
- [19] P. Monaghan, A. Bel, A *Plasmodium falciparum* FK506-binding protein (FKBP) with peptidyl-prolyl *cis-trans* isomerase and chaperone activities, *Mol. Biochem. Parasitol.* 139 (2005) 185–195.
- [20] A. Khoklatchev, S. Xu, J. English, P. Wu, E. Schaefer, M.H. Cobb, Reconstitution of mitogen-activated protein kinase phosphorylation cascades in bacteria, *J. Biol. Chem.* 272 (1997) 11057–11062.
- [21] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, *Nature* 411 (2001) 494–498.
- [22] J. Harborth, S.M. Elbashir, K. Bechert, T. Tuschl, K. Weber, Identification of essential genes in cultured mammalian cells using small interfering RNAs, *J. Cell Sci.* 114 (2001) 4557–4565.
- [23] A.H. Cory, T.C. Owen, J.A. Barltrop, J.G. Cory, Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture, *Cancer Commun.* 3 (1991) 207–212.
- [24] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods* 65 (1983) 55–63.

- [25] A. Galat, Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity-targets-functions, *Curr. Top. Med. Chem.* 3 (2003) 1315–1347.
- [26] K.M. Pedersen, B. Finsen, J.E. Celis, N.A. Jensen, muFKBP38: a novel murine immunophilin homolog differentially expressed in Schwannoma cells and central nervous system neurons in vivo, *Electrophoresis* 20 (1999) 249–255.
- [27] J.P. Griffith, J.L. Kim, E.E. Kim, M.D. Sintchak, J.A. Thomson, M.J. Fitzgibbon, M.A. Fleming, P.R. Caron, K. Hsiao, M.A. Navia, X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex, *Cell* 82 (1995) 507–522.
- [28] M. Weiwad, F. Edlich, F. Erdmann, F. Jarczowski, S. Kilka, M. Dorn, A. Pechstein, G. Fischer, A reassessment of the inhibitory capacity of human FKBP38 on calcineurin, *FEBS Lett.* 579 (2005) 1591–1596.
- [29] A. Galat, Sequence diversification of the FK506-binding proteins in several different genomes, *Eur. J. Biochem.* 267 (2000) 4945–4959.
- [30] M.C. Bassik, L. Scorrano, S.A. Oakes, T. Pozzan, S.J. Korsmeyer, Phosphorylation of BCL-2 regulates ER Ca (2+) homeostasis and apoptosis, *EMBO J.* 23 (2004) 1207–1216.
- [31] M.V. Blagoskonny, Unwinding the loop of Bcl-2 phosphorylation, *Leukemia* 15 (2001) 869–874.
- [32] K. Breitschopf, J. Haendeler, P. Malchow, A.M. Zeiher, S. Dimmeler, Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway, *Mol. Cell. Biol.* 20 (2000) 1886 – 1896.
- [33] H.Q. Wang, Y. Nakaya, Z. Du, T. Yamane, M. Shirane, T. Kudo, M. Takeda, K. Takebayashi, Y. Noda, K.I. Nakayama, M. Nishimura, Interaction of presenilins with FKBP38 promotes apoptosis by reducing mitochondrial Bcl-2, *Hum. Mol. Genet.* 14 (2005) 1889–1902.
- [34] C. Sich, S. Improta, D.J. Cowley, C. Guenet, J.-P. Merly, M. Teufel, V. Saudek, Solution structure of a neurotrophic ligand bound to FKBP12 and its effects on protein dynamics, *Eur. J. Biochem.* 267 (2000) 5342–5354.

List of Figures

- Fig. 1 Expression of FKBP38 in different cancer cell lines. (A) Expression of FKBP38 was evaluated in cancer cells as indicated. The lysates of different cancer cell lines were separated on 12% SDS–PAGE followed by Western blot using anti-FKBP38 antisera. (B) Localization of endogenous FKBP38 at the mitochondria in HeLa cells. Cells were stained with anti-FKBP38 antisera (red) and MitoTracker (blue). Shown also is the superimposed image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)
- Fig. 2 Purification of FKBP38 and FKBP38NTD. (A) Co-expression of FKBP38 with Bcl-2 and Bcl-Xl. FKBP38 and FKBPNTD were expressed and purified as described before [17]. Shown are samples separated on 12% SDS-PAGE after Ni-NTA and Sephacryl-S200 steps, and visualized by Coomassie staining. (B) FKBP38 and FKBP38 were subjected to Western blot analysis with anti-FKBP38 antisera.
- Fig. 3 Chaperone activity assays of FKBP38. (A) The FKBP38 with his-tag and Bcl-2 with no-tag were co-expressed in *E. coli* BL21(DE3) cells to check complex formation of FKBP38 with Bcl-2. The loading sample (L) and eluted fraction (E) from Ni²⁺–NTA were analyzed by Western blot with antisera to FKBP38 and Bcl-2. (B) The effect of FKBP38 on the solubility of Bcl-2 was probed by expressing Bcl-2 in the absence or presence of FKBP38. Samples are analyzed on 12% SDS–PAGE. T, total protein lysate; S, supernatant; P, pellet. (C) Effect of FKBP38 in preventing aggregation of citrate synthase. The citrate synthase was mixed with FKBP38 or FKBP12 or BSA, incubated at 42 °C for 20 min, and then absorbance at 360 nm was measured.
- Fig. 4 Phosphorylation of FKBP38 and its effect on calcineurin activity. (A) Potential phosphorylation of FKBP38 was evaluated in vitro using ERK2 and JNK. FKBP38 was mixed with JNK or ERK2 as described under Materials and methods. Phosphorylated proteins were separated on 12% SDS–PAGE and then visualized by autoradiography. (B) Effect of FKBP38 on the activity of calcineurin. To check the influence of FKBP38 on the phosphatase activity of calcineurin, the synthetic substrate was derived from the RII as described under Materials and methods. The activities were measured by addition of malachite green reagent in the absence or presence of FK-506.

- Fig. 5 The FKBP domain of FKBP38 (FKBPNTD) does not bind FK-506 in NMR-based binding assays. Chemical shift perturbations to ^{15}N -labeled FKBP38NTD (A) and FKBP12 (B) were monitored with ^{15}N -heteronuclear single quantum correlation spectroscopy (HSQC) upon addition of FK-506. The HSQC spectra with or without FK506 are shown in red or blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)
- Fig. 6 Suppression of FKBP38 downregulates Bcl-2 protein. (A) To probe the effect of FKBP38 on the stability of Bcl-2, RNAi of FKBP38 was carried out. HeLa cells were treated by the FKBP siRNA described under Materials and methods. After 48 h later, the protein levels were checked by Western blot analyses. (B) The mRNA levels of FKBP38, Bcl-2, and Bcl-Xl were checked by a semi-quantitative RT-PCR analysis. GAPDH was used as an internal control.
- Fig. 7 Silencing of FKBP38 reduces cell viability. To check the effect of RNAi of FKBP38, HeLa cells were treated with FKBP38 or Bcl-2-specific siRNAs, respectively. After 48 h later, cell viability was measured as described under Materials and methods.
- Fig. 8 Suppression of FKBP38 induces caspase-3 dependent apoptosis. To investigate the effect of downregulation of FKBP38 by RNAi of FKBP38, HeLa cells were treated with FKBP- and Bcl-2-specific siRNAs, respectively. After 48 h later, apoptotic markers including the activation of DEVDase (A), cleavage of PARP (B), and condensation of nucleus (C) were measured as described under Materials and methods.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8