A Novel Calmodulin-Ca\(^{2+}\) Target Recognition Activates the Bcl-2 Regulator FKBP38\(^*\)\(^{[5]}\)

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The FK506-binding protein 38 (FKBP38) affects neuronal apoptosis control by suppressing the anti-apoptotic function of Bcl-2. The direct interaction between FKBP38 and Bcl-2, however, requires a prior activation of FKBP38 by the Ca\(^{2+}\) sensor calmodulin (CaM). Here we demonstrate for the first time that the formation of a complex between FKBP38 and CaM-Ca\(^{2+}\) involves two separate interaction sites, thus revealing a novel scenario of target protein regulation by CaM-Ca\(^{2+}\). The C-terminal FKBP38 residues Ser\(^{290}\)–Asn\(^{313}\) bind to the target protein-binding cleft of the Ca\(^{2+}\)-coordinated C-terminal CaM domain, thereby enabling the N-terminal CaM domain to interact with the catalytic domain of FKBP38 in a Ca\(^{2+}\)-independent manner. Only the latter interaction between the catalytic FKBP38 domain and the N-terminal CaM domain activates FKBP38 and, as a consequence, also regulates Bcl-2.

FKBP38 is a member of the peptidyl prolyl cis/trans-isomerase (PPIase)\(^3\) family that belongs to the FK506-binding proteins (FKBP) family. Its active form was found to inhibit Bcl-2-controlled neuronal cell death (1). The PPIase activity of FKBP38 is induced by the formation of a FKBP38/CaM-Ca\(^{2+}\) complex, which apparently affects the conformation of FKBP38. Thereby the active site of FKBP38 becomes accessible for substrate proteins, such as Bcl-2. Active site-directed ligands of FKBP38, on the other hand, can prevent the interaction between active FKBP38 and Bcl-2. As a consequence, active site-directed inhibition of FKBP38/CaM-Ca\(^{2+}\) (i) significantly increases cell survival rates of neuroblastoma cells after apoptosis induction by different apoptotic stimuli and (ii) shows substantial neuroprotective and neuroregenerative effects in the rat ischemia model of endothelin-1-induced cerebral middle artery occlusion (2). Furthermore, FKBP38 plays a role in cell size regulation (3) and participates in the development of murine neuronal tissues by regulating sonic hedgehog signaling (4). FKBP38 also interacts with the HIF1a- and the anti-apoptotic function of Bcl-2. As a consequence, active site-directed inhibition of FKBP38/CaM-Ca\(^{2+}\) not only promotes cell survival but also regulates Bcl-2. Furthermore, FKBP38 participates in the development of murine neuronal tissues by regulating sonic hedgehog signaling (4). FKBP38 also interacts with the HIF1a- and HIF1b-regulated complex-1 (5a, 5b, 6a; the latter interaction, however, occurs only in the presence of CaM-Ca\(^{2+}\).

FKBP38 contains two domains: (i) a PPIase domain in the N-terminal region and (ii) a C-terminal tetra-tricopeptide repeat (TPR) domain. The PPIase domain folds to a FKBP12-like structure consisting of five anti-parallel \(b\)-strands and an \(\alpha\)-helix (7). In contrast to the prototypic FKBP12, the rim of the FKBP38 active site is more negatively charged, the bulge in the \(\beta\)-strands D is shortened, and the flexible loop between \(\beta\)-strands E and F is slightly extended (7). The TPR domain in the closely related proteins FKBP51 and FKBP52 comprises three TPR motifs (8, 9). Compared with FKBP51 and FKBP52, whose sequences end with an \(\alpha\)-helix following the TPR motifs, FKBP38 additionally contains a membrane anchor that localizes the protein to the membranes of the endoplasmic reticulum and mitochondria (1). Although several FKBP family members share the same domain composition, FKBP38 is the only CaM-Ca\(^{2+}\)-regulated PPIase known to date.

The small Ca\(^{2+}\)-sensing protein CaM consists of two globular domains connected by a linker that is rather flexible in solution (10). Each domain contains two helix-loop-helix elements, referred to as EF-hand motifs, that can bind Ca\(^{2+}\) among other positively charged ions (11). Upon Ca\(^{2+}\) binding, each CaM domain undergoes structural changes that lead to a concerted exposure of hydrophobic groups in a methionine-rich cleft (12, 13). Each EF-hand in the C-terminal domain binds one calcium ion with a \(K_D\) value of about 0.2 \(\mu M\), whereas the EF-hand motifs of the N-terminal domain show 10-fold lower Ca\(^{2+}\) affinities (14). The conformational change between the Ca\(^{2+}\)-free and -complexed form of CaM mediates the signal of high cellular Ca\(^{2+}\) concentration for a plethora of target proteins, which participate in physiological processes such as control of muscle contraction, fertilization, cell proliferation, vesicular fusion, and apoptosis. In response to increased cellular Ca\(^{2+}\) concentrations, for example, CaM usually interacts with short amphiphilic helices of \(~20\) residues that are exposed in the target protein structures, as is the case for CaM kinases such as the CaM kinase II and myosin light chain kinases (15). In many target enzymes, like CaM kinase II and calcineurin A, the interaction with CaM displaces autoinhibitory elements from their active sites (16, 17). In contrast to proteins that interact only with CaM-Ca\(^{2+}\), the Ca\(^{2+}\) release channel ryanodine receptor 1 binds CaM in both the presence and the absence of Ca\(^{2+}\) (13).
18). Neuromodulin and neurogranin, on the other hand, have a higher affinity for Ca\(^{2+}\)-free CaM (18–20). Finally, CaM can also interact with nonhelical sites in target proteins. For instance, the edemia factor from *Bacillus anthracis* not only binds to the Ca\(^{2+}\)-coordinated C-terminal domain but also forms a complex with the surface of the Ca\(^{2+}\)-free N-terminal CaM domain, thereby restructuring the active site of the edemia factor (21–23).

In the present study we show that two distinct binding sites exist between FKBP38 and CaM-Ca\(^{2+}\); (i) a helical motif, located in the FKBP38 sequence between the TPR motifs and the membrane anchor, forms a complex with the C-terminal CaM domain only in the presence of Ca\(^{2+}\), and (ii) the catalytic domain of FKBP38 binds to the N-terminal domain of CaM in a Ca\(^{2+}\)-independent manner. Hence, these results reveal a hitherto unknown scenario of Ca\(^{2+}\)-independent enzyme activation by CaM.

**MATERIALS AND METHODS**

Human FKBP38 lacking the membrane anchor (FKBP38\(^{1–336}\), the PPlase domain of FKBP38 (FKBP38\(^{35–153}\)), human FKBP12, human CaM, the N-terminal domain of human CaM (CaM\(^{1–75}\)), the C-terminal domain of human CaM (CaM\(^{76–148}\)), and human Bcl-2 were all expressed by using a pET28a vector in Rosetta\textsuperscript{TM} cells, with the C-terminal CaM domain His\(_6\)-tagged. The maltose-binding protein (MBP)-Bcl-2 fusion protein was purchased from Sigma. An affinity-purified section 4 polyclonal antibody from rabbit against the purified FKBP38 domain (FKBP38\(^{1–165}\)) was employed. Additional antibodies used were monoclonal hamster anti-Bcl-2 (BD PharMingen, San Diego, CA) and polyclonal rabbit anti-CaM antibodies. Competing CaM variants were applied in 5-fold excess.

**Western Blot Analysis of CaM Interaction with the Peptide Array**

Before Western blot screening, the dry peptide array membranes were rinsed for 10 min in methanol and for 3×20 min in TBS buffer (30 mM Tris/HCl, pH 7.6, 170 mM NaCl, 6.4 mM KCl). CaM solution (100 nM) in TBS buffer was allowed to react with peptide array membranes in the presence of either 2 mM CaCl\(_2\) or 2 mM EGTA for 4 h at 4 °C under gentle shaking. The membrane was subsequently washed three times with TBS buffer before bound protein was blotted onto nitrocellulose membranes and analyzed using rabbit CaM antibodies.

**PPlase Activity Measurements**

PPlase activity was determined using a protease-coupled assay, with the oligopeptide succinyl-ALPF-4-nitroanilide as substrate (1). Typically, the PPlase activity of FKBP38 was measured in a reaction mixture containing 1 μM of either FKBP38\(^{1–336}\) or FKBP38\(^{35–153}\), with 1.5 or 15 μM recombinant human CaM, respectively, and 1 mM CaCl\(_2\). Insensitivity of CaM to proteolytic digestion by a-chymotrypsin was verified in the time range of the kinetic experiments.

**Protein-Protein Interaction Assays**

**Co-immunoprecipitation**—The cells were grown in flasks, incubated for 16 h with 50 μM etoposide, and finally harvested. The cell lysis and co-immunoprecipitation experiments were performed according to manufacturer protocols of the immunoprecipitation starter kit (GE Healthcare, Upplaka, Sweden). Prior to incubation, 0.5 μM EGTA was added to the samples. In addition, 1 μM CaCl\(_2\) was added to those samples incubated in the presence of Ca\(^{2+}\).

**CaM Binding Assay**—CaM-Sepharose (GE Healthcare) was pre-equilibrated in 25 mM Tris/HCl buffer (pH 7.5, 200 mM NaCl, 1 mM dithiothreitol) in the presence of either 2 mM CaCl\(_2\) or 2 mM EGTA. Subsequently, 30 μg of recombinant FKBP38\(^{1–336}\) was incubated with CaM-Sepharose. The Sepharose was washed, and the bound proteins were analyzed by SDS-PAGE and Western blotting using polyclonal rabbit anti-FKBP38 antibodies. Competing CaM variants were applied in 5-fold excess.

**Bcl-2 Binding Assay**—40 μl of 6 μM MBP-Bcl-2 fusion protein was subjected to 40 μl of amylase-resin (New England Biolabs, Beverly, MA) and incubated for 30 min. Thereafter, the beads were washed twice with 25 mM Tris/HCl buffer (pH 7.5, 200 mM NaCl, 1 mM dithiothreitol) and subsequently incubated for 1 h with 40 μl of reaction mixture containing 200 μM of a CaM variant and either 50 μM FKBP38\(^{35–153}\) or 100 μM FKBP38\(^{35–153}\). After three washing steps with 25 mM Tris/HCl buffer (pH 7.5, 200 mM NaCl, 1 mM dithiothreitol), the samples were boiled in Laemmli buffer and subjected to SDS-PAGE. Binding of FKBP38\(^{1–336}\) was analyzed using polyclonal rabbit anti-FKBP38 antibodies. According to a competition model using DynaFit software, the presence of 1 mM EGTA causes the content of CaM-Ca\(^{2+}\) complex in the sample to drop below 0.6% of the CaM concentration under any chosen condition and is thus referred to as Ca\(^{2+}\)-free.

**Isothermal Titration Calorimetry**

ITC experiments were performed at 25 °C using a MicroCal VP-ITC microcalorimeter. FKBP38 and CaM variants were diluted in 10 mM MES buffer (pH 6.8, 100 mM NaCl, 0.05% NaN\(_3\)) with either 1 mM EGTA or 2 mM CaCl\(_2\). The FKBP38\(^{1–336}\) solutions ranged in concentration from 35 to 70 μM; FKBP38\(^{35–153}\) was applied in a concentration range from 0.1 to 1.0 mM; the concentrations of the various CaM variants ranged from 0.2 to 2.0 mM in different experiments. The 24-mer peptide (0.25 mM) was applied in 10 mM MES buffer with 2 mM CaCl\(_2\).

Usually the FKBP38 solution was placed in the calorimeter cell, and the CaM solution was loaded into the syringe injector. The titrations were carried out in 5–10–μl aliquots, with a 240-s delay between each injection. Calorimetric titration data were fitted using the Origin 5.0 program supplied with the Microcal VP-ITC instrument, to obtain the stoichiometry (N), the association constant (K), the binding enthalpy (ΔH), and the bind-
ing entropy ($\Delta S$). The reported binding constants are an average of duplicate measurements. As a control, the protein or peptide solutions were titrated into buffer.

**Fluorescence and NMR Spectroscopy**

Steady-state fluorescence spectra were recorded with a PerkinElmer Life Sciences FluoroMax2 fluorescence spectrometer, using a 1 x 1-cm cuvette, an excitation wavelength of 280 nm, and excitation and emission slit widths of 5 and 3 nm, respectively. The protein samples were applied in 10 mM MES buffer with either 2 mM CaCl$_2$ or 1 mM EDTA. The binding constant ($K_D$) was calculated from the fluorescence intensity by using the equation,

$$P_0 \cdot \alpha = \frac{C_0 \cdot \alpha}{n(1-\alpha)} - \frac{K_D}{n}$$

(Eq. 1)

where $P_0 =$ total protein concentration, $\alpha = (F_{\text{max}} - F)/F_{\text{max}}$, $F_{\text{max}} =$ fluorescence intensity at saturation, $F_0 =$ initial fluorescence intensity, $n =$ number of independent binding sites, $C_0 =$ total CaM concentration at each addition, and $K_D =$ dissociation constant.

All of the NMR spectra were acquired at 25 °C, using a Bruker DRX 500 spectrometer as previously described (7). The NMR sample contained 0.5 mM 15N-labeled CaM in 10 mM MES buffer (pH 6.8, 100 mM KCl, 6 mM CaCl$_2$, 0.05% NaN$_3$). FKBP38$_{290-313}$ was added to the sample in 3-fold excess (1.5 mM). The backbone amide peaks were picked with the program Felix 2000 (Accelrys Inc., San Diego, CA) and assigned via 15N-edited three-dimensional spectra with the help of a previously reported assignment of CaM-Ca$^{2+}$ (36). The backbone amide assignments are listed in supplemental Table S1.

**RESULTS**

The FKBP38 Segment Ser$_{290}^{313}$ Interacts with CaM in a Ca$^{2+}$-dependent Manner.—The interaction between FKBP38 and CaM is dependent on the Ca$^{2+}$ concentration both in vivo and in vitro (1). A peptide scan was performed to identify the CaM-binding site of FKBP38; CaM bound to two clusters of peptides in the presence of Ca$^{2+}$, whereas in the absence of Ca$^{2+}$, no binding of CaM to the array of FKBP38 peptides was detected (Fig. 1A). The two clusters of peptides, which interact

FIGURE 1. CaM interacts with the FKBP38 residues Ser$_{290}^{313}$ only in the presence of Ca$^{2+}$ ions. A, an array of 13-mer peptides spanning the FKBP38 sequence was synthesized with forward shifts by one amino acid. CaM interaction with the peptide array in the presence of 1 mM EGTA (upper panel) and 2 mM CaCl$_2$ (lower panel) was analyzed by Western blot. The CaM-Ca$^{2+}$ interaction pattern is marked by boxes. The respective binding motifs i and ii, comprising the peptides l21–23 and m14–17, correspond to segments Ile$_{285}^{298}$–Ala$_{298}^{301}$ and Glu$_{303}^{316}$–Arg$_{316}^{319}$ in the C-terminal FKBP38 domain, located between the TPR motifs and the membrane anchor. B, sequence of the FKBP38 segment Pro$_{280}^{283}$–Cys$_{321}^{324}$, featuring the Ca$^{2+}$-dependent CaM-binding motifs identified in the FKBP38 protein sequence (boxes). Probability values (ranging from 0 for low and 9 for high) for an interaction with CaM according to the CaM Target Data Base (calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html) are shown below the FKBP38 sequence. C, Western blot analysis of endogenous proteins from SH-SY5Y cell lysate interacting with a biotin-labeled FKBP38$_{290-313}$ peptide-bound streptavidin matrix in the presence of either 0.5 mM EGTA or 1 mM Ca$^{2+}$, using CaM antibodies. The bound protein was dissected from SDS-PAGE, digested with trypsin, and analyzed by MALDI-TOF. D, Western blot analysis of FKBP38 binding to CaM-Sepharose in the presence of 2 mM Ca$^{2+}$ ions. The flow through is displayed on the left, whereas eluted proteins are shown on the right. The FKBP38$_{290-313}$ peptide competes with FKBP38 for CaM binding. E, PPIase activity assay, showing FKBP38$_{3536}^{3537}$-CaM-Ca$^{2+}$ inhibition by the FKBP38$_{290-313}$ peptide. The solid line is fitted according to the dose-dependent decrease in PPIase activity.
with CaM-Ca\(^{2+}\) indicate minimal binding motifs corresponding to the FKBP38 segments Ile\(^{285}\)–Ala\(^{298}\) and Glu\(^{303}\)–Arg\(^{316}\) (Fig. 1B). Both segments are located in the C-terminal FKBP38 domain between the TPR motifs and the membrane anchor. The sequences of both segments consist largely of positively charged and hydrophobic amino acids and may therefore constitute an amphipathic \(\alpha\)-helix. Interestingly, the putative CaM-binding site predicted by the CaM Target Data Base covers both segments nearly completely (Fig. 1B).

Because the matrix-bound peptides (i) exhibit the best accessibility and (ii) adopt the correct secondary structure generally in the central positions, we focused on the middle section of the identified motif. As a consequence, the peptide biotinyl-SKLVKHAAQRSTETALYRKMLGN-NH\(_2\), which corresponds to FKBP38 residues Ser\(^{290}\)–Asn\(^{313}\) (FKBP38\(^{290–313}\)), was immobilized on streptavidin beads and subsequently tested for binding to endogenous CaM from SH-SY5Y cell lysate. CaM bound to the loaded affinity matrix only in the presence of Ca\(^{2+}\), as confirmed by MALDI-TOF analysis and Western blot (Fig. 1C). Moreover, the FKBP38\(^{290–313}\) peptide competed with FKBP38\(^{1–336}\) for binding to CaM-Sepharose (Fig. 1D). To quantify the interference of the peptide with the activity of FKBP38\(^{35–153}\), FKBP38\(^{290–313}\) was applied in a PPIase activity assay. The peptide competed with FKBP38\(^{35–153}\) for CaM-Ca\(^{2+}\) binding, thus dissociating the FKBP38\(^{35–153}\)/CaM-Ca\(^{2+}\) complex into the PPIase-inactive constituents with an IC\(_{50}\) value of 1.4 ± 0.13 \(\mu\)M (Fig. 1E). Taken together, these results indicate a Ca\(^{2+}\)-dependent complex formation between CaM and FKBP38\(^{290–313}\).

The Catalytic Domain of FKBP38 Features a Second CaM-binding Site—Given the interaction between FKBP38\(^{290–313}\) and CaM-Ca\(^{2+}\), we furthermore examined whether CaM interacts with an FKBP38 construct that lacks the C-terminal CaM-binding site. To this end, the PPIase activity of the catalytic FKBP38 domain (FKBP38\(^{35–153}\)) was examined in the presence of CaM. Surprisingly, FKBP38\(^{35–153}\) showed PPIase activity when 10 \(\mu\)M CaM was added, although residues Ser\(^{290}\)–Asn\(^{313}\), which form the CaM-binding site in FKBP38, were not present (Fig. 2A). Hence, CaM interacts with a second CaM-binding site located in the catalytic domain of FKBP38. Moreover, the presence of CaM induced the activity of FKBP38\(^{35–153}\) to the same extent in both the presence and the absence of Ca\(^{2+}\), as demonstrated by similar k\(_{cat}/K_M\) values, which amends the previously reported Ca\(^{2+}\)-dependent activation of FKBP38 (1).
The fact that this second binding site was not detected in the peptide array suggests a nonlinear binding motif.

These results reveal a Ca\(^{2+}\)-independent interaction between CaM and the catalytic domain of FKBP38, which apparently induces the active conformation of FKBP38. Remarkably, the dose response curves do not adopt hyperbolic character, as one might expect. This effect is due to the adsorption of FKBP38 and CaM variants at the cuvette surface and was successfully suppressed in the fluorescence assays described below.

The fluorescence measurements were performed to investigate the CaM interaction with FKBP38\(^{35-153}\) in the absence of Ca\(^{2+}\). Fig. 2B shows the fluorescence spectra of both proteins in the absence of Ca\(^{2+}\) ions. The spectrum of the FKBP38\(^{35-153}\)/CaM complex is red-shifted by 4 nm, and its amplitude is reduced by 8.5% compared with the combined spectrum of the isolated proteins. A titration experiment shows the changes in the protein fluorescence of FKBP38\(^{35-153}\) in dependence of the CaM concentration, revealing a \(K_D\) value of 6.63 ± 0.95 mM for this interaction (Fig. 2C). These data again indicate a Ca\(^{2+}\)-independent, specific binding of FKBP38\(^{35-153}\) to CaM. In contrast, the combined fluorescence spectrum of separately measured FKBP12 and CaM is nearly identical to the spectrum of FKBP12 in the presence of CaM (supplemental Fig. S1).

To further investigate the consequences of this interaction between the catalytic FKBP38 domain and CaM on the binding of Bcl-2, we analyzed the binding of either FKBP38\(^{1-336}\) or FKBP38\(^{35-153}\) to a Bcl-2 loaded affinity matrix. Fig. 2D shows that FKBP38\(^{35-153}\) bound to Bcl-2 both in presence and absence of Ca\(^{2+}\) whenever CaM was present, whereas no binding of FKBP38\(^{35-153}\) to Bcl-2 was detected in the absence of CaM. FKBP38\(^{1-336}\) and FKBP38\(^{35-153}\) competed for Bcl-2 binding in the presence of Ca\(^{2+}\)-free CaM, but when Ca\(^{2+}\) was present the FKBP38\(^{1-336}/\text{CaM-Ca}^{2+}\) complex prevented the binding of FKBP38\(^{35-153}\) to the Bcl-2 matrix. Taken together, these results (i) demonstrate the existence of a Ca\(^{2+}\)-independent CaM-binding site in the catalytic domain of FKBP38 and (ii) indicate a higher affinity between the two proteins when both binding sites of FKBP38 are present.

The N-terminal Domain of CaM Interacts with the Catalytic Domain of FKBP38

To analyze the respective counterparts in CaM that interact with the two binding sites in FKBP38, a tryptic digest was performed to separate both CaM domains and analyze their interactions with FKBP38. The digest resulted in two fragments with a cleavage site between the CaM residues by Western blot using anti-FKBP38 and mouse anti-Bcl-2 antibodies. B, co-immunoprecipitation of endogenous FKBP38 and Bcl-2 applying hamster anti-Bcl-2 antibody. Antibody/protein complexes were analyzed by Western blot using anti-Bcl-2 and anti-FKBP38 antibodies. C, FKBP38\(^{1-336}\) (10 μM input) was applied in the absence and presence of 20 mM CaM variants and 2 mM Ca\(^{2+}\) to MBP-Bcl-2 immobilized on amylose resin. Bound protein was eluted by 200 mM maltose and analyzed by Western blot using anti-FKBP38 antibodies. D, fluorescence measurements at 278 nm of 1 mM FKBP38\(^{35-153}\) (dotted line), 1 mM N-terminal CaM domain (solid line), and a 1:1 mixture (dotted line) in the presence of 1 mM EGTA. In addition, the calculated spectrum (dashed-dotted line) represents the sum of the individual protein spectra, as it should appear when the components do not interact. E, activity measurements of 1 μM FKBP38\(^{35-153}\) using the PPIase assay, in the presence of 2 mM CaCl\(_2\) and various concentrations of either the N-terminal (■) or the C-terminal (○) domain of CaM. The dose-response curves are displayed for the presence of the N-terminal (dashed line) and the C-terminal (dotted line) CaM domain.
Lys\textsuperscript{75} and Met\textsuperscript{76}, according to Edman digest analysis (data not shown). Therefore, two CaM fragments were cloned and expressed in *E. coli*. The fragment of the N-terminal CaM domain comprised residues Ala\textsuperscript{1}–Lys\textsuperscript{75} (CaM\textsuperscript{1–75}), whereas the fragment corresponding to the C-terminal CaM domain consisted of the sequence Met\textsuperscript{76}–Lys\textsuperscript{148} (CaM\textsuperscript{76–148}).

First, we used an affinity matrix loaded with the peptide that corresponds to FKBP38\textsuperscript{290–313} to bind endogenous CaM from SH-SY5Y cell lysate. Endogenous CaM was sequestered to the matrix and thus removed from the cell lysate (supplemental Fig. S2). Next, co-immunoprecipitation experiments were performed to analyze the interactions of endogenous FKBP38 and Bcl-2 in the presence of different CaM variants. When Ca\textsuperscript{2+} was present, FKBP38 interacted with Bcl-2 in the lysate of SH-SY5Y cells, but in CaM-depleted cell lysate no such interaction was observed (Fig. 3, A and B). The addition of full-length CaM to the CaM-depleted cell lysate restored the ability of endogenous FKBP38 to interact with Bcl-2. Upon the addition of the separate CaM domains, however, FKBP38/Bcl-2 interactions were restored only in the presence of CaM\textsuperscript{1–75}, whereas no interactions between FKBP38 and Bcl-2 occurred in the presence of CaM\textsuperscript{76–148}. These results indicate that it is the N-terminal domain of CaM that interacts Ca\textsuperscript{2+}–independently with the catalytic FKBP38 domain.

Thus, we subsequently also investigated the interaction of FKBP38\textsuperscript{35–153} with a Bcl-2 affinity matrix in the presence of different CaM variants. FKBP38\textsuperscript{35–153} interacted with Bcl-2 in the presence of full-length CaM or CaM\textsuperscript{1–75}, but no FKBP38\textsuperscript{35–153} bound to the Bcl-2 affinity matrix when only CaM\textsuperscript{76–148} was present (Fig. 3C). Hence, the N-terminal CaM domain mediates the interactions with the catalytic FKBP38 domain, as is further evidenced by a decrease of the protein fluorescence amplitude by 17.8 ± 0.1% and a 1-nm red shift observed in the spectrum of FKBP38\textsuperscript{35–153} in the presence of CaM\textsuperscript{1–75} (Fig. 3D). Fluorescence measurements of the FKBP38\textsuperscript{35–153}/CaM\textsuperscript{1–75} complex in the presence and absence of Ca\textsuperscript{2+} demonstrated that (i) the interaction between these two protein variants occurs both with and without Ca\textsuperscript{2+} and (ii) the spectral changes in the catalytic FKBP38 domain are identical under both conditions (supplemental Fig. S3).

Further, we tested the influence of the two separate CaM domains on the PPIase activity of the catalytic FKBP38 domain (Fig. 3E). Consistent with the previous data, only CaM\textsuperscript{1–75} caused an increase in the PPIase activity of FKBP38\textsuperscript{35–153}, thus demonstrating that this interaction between the N-terminal domains is required and sufficient for the activation of FKBP38. In contrast, the addition of the C-terminal CaM domain did not alter the catalytic activity of FKBP38\textsuperscript{35–153}.

### Table 1: Parameters for FKBP38 and CaM-Ca\textsuperscript{2+} Interaction

| Titration          | Ca\textsuperscript{2+} | 1/K_D \(\mu M\) | \(\Delta H\) \(\text{kcal mol}^{-1}\) | \(\Delta G\) \(\text{kcal mol}^{-1}\) | Apparent stoichiometry | Binding model |
|--------------------|------------------------|-----------------|--------------------------|--------------------------|---------------------------|---------------|
| FKBP38<sup>35–153</sup> vs. CaM<sup>1–75</sup> | – | 7.0 ± 0.55 | –28.3 ± 0.28 | –28.9 ± 0.28 | 0.97 ± 0.01 | F<sub>F1</sub> + C<sub>1</sub> ⇔ F<sub>F1</sub>C<sub>1</sub> |
| FKBP38<sup>35–153</sup> vs. CaM<sup>76–148</sup> | + | 2.2 ± 0.24 | 6.8 ± 0.61 | 317.7 ± 0.72 | 38.5 | 1.07 ± 0.13 | F<sub>F2</sub> + C<sub>2</sub> ⇔ F<sub>F2</sub>C<sub>2</sub> |
| FKBP38 vs. CaM first binding site | + | 2.2 ± 0.22 | 10.3 ± 0.34 | 3.18 ± 0.79 | 42.1 | 1.16 ± 0.17 | F<sub>F1</sub> + C<sub>1</sub> ⇔ F<sub>F1</sub>C<sub>1</sub> |
| FKBP38 vs. CaM second binding site | + | 4.9 ± 0.97 | –24.9 ± 0.97 | –29.8 ± 1.02 | –5.0 | 0.95 ± 0.25 | F<sub>F2</sub> + C<sub>2</sub> ⇔ F<sub>F2</sub>C<sub>2</sub> |

Isothermal titration calorimetry (ITC) measurements with FKBP38<sup>35–153</sup> and CaM<sup>1–75</sup> yielded nearly identical results in both the presence and the absence of Ca\textsuperscript{2+}. In both cases, the data revealed 1:1 stoichiometries with \(K_D\) values of 6.9 ± 0.92 and 7.0 ± 0.55 \(\mu M\), respectively, according to a one-binding-site model (Table 1 and supplemental Fig. S4).

**FKBP38<sup>290–313</sup> Binds to the C-terminal Domain of CaM**—Given the interaction between the catalytic FKBP38 domain and the N-terminal CaM domain, we were interested in identifying the part of CaM that binds to the Ca\textsuperscript{2+}–dependent binding site of FKBP38. Therefore, we analyzed the influence of different CaM variants on the interaction between FKBP38<sup>35–153</sup> and CaM-Sepharose, because FKBP38 binds in a Ca\textsuperscript{2+}–dependent manner to CaM-Sepharose. Fig. 4A shows that in addition to full-length CaM also CaM<sup>76–148</sup> competed with CaM-Sepharose for FKBP38<sup>35–153</sup> binding, whereas the presence of CaM<sup>1–75</sup> did not significantly reduce the amount of matrix-bound FKBP38<sup>35–153</sup>. Hence, the C-terminal CaM domain interacts with FKBP38<sup>290–313</sup>.

Furthermore, PPIase activity measurements were performed to test whether CaM<sup>76–148</sup> interferes with the activation of FKBP38 by CaM–Ca\textsuperscript{2+}. Indeed, CaM<sup>76–148</sup> lowered the activity of the FKBP38<sup>35–153</sup>/CaM-Ca\textsuperscript{2+} complex, thus demonstrating a competition with full-length CaM for binding to FKBP38<sup>35–153</sup> (Fig. 4B). Thereby, CaM<sup>76–148</sup>/Ca\textsuperscript{2+} bound to FKBP38<sup>35–153</sup> with a \(K_D\) value of 2.01 ± 0.71 \(\mu M\). Both CaM domains were subsequently used in ITC experiments to determine binding to FKBP38<sup>290–313</sup>. Only CaM<sup>76–148</sup> interacted with the peptide that corresponds to FKBP38<sup>290–313</sup>, revealing a 1:1 complex with a \(K_D\) value of 2.2 ± 0.24 \(\mu M\) according to a one-binding-site model (Table 1 and supplemental Fig. S5).

The interaction between FKBP38<sup>290–313</sup> and CaM<sup>76–148</sup> on the one hand and the interaction between the catalytic FKBP38 domain and the N-terminal CaM domain on the other hand were also detected when ITC measurements were performed with FKBP38<sup>35–153</sup> and CaM in the presence of Ca\textsuperscript{2+} (supplemental Fig. S6). These measurements revealed two binding sites, each with a 1:1 molar ratio between both binding partners according to a two-binding-site model (Table 1). Thereby, one binding site interacted with a \(K_D\) of 2.2 ± 0.22 \(\mu M\), whereas the other binding site showed a \(K_D\) value of 4.9 ± 0.97 \(\mu M\). Both binding constants are similar to those derived from measurements with the single domains, although the interaction between the isolated N-terminal domains shows slightly lower affinity than the second binding site of the full-length proteins (Table 1).

**TABLE 1: Parameters for FKBP38 and CaM-Ca\textsuperscript{2+} Interaction**

Parameters for this interaction between FKBP38 and CaM-Ca\textsuperscript{2+} at two different binding sites, measured in 10 mM MES (pH 6.8), 100 mM KCl at 20 °C in the presence of either 1 mM EGTA or 2 mM CaCl\textsubscript{2}. In each respective binding model, F1 and F2 represent the N-terminal and C-terminal binding sites of FKBP38, respectively, whereas C1 and C2 stand for the corresponding CaM-binding sites.
Finally, NMR spectra were collected to locate the site where FKBP38<sup>290–313</sup> binds to CaM. <sup>1H</sup>/<sup>15N</sup>-HSQC spectra of <sup>15N</sup>-labeled CaM-Ca<sup>2+</sup>/H<sub>11001</sub> (Fig. 4C), collected both in absence and presence of FKBP38<sup>290–313</sup>, revealed significant chemical shift perturbations for several residues in the C-terminal CaM domain, comprising segments Val<sup>91</sup>–Phe<sup>92</sup>, Glu<sup>104</sup>–Val<sup>108</sup>,...
FKBP38 domain that comprises residues Ser290–Asn313 is a novel scenario of CaM target activation. This interaction is mediated by two distinct interaction sites in both proteins, revealing a novel scenario of CaM target activation.

One interaction involves a short sequence in the C-terminal FKBP38 domain that comprises residues Ser290–Asn313 and a CaM-loaded Ca2+-independent affinity matrix and (ii) inhibit PPase activity of the FKBP38-Ca2+-CaM-Ca2+ complex. Heteronuclear two-dimensional [1H, 13C]-HSQC experiments showed an interaction between the FKBP38290–313 peptide and the target protein-binding cleft in the C-terminal domain of CaM-Ca2+, suggesting binding site recognition of FKBP38290–313 by CaM76–148 that is comparable to the previously described CaM complexes (15, 24). Hence, the interaction between the C-terminal domains of FKBP38 and CaM-Ca2+ is in good agreement with previous observations of the Ca2+-dependent interaction between FKBP38 and CaM (1).

Moreover, our study discovered a second interaction in the FKBP38/CaM-Ca2+ complex that occurs between the N-terminal domains of both proteins. PPase measurements revealed enzymatic activity of the catalytic FKBP38 domain in the presence of either Ca2+-free CaM or CaM-Ca2+. Furthermore, co-immunoprecipitation experiments using CaM-depleted lysate from SH-SYSY cells confirmed that interactions between FKBP38 and Bcl-2 are induced only in the presence of either full-length CaM or CaM1–75, whereas CaM76–148 did not cause Bcl-2/FKBP38 interactions. These results clearly demonstrate that only CaM1–75 is responsible for the activation of FKBP38, independent of Ca2+. This interaction between the catalytic FKBP38 domain and the N-terminal CaM domain is therefore crucial for the participation of FKBP38 in the regulation of programmed cell death via its interaction with Bcl-2. These results suggest a novel scenario where the two CaM domains bind to separate interaction sites in FKBP38, one of which requires Ca2+, whereas the other does not.

Many CaM-regulated proteins, such as myosin light chain kinases or CaM-dependent kinases, interact with CaM only in the presence of Ca2+ (25), whereas Ca2+-free CaM has only a low affinity for these particular target proteins (13). In the CaM-Ca2+ complex of the edema factor from B. anthracis, only the C-terminal CaM domain coordinates Ca2+, whereas no calcium ions are found in the N-terminal CaM domain (22, 23). Furthermore, CaM binds with its Ca2+-free N-terminal domain to the Ca2+-ATPase in the membrane of erythrocytes (26). Finally, several CaM-binding partners are regulated by CaM in a Ca2+-independent fashion (18). For instance, the cGMP-dependent protein kinase is regulated by CaM, but the presence or absence of Ca2+ does not influence the regulation of this enzyme (27). Such a Ca2+-independent regulation by CaM was also identified for the adenylyl cyclase from Bordetella pertussis (28). The interaction of FKBP38 and CaM-Ca2+ therefore shares certain properties previously described for other CaM-binding partners but features a novel combination of both a Ca2+-dependent and a Ca2+-independent interaction to regulate enzymatic activity.

The interaction between FKBP3835–153 and CaM was also detected by protein fluorescence. Moreover, ITC measurements revealed a 1:1 complex for the interaction between the catalytic FKBP38 domain and the N-terminal CaM domain, with a KD value of 7.0 ± 0.55 µM. The binding between the C-terminal interaction sites of both proteins, however, increases the affinity significantly to a KD of 1.4 µM (1). These results apparently favor a 1:1 stoichiometry between FKBP38 and CaM-Ca2+, because FKBP38 is activated in cells in a Ca2+-dependent manner with higher affinity than the binding constants of the single interaction sites (Fig. 5). However, a 1:2 stoichiometry could theoretically also be possible, if allosteric effects induced by the C-terminal interaction assist the interaction of the N-terminal binding sites or other as yet unknown factors facilitate the binding of two CaM molecules to FKBP38.

The interaction between FKBP38290–313 and CaM76–148 is entropically driven, as also found for other CaM-Ca2+-binding sites, because of the dehydration of the binding interface caused by the dominant role of hydrophobic interactions (29). The higher affinity of the C-terminal interaction is essential for the regulation of FKBP38 under physiological conditions, because
the availability of free CaM-Ca\(^{2+}\) limits the activation of target proteins in the cell (30, 31). Considering the high cellular abundance of CaM, reaching 0.5% of the total protein content in the brain (32), this appears surprising, but the cellular concentration of free CaM-Ca\(^{2+}\) does not exceed 1 pm (13). Thus, the CaM-Ca\(^{2+}\) concentration is far below the concentration of cellular CaM-Ca\(^{2+}\) target proteins, implying a coupling of CaM-dependent enzyme activities by the competition for the limiting CaM-Ca\(^{2+}\) species (33, 34). This competition with other CaM target proteins apparently results in a Ca\(^{2+}\)-dependent interaction between FKBP38 and CaM, as indicated by the increased affinity of the full-length proteins.

Interestingly, in neuronal cells large amounts of CaM are sequestered to the endoplasmic reticulum and mitochondria by proteins such as neuromodulin and neurogranin, which exhibit a higher CaM affinity in the absence of Ca\(^{2+}\), thus forming a CaM reservoir (19, 20). This CaM pool is released in the presence of Ca\(^{2+}\), resulting in particularly high concentrations of free CaM-Ca\(^{2+}\) at the endoplasmic reticulum and mitochondria membranes of neuronal cells, where FKBP38 is located in the cell (1).

Therefore we propose that a cellular increase in the Ca\(^{2+}\) concentration initiates the formation of a complex between the C-terminal domains of FKBP38 and CaM, subsequently leading to an activation of FKBP38 as a result of the interaction between the N-terminal CaM domain and the catalytic FKBP38 domain. Hence, a high cytosolic Ca\(^{2+}\) concentration is presumably favorable for the activation of FKBP38 by CaM and thus also for the regulation of Bcl-2 by FKBP38/CaM-Ca\(^{2+}\) in neuronal apoptosis.

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