FKBP38 is a negative effector of the anti-apoptotic Bcl-2 protein in neuroblastoma cells. The interaction with Bcl-2 and the enzyme activity of FKBP38 depend on prior binding of calmodulin-Ca"{2+} (CaM-Ca"{2+}) at high Ca"{2+} concentrations. The FKBP38 protein structure contains three tetratricopeptide repeat (TPR) motifs corresponding to the Hsp90 interaction sites of other immunophilins. In this study we show that the TPR domain of FKBP38 interacts with the C-terminal domain of Hsp90, but only if the FKBP38-CaM-Ca"{2+} complex is preformed. Hence, FKBP38 is the first example of a TPR-containing immunophilin that interacts cofactor-dependently with Hsp90. In the ternary Hsp90-FKBP38-CaM-Ca"{2+} complex the active site of FKBP38 is blocked, thus preventing interactions with Bcl-2. The dual control of the active site cleft of FKBP38 by CaM-Ca"{2+} and Hsp90 highlights the importance of the enzyme activity of the FKBP38-CaM-Ca"{2+} complex in the regulation of programmed cell death.

The human FKBP38 (FK506-binding protein 38), the product of the FKBP8 gene, belongs to the enzyme class of peptidyl-prolyl cis/trans isomerases (PPIases, EC 5.2.1.8) that assist protein folding by catalyzing the slow interconversion of cis and trans isomers of the peptide bond preceding proline in polypeptide chains. This isomerization reaction is involved in both de novo protein folding and regulation of biological activity of native proteins. The accelerated isomerization catalyzed by PPIases is essential in signal transduction pathways (1, 2).

FKBP38 was found to play an important role in the apoptosis regulation of neuroblastoma cells by inhibiting interactions of Bcl-2 with its cellular targets, such as calcineurin and Bad (3, 4). The negative regulation of Bcl-2 by FKBP38 depends on functional integrity of the FKBP domain, which is controlled by the cellular Ca"{2+} concentration and the interaction with the Ca"{2+} sensor CaM, characterizing FKBP38 as the first cofactor-regulated PPIase (3). Potent neuroprotective and neuroregenerative effects of specific low molecular weight FKBP38 inhibitors and FKBP38 short interfering RNA constructs in neuroblastoma cells outline the importance of FKBP38 activity in the control of neuronal apoptosis. Furthermore, the administration of N-(N',N"{'-dimethylcarboxamidomethyl)-cycloheximide, a low molecular weight FKBP38 inhibitor, to an endothelin-1-induced middle cerebral artery occlusion stroke model in the rat brain demonstrated the potency of FKBP38 inhibition for neuroregeneration and neuroprotection in rat brain tissue (5). The crucial role of FKBP38 in the control of neuronal apoptosis is indicated as well by a pronounced FKBP38 expression in neuronal cells (5, 6). Additionally, FKBP38 is also involved in the apoptosis control of other cell types, as recently shown by the anti-apoptotic effect resulting from the interaction of FKBP38 and the NSSA protein of the hepatitis C virus in hepatoma cells (7, 8). The FKBP38 ortholog in mice was described to be involved in the regulation of neuronal development by affecting sonic hedgehog signaling (9). In addition, it displayed specific antitumor effects by the regulation of anti-invasive syn-1 gene expression and suppression of the proinvasive MMP9 gene (10). Human FKBP38 was also found to be involved in cell size regulation (11).

FKBP38 contains the second messenger-regulated PPlase domain in its N-terminal region and a C-terminal tetratricopeptide repeat (TPR) domain, harboring three TPR motifs, a putative CaM-binding site, and a membrane anchor. Human FKBP5, with similar domain composition and significant sequence homology to FKBP38 were shown to play a role in homologous chromosome pairing during male meiosis (12), are involved in steroid hormone receptor signaling (13–15), and regulate cellular dioxin response (16). The XAP2 protein (X protein-associated protein2, FKBP37.7), for instance, which contains, analogous to FKBP38, an N-terminal FKBP domain and a C-terminal TPR domain with three TPR motifs, is a component of the aryl hydrocarbon receptor complex and thus is involved in cellular detection of dioxins and subsequent cellular response on xenobiotics. Within the receptor complex, XAP2 interacts not only with the aryl hydrocarbon receptor but also via its TPR domain with the 12-kDa C-terminal (C90) domain of Hsp90 (16, 17).

Similar interactions between Hsp90 and the TPR-containing PPIases FKBP51 and FKBP52 were identified in steroid hormone receptor complexes (13, 18). The interaction of TPR-containing proteins to Hsp90 is mutually exclusive (18, 19) and is conferred by a binding moiety that is formed by the dimerized C90 domains (14). The TPR domain of FKBP38 shares significant sequence homology with the corresponding domains in FKBP51, FKBP52, and XAP2.
Hsp90 Inhibits FKBP38-CaM-Ca$$^{2+}$$

The heat shock protein Hsp90, which exists in two isoforms (Hsp90α and Hsp90β) with about 86% identity in their amino acid sequences, occurs largely in homodimers, but heterodimers and monomers were reported as well (20–22). The C90 domain mediates the anti-parallel dimerization of Hsp90 monomers with a dissociation constant in the low nanomolar range (23, 24). Like FKBP38, Hsp90 binds CaM in a Ca$$^{2+}$$-dependent manner (25, 26). The CaM-binding site in Hsp90 is a short peptide in the C-terminal part of the protein, which likely adopts an α-helix (26), and therefore shares features common to CaM-binding sites (27). CaM binding is involved in the regulation of calcineurin, nitric-oxide synthase (NOS), CaM-dependent kinases, inositol 1,4,5-trisphosphate receptors, plasma membrane Ca$$^{2+}$$ pumps, Ras guanine nucleotide exchange factors, and rhodopsin protein phosphatase (28). In case of the different forms of NOS, for instance, Hsp90 was reported to facilitate CaM binding to NOS and to activate NOS further by direct binding (29–32). Hence, Hsp90 is thought to play a role in the CaM-mediated control of a plethora of biological processes, such as muscle contraction, fertilization, cell proliferation, vesicular fusion, and apoptosis, where CaM transfers highly regulated changes in the cellular concentration of the second messenger Ca$$^{2+}$$ to target proteins and subsequently regulates their activities.

In this study, we demonstrate that the CaM-dependent PPlase FKBP38 interacts with Hsp90 via binding of the TPR domain to the C90 domain of Hsp90. This interaction, however, depends in contrast to other interactions of Hsp90 with TPR-containing proteins on the previous formation of the FKBP38-CaM-Ca$$^{2+}$$ complex. The resulting ternary complex is enzymatically inactive, and the association of Bcl-2 and the PPlase site of FKBP38 is prevented.

MATERIALS AND METHODS

Sources of enzymes used in the experiments are as follows: recombinant human FKBP38, lacking the membrane anchor (FKBP38-(1–336)), recombinant human FKBP12, recombinant human Hsp90 C90 (Hsp90-(590–730)), recombinant human calmodulin, and human Bcl-2 were expressed by using a pET28a-vector in Escherichia coli RosettaTM cells. Human Hsp90 protein was purchased from StressGen. MBP-Bcl-2 fusion protein was purchased from Sigma. The FKBP38 antibody was an affinity-purified section 4 polyclonal antibody from rabbit against the purified FKBP38 domain (amino acids 1–165). Additional antibodies used are as follows: polyclonal rabbit anti-actin (Sigma), monoclonal hamster anti-Bcl-2 (Pharmingen), and monoclonal rat anti-Hsp90 (StressGen).

Peptide substrates used were obtained from Bachem (Heidelberg, Germany). FK506 was purchased from Calbiochem. Low molecular weight substances were synthesized according to standard procedures (33) and characterized by electrospray ionization-mass spectrometry.

Measurement of PPlase Activity

PPlase activity was determined using protease-coupled assays as described elsewhere (34, 35). Typically, FKBP38 PPlase activity was measured in a reaction mixture containing 1 μM FKBP38, 5 μM CaM, and 1 mM CaCl$$_2$$. FKBP38 was tested using the oligopeptide succinyl-AFPF-4-nitroanilide as substrate. In the case of Hsp90 and CaM insensitivity toward proteolytic digestion by α-chymotrypsin in the time range of the kinetic experiments was verified. The results are the means of three replicates.

Protein-Protein Interaction

Co-immunoprecipitation—Cells were grown in flasks for 16 h and harvested. Cell lysis and co-immunoprecipitation experiments were performed according to the manufacturer’s protocols of the immunoprecipitation starter kit (GE Healthcare). Prior to incubation, 0.5 μM EGTA was added to the samples. 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), either in the presence of 2 mM CaCl$$_2$$ or 2 mM EGTA. Subsequently, 20 μg of recombinant FKBP38 in the presence and absence of 50 μg of Hsp90 was incubated with CaM-Sepharose. Sepharose was washed, and bound proteins were analyzed by 12.5% SDS-PAGE and Coomassie Blue staining.

Bcl-2 Binding Assay—40 μl of 6 μM MBP-Bcl-2 fusion protein (MBP-Bcl-2) was subjected to 40 μl of amyllose-resin (New England Biolabs, Beverly, MA) and incubated for 30 min. Subsequently, beads were washed twice with buffer, 25 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM dithiothreitol, and then incubated for 1 h with 40 μl of reaction mixture containing 10 μM FKBP38 and 40 μM CaM in the presence of different concentrations of Hsp90 C90 protein.

CD Spectroscopy—Near-UV CD spectra were recorded at 25 °C on a Jasco J-710 circular dichroism spectrometer in 10 mM HEPES, pH 7.8, 2 mM CaCl$$_2$$ containing 1 μM FKBP38 and 5 μM CaM either separated or mixed in a 10-mm tandem cuvette. Interaction of 1 μM FKBP38 and 1 μM Hsp90 was measured in the presence of 1 mM CaCl$$_2$$ and 5 μM CaM in a tandem cuvette as well.

Fluorescence Methods—1 μM FKBP38, 1 μM FKBP12, 1 μM FKBP52, 1 μM Hsp90, and 2 μM CaM were used in 10 mM HEPES, pH 7.5, 2 mM CaCl$$_2$$ for fluorescence measurements and the fluorometric titration of FKBP38. The concentration of the protein was measured by UV-visible spectroscopy. The experiments were performed after equilibrating at 25 °C for 10 min. The samples were excited at 278 nm, and the fluorescence was scanned from 285 to 500 nm for fluorescence measurements. The fluorescence titration was performed with an excitation at 278 nm and monitored at 325 nm. Fluorescence measurements, using 1-ml quartz cuvettes with a 0.5-cm path length, were performed on a Jobin Yvon Instruments Fluoromax 2. The results are means of three replicates.

The binding constant (EC$$\text{50}$$) was calculated from the fluorescence intensity by Sigma blot software using sigmoidal dose-response ligand binding fit with Equation 1,

$$F = F_0 + \frac{F_{\text{max}} - F_0}{1 + 10^{\Delta EC_50} \text{replicant}}$$

where F is fluorescence intensity; F$$_0$$ = initial fluorescence intensity.
RESULTS

FKBP38 Interacts with Hsp90 in the Presence of CaM-Ca²⁺—TPR-containing FKBP types have been described previously to form complexes with Hsp90; hence, we were interested in the identification of possible interactions between FKBP38 and the heat shock protein. First, co-IP experiments were performed using the endogenous proteins of SH-SY5Y cells, showing, indeed, an interaction between FKBP38 and Hsp90 (Fig. 1A). However, the results point to a Ca²⁺-dependence of this interaction, because significant amounts of FKBP38 were only found to bind Hsp90 in the presence of Ca²⁺ in samples immunoprecipitated with either anti-Hsp90 or anti-FKBP38 antibodies. The requirement of calcium ions for the interaction of FKBP38 and Hsp90 in SH-SY5Y crude cell lysate indicates a possible involvement of the Ca²⁺ sensor CaM in this interaction, because free FKBP38 and the CaM-Ca²⁺-complexed form of the PPIase differ in their biological activity. To investigate whether both forms of FKBP38 also differ in their binding to Hsp90, co-IP experiments using recombinantly expressed FKBP38 and Hsp90 were performed (Fig. 1B). In fact, FKBP38 was found to interact with Hsp90 only in the presence of the CaM-Ca²⁺ complex, whereas calcium ions alone did not suffice to induce interactions between FKBP38 and Hsp90. These data demonstrate that only the FKBP38-CaM-Ca²⁺ complex interacted with Hsp90.

Furthermore, it was analyzed whether FKBP38 is a client substrate for Hsp90 chaperone activity using ATP and geldanamycin in the co-IP experiments. However, neither the addition of 5 μM ATP nor the presence of 50 μM geldanamycin interfered with the interaction of FKBP38 and Hsp90, excluding the possibility that the interaction between FKBP38-CaM-Ca²⁺ and Hsp90 involves the protein folding activity of the chaperone (Fig. 1B). An interaction between Hsp90 and the active site of the FKBP38-CaM-Ca²⁺ complex was also tested using the low molecular weight inhibitor FK506. The presence of 5 μM FK506 did not interfere with the formation of the Hsp90-FKBP38-CaM-Ca²⁺ complex, demonstrating that Hsp90 does not interact with the active site of FKBP38 (Fig. 1B).

Then native gel electrophoresis was performed to analyze the migration pattern of FKBP38 and Hsp90 in the presence and absence of each other and CaM using recombinant FKBP38 and purified human Hsp90. The protein migration was analyzed by Western blot using antibodies against Hsp90 and FKBP38, respectively (Fig. 1C). The experiment revealed only a shift in migration of FKBP38 protein in the presence of Hsp90 and CaM-Ca²⁺, whereas no shift in the FKBP38 migration occurred in the presence of Hsp90 and absence of CaM-Ca²⁺ (Fig. 1C, right panel). The same pattern was found for Hsp90 (Fig. 1C, left panel), where the two bands of Hsp90, likely representing monomeric and dimeric form, shifted to a band that contains FKBP38 protein as well. Interestingly, the data shows that the monomer/dimer ratio of Hsp90 is changed toward the dimer, suggesting an influence of FKBP38-CaM-Ca²⁺ on the dimerization of Hsp90. The results point to Hsp90 interactions only with the enzymatically active FKBP38-CaM-Ca²⁺ complex.

Hsp90 Interacts with the FKBP38-CaM-Ca²⁺ Complex

FIGURE 1. Hsp90 only interacts with the FKBP38-CaM-Ca²⁺ complex. A, co-IP of FKBP38 and Hsp90 was performed using total SH-SY5Y cell lysate. The cell lysate (20 μg) was incubated with anti-Hsp90 (upper panel) and anti-FKBP38 (lower panel) antibodies, respectively, before being incubated on protein G-Sepharose either with 2 mM EGTA or 2 mM Ca²⁺. Subsequently, samples were analyzed by Western blot using antibodies against FKBP38 (upper panel) and Hsp90 (lower panel), respectively. Protein G-Sepharose in the presence of 2 mM Ca²⁺ served as control. The applied endogenous protein from SH-SY5Y cell lysate is shown on the right (input). B, co-IP of FKBP38 and Hsp90 was performed with isolated proteins. Hsp90 was applied to anti-Hsp90 antibodies immobilized on protein G-Sepharose. Subsequently samples were incubated with FKBP38 (5 μM) in the presence of 2 mM CaCl₂ and CaM (10 μM). The effect of low molecular weight ligands was tested using final concentrations of 5 μM FK506, 50 nM geldanamycin, and 5 μM ATP. After incubation, the samples were analyzed by Western blot using antibodies against FKBP38 and Hsp90. C, native gel electrophoresis of recombinantly expressed FKBP38 and Hsp90 in the presence and absence of CaM and 2 mM Ca²⁺. Proteins were incubated for 1 h at 4 °C, and −2 μg were subjected to native PAGE. Subsequently, the protein migration was analyzed by Western blot using antibodies against Hsp90 (left panel) and FKBP38 (right panel).

intensity; \( F_{\text{max}} \) is fluorescence intensity at saturation; \( d \) is Hill slope; \( IC_{50} \) = total Hsp90 concentration at each addition; and \( EC_{50} \) = dissociation constant.
The TPR Domain of FKBP38 Interacts with the C90 Domain of Hsp90—Except for FKBP38, human TPR-containing FKBP proteins interact with Hsp90 by recruiting its C90 domain. A cofactor, such as CaM-Ca^2+/, is not required for this interaction. In contrast, our results show that the interaction of Hsp90 and FKBP38 occurs only in the presence of CaM-Ca^2+/, introducing the possibility that CaM links FKBP38 and Hsp90, because both proteins interact with CaM-Ca^2+/ individually. Therefore, we addressed the question whether FKBP38 and Hsp90 interact directly with one another in the ternary complex.

Initially, we investigated whether Hsp90 and FKBP38 compete for CaM-Ca^2+ binding by applying recombinant FKBP38 to CaM-Sepharose in the presence and absence of recombinant Hsp90 (Fig. 2A). The addition of Hsp90 did not interfere with the formation of the FKBP38-CaM-Ca^2+ complex, indicating that these two proteins do not compete for the same interaction site of CaM-Ca^2+.

Next, we performed fluorescence spectroscopy measurements, assay the CaM-Ca^2+ complexes of recombinant FKBP38 and Hsp90 to identify spectral changes caused by direct interaction between FKBP38 and Hsp90 upon complex formation (Fig. 2B). The fluorescence spectrum of the Hsp90-FKBP38-CaM-Ca^2+ complex showed a maximum at 330 nm. The spectrum of the FKBP38-CaM-Ca^2+ complex, however, had its maximum at 321 nm in the absence of Hsp90,
indicating that the four tryptophan residues in FKBP38 were not solvent-accessible, whereas the spectrum of Hsp90 pointed to positions for the tryptophan side chains on the protein surface as indicated by the maximum at 338 nm. The composite spectrum of the individual spectra of the FKBP38-CaM-Ca\(^{2+}\) complex and Hsp90 showed a maximum at 324 nm. Therefore, the fluorescence of the three proteins is red-shifted by 6 nm upon formation of the ternary complex. In addition, the signal amplitude was reduced by 23%. It is noteworthy that the interactions of both proteins with CaM-Ca\(^{2+}\) induces change in the protein fluorescence of FKBP38 and Hsp90 itself, whereby the maxima in the spectra experienced a blue shift of 2 and 3 nm, respectively, and the fluorescence signal of both complexes was increased by about 10% compared with the composite spectra of the individual proteins (supplemental Figs. 3 and 4). In contrast to FKBP38, no major differences between the fluorescence spectrum of FKBP12 in the presence of Hsp90-CaM-Ca\(^{2+}\) and the combination of spectra of the separated proteins were observed (supplemental Fig. 1). The maximum of the spectrum of FKBP52 in complex with Hsp90, however, is reduced by 30% and red-shifted by 9 nm to longer wavelengths (supplemental Fig. 2). The Hsp90 concentration-dependent change in the fluorescence signal of FKBP38 and Hsp90 indicating the formation of the Hsp90-FKBP38-CaM-Ca\(^{2+}\) complex was monitored at 325 nm (Fig. 2C). The resulting curve indicates that the observed spectral shift depends on the applied amount of Hsp90 protein. The data indicate an EC\(_{50}\) value of about 140 nM for interaction between FKBP38-CaM-Ca\(^{2+}\) and Hsp90.

Previously we observed that the interaction of FKBP38 and the CaM-Ca\(^{2+}\) complex caused shifts in the near-UV CD spectrum, thus pointing to environmental changes of aromatic side chains in the FKBP38 protein, likely caused by alterations in the tertiary structure of FKBP38 (3). Based on these findings, we were interested in analyzing whether the formation of Hsp90-FKBP38-CaM-Ca\(^{2+}\) alters the near-UV CD spectrum of the proteins and perhaps reverses the effect of CaM binding to FKBP38. When comparing the near-UV CD spectra of FKBP38 and CaM measured in tandem silica cells in the presence of 2 mM CaCl\(_2\) either separated or mixed significant spectral changes occur in the range between 268 and 280 nm (supplemental Fig. 5). Thereby the spectral contributions of FKBP38 dominate the composite spectrum in the indicated range of wavelength, which makes it possible to correlate differences between both spectra with spatial re-positioning in the aromatic side chains of FKBP38.

Fortunately, the near-UV CD spectrum of Hsp90 also exhibits amplitudes that are 10-fold smaller compared with the spectrum of FKBP38 at a molar ratio of 1:1, again making a correlation between spectral shifts and positional changes of side chains of aromatic FKBP38 residues possible (Fig. 2D). The comparison of the near-UV CD spectrum of Hsp90-FKBP38-CaM-Ca\(^{2+}\) and the combined spectrum of the individual proteins in the presence of CaM-Ca\(^{2+}\), however, reveals no major differences between the spectra, indicating the absence of spatial re-positioning in the aromatic side chains of FKBP38 when the Hsp90-FKBP38-CaM-Ca\(^{2+}\) complex is formed (Fig. 2F).

To determine whether the C90 domain of Hsp90 is sufficient to interact with FKBP38-CaM-Ca\(^{2+}\), a binding assay was performed using matrix-bound recombinant C90-GST fusion protein. FKBP38 interacted with the C90 domain of Hsp90 in a CaM-Ca\(^{2+}\)-dependent fashion that was not affected by FK506 (Fig. 2E), but CaM-Ca\(^{2+}\) alone did not interact with the matrix-bound C90-GST protein (data not shown). These data exclude the possibility of FKBP38 and Hsp90 being linked by CaM-Ca\(^{2+}\) and imply direct interactions between FKBP38-CaM-Ca\(^{2+}\) and the C90 domain of Hsp90 in the ternary complex. Furthermore, the addition of soluble C90 protein reduced the portion of matrix-bound FKBP38, likely competing with C90-GST at the glutathione-Sepharose. The FKBP38D variant containing the amino acids that assemble the FKBP domain of FKBP38 showed no interaction with C90-GST protein bound to glutathione-Sepharose, suggesting that the C-terminal TPR domain of FKBP38 is required for interactions with Hsp90. Taken together, the formation of the Hsp90-FKBP38-CaM-Ca\(^{2+}\) complex is mediated by the C90 domain of Hsp90 and the TPR domain of FKBP38, including direct interactions between both proteins.

**Hsp90 Binding Controls the Active Site of FKBP38**—To examine functional consequences associated with the formation of the Hsp90-FKBP38-CaM-Ca\(^{2+}\) complex, the PPlase activity of the FKBP38-CaM-Ca\(^{2+}\) complex was assayed in the presence of Hsp90. Fig. 3A shows the decrease of the CaM-Ca\(^{2+}\)-regulated PPlase activity of FKBP38 at increasing Hsp90 concentrations. As shown in co-IP experiments, the active site-blocking ligand FK506 did not interfere with the formation of the ternary complex. The results point to a role for Hsp90 as a negative regulator of the biological activity of FKBP38 because Hsp90 fully inhibits the PPlase activity of the FKBP38-CaM-Ca\(^{2+}\) complex. Interestingly, the C90 domain did not suffice to inhibit the catalytic activity of FKBP38-CaM-Ca\(^{2+}\) (Fig. 3A).

To analyze the effect of Hsp90-mediated FKBP38-CaM-Ca\(^{2+}\) inhibition on the interaction of the active site of FKBP38 with Bcl-2, we performed native gel analysis with recombinant proteins analyzed by Western blot using anti-Bcl-2 antibodies (Fig. 3B). The Bcl-2 protein migrates in two bands that likely represent Bcl-2 monomers and dimers. FKBP38 only interacts with Bcl-2 in the presence of CaM-Ca\(^{2+}\), as described previously (3). The formation of Bcl-2-FKBP38-CaM-Ca\(^{2+}\) complexes changes the migration of Bcl-2 in the native gel. In the presence of Hsp90, however, the Bcl-2 migration is shifted back to a pattern observed for Bcl-2 in the absence of FKBP38. These results show that Hsp90 prevents interactions of FKBP38 and Bcl-2 and thus interferes with the formation of the Bcl-2-FKBP38-CaM-Ca\(^{2+}\) complex.

To test the effect of only the C90 domain on FKBP38 interactions with Bcl-2, we performed a Bcl-2 binding assay using recombinant FKBP38-CaM-Ca\(^{2+}\) applied to MBP-Bcl-2-bound maltose-Sepharose in the presence of different concentrations of recombinant Hsp90 C90 domain protein (Fig. 3C). FKBP38-CaM-Ca\(^{2+}\) bound to the Sepharose when MBP-Bcl-2 was present, whereas the C90 protein reduced the amount of Bcl-2-bound FKBP38 protein in a concentration-dependent manner. Thus, the interaction between FKBP38-CaM-Ca\(^{2+}\) and the protein substrate Bcl-2 is inhibited by the C90 domain. Hsp90 not only inhibits the enzymatic activity of FKBP38, it also prevents the interaction of FKBP38-CaM-Ca\(^{2+}\) with Bcl-2,
suggesting a role for Hsp90 as the negative FKBP38 regulator in the control of neuronal apoptosis.

To analyze further the influence of the Hsp90-FKBP38-CaM-Ca\(^{2+}\)/H11001 complex formation on the accessibility of the active site cleft of FKBP38, we performed a competition experiment using FKBP12 and FKBP38 as described previously (3). The PPIase activity of FKBP12 is inhibited by FK506 and because of competition with the active site of FKBP38 recovered in the presence of FKBP38-CaM-Ca\(^{2+}\) (Fig. 3D). The addition of Hsp90 reduced the measured FKBP12 activity, pointing to a diminished accessibility of the FKBP38 active site for FK506 in the Hsp90-FKBP38-CaM-Ca\(^{2+}\) complex. According to the direct PPIase measurements (Fig. 3A), the C90 domain of Hsp90 caused no reduction in the FK506 competition between the active sites of FKBP38 and FKBP12 (Fig. 3D). Therefore, full-length Hsp90 conceals the active site of the FKBP38-CaM-Ca\(^{2+}\) complex and thus prevents interactions with its substrates and inhibitors.

**DISCUSSION**

The CaM-Ca\(^{2+}\)-dependent FKBP38 interacts with Hsp90, as shown by immunoprecipitation experiments, native gels, and fluorescence spectroscopy. Thereby the TPR domain of FKBP38 mediates the interaction to Hsp90 (Fig. 2E). Similar interactions to Hsp90 have been shown between the TPR domains of other human immunophilins, such as FKBP51, FKBP52, Cyp40, and XAP2 (13, 17, 18, 23, 44). The TPR domain of FKBP38 therefore shares the property of Hsp90 binding with the TPR domains of other PPIases.

However, the interaction between the TPR domain of FKBP38 and Hsp90 differs from previously described interactions between immunophilins and Hsp90 in a strict dependence on the presence of CaM-Ca\(^{2+}\). Our results demonstrate that FKBP38 interacts only with Hsp90, when the catalytically active FKBP38-CaM-Ca\(^{2+}\) complex is preformed (Fig. 1). These data imply that the formation of the FKBP38-CaM-Ca\(^{2+}\) complex increases the affinity of the TPR domain for Hsp90 leading to a ternary Hsp90-FKBP38-CaM-Ca\(^{2+}\) complex. Thus, the TPR domain of FKBP38 undergoes structural changes upon formation of the FKBP38-CaM-Ca\(^{2+}\) complex. The assembly of Hsp90-FKBP38-CaM-Ca\(^{2+}\) via CaM-Ca\(^{2+}\) can be excluded, because the C90 domain of Hsp90 that cannot interact individually with CaM-Ca\(^{2+}\) binds also FKBP38-CaM-Ca\(^{2+}\) exclusively (Fig. 2E). To our knowledge, FKBP38 is the...
first example of a TPR-containing protein that requires a cofactor-dependent activation for interaction with Hsp90.

Besides Hsp90 interaction, FKBP38 has more characteristic properties that differ greatly between the free form of the protein and its complex with CaM-Ca²⁺, such as PPlase activity, binding of active site-directed ligands, and interaction with Bcl-2 (3). Thus, FKBP38 undergoes changes in its tertiary structure upon CaM-Ca²⁺ binding, which may involve restructuring events in the TPR domain of FKBP38 stabilizing a conformation that contributes to the assembly of the Hsp90-FKBP38-CaM-Ca²⁺ complex. Interestingly, the TPR domain of the Hsp90 interaction partner Cyp40 has been shown to undergo conformational dynamics in the free form of the protein (45). In contrast to FKBP38, Cyp40 does not require binding to CaM-Ca²⁺ in order to interact with Hsp90. Furthermore, Hsp90 binding was shown to induce re-folding events in the TPR domain of protein phosphatase 5 (PP5), and this TPR domain exhibits a conformational diversity even in complex with Hsp90 (46, 47). The TPR domain of FKBP38, however, requires interactions with CaM-Ca²⁺ to adopt a fold that exhibits an interaction site for Hsp90. The conformational re-arrangements of the FKBP38 TPR domain extend our knowledge of structural dynamics in TPR domain proteins.

PPlase measurements and binding assays revealed that the C-terminal domain of Hsp90 is sufficient for the formation of the ternary complex. The presence of ATP and the ATPase inhibitor geldanamycin does not influence the interaction of Hsp90 with the FKBP38-CaM-Ca²⁺ complex, indicating that only the C-terminal domain of Hsp90 is involved in FKBP38 binding (Fig. 1B). Therefore, our data point to binding of the FKBP38 TPR domain to the C90 domain of Hsp90, which corresponds to other immunophilin-Hsp90 interactions (13, 17, 18, 23, 44).

In contrast to Hsp90 interactions with other TPR-containing PPlases, the formation of the Hsp90-FKBP38-CaM-Ca²⁺ complex participates in the regulation of the active site of FKBP38. Activity measurements and the inhibitor competition assay show that the affinity of FKBP38-CaM-Ca²⁺ for the low molecular weight ligand is diminished in the presence of Hsp90, whereas the C90 domain of Hsp90 does not inhibit the catalytic activity of the FKBP38-CaM-Ca²⁺ complex. However, the binding of the C90 domain to FKBP38-CaM-Ca²⁺ prevents the interaction with Bcl-2 (Fig. 3C). Thus, Hsp90 masks the catalytic cleft of the FKBP38-CaM-Ca²⁺ complex, as shown by the FK506 competition experiment monitoring FKBP12 activity (Fig. 3D). On the other hand the interaction between the FKBP38-CaM-Ca²⁺ complex and Hsp90 may also influence Hsp90 by shifting the ratio between monomer and dimer toward the Hsp90 dimer (Fig. 1C). After formation of the Hsp90-FKBP38-CaM-Ca²⁺ complex, FKBP38 has a similar structural topology compared with the FKBP38-CaM-Ca²⁺ complex, as shown by the highly structured near-UV CD spectra of both complexes. These results imply that Hsp90 binding does not reverse structural re-arrangement in FKBP38 leading to PPlase activity of FKBP38-CaM-Ca²⁺. In the ternary complex Hsp90 and FKBP38 do not compete for CaM-Ca²⁺ interaction, although FKBP38 and Hsp90 bind the CaM-Ca²⁺ complex separately (3, 26). Interestingly, Hsp90 was found to interact with CaM-Ca²⁺-dependent enzymes, such as the protein phosphatase calcineurin and the NOS, and to regulate their activity (30, 31, 48, 49).

The binding of Hsp90 to FKBP38-CaM-Ca²⁺ inhibits not only its enzymatic activity, but also interactions with Bcl-2 (Fig. 3). This observation is not unexpected because of the following: (i) Bcl-2 binds to the active site of FKBP38 and (ii) the active site-directed inhibition of FKBP38-CaM-Ca²⁺ prevents interactions between Bcl-2 and the immunophilin (3). Therefore, the Hsp90-mediated prevention of interactions between FKBP38-CaM-Ca²⁺ and Bcl-2 is similar to the effect recently observed for the hepatitis C virus NS5A protein that interacts with FKBP38 also preventing Bcl-2-FKBP38 interactions (7).

However, elevated Hsp90 and Bcl-2 levels increase individually the Bcl-2 population that escapes the regulation by FKBP38-CaM-Ca²⁺ (Fig. 4). Interestingly, some studies provided evidence that Hsp90 and Bcl-2 expression can be up-regulated in concert (42, 43). Furthermore, Hsp90 was already shown to participate in the regulation of apoptosis that can be linked to Bcl-2-dependent pathways, and the co-administration of apoptotic stimuli and Hsp90 inhibitors potentiates the level of apoptosis compared with results in the absence of Hsp90 inhibitors (40, 50–52).

Taken together our results introduce Hsp90 as a cellular regulator of FKBP38. The formation of the Hsp90-FKBP38-CaM-Ca²⁺ complex restores Bcl-2 binding capacity for its cellular interaction partners and thus prevents apoptosis of neuronal cells (Fig. 4). Hsp90 plays a similar role as active site-directed ligands of FKBP38 in this regulatory pathway providing the basis of a physiological control of FKBP38 activity.

---

**FIGURE 4.** Hsp90 controls the FKBP38-Bcl-2 interaction in neuroblastoma cells. After Ca²⁺ influx in the cytosol, the CaM-Ca²⁺ complex is formed. The CaM-Ca²⁺ complex binds to FKBP38 and thus activates the PPlase. Only the active FKBP38-CaM-Ca²⁺ complex interacts with Bcl-2 (left panel). The interaction between FKBP38 and Bcl-2 prevents the control of pro-apoptotic proteins, e.g. Bad, by heterodimer formation with Bcl-2 and therefore the pro-apoptotic binding partners of Bcl-2 promote apoptosis. In case of high cellular Hsp90 concentrations, the formation of the Hsp90-FKBP38-CaM-Ca²⁺ complex prevents interactions between Bcl-2 and its negative regulator FKBP38-CaM-Ca²⁺ (right panel). Thus, Bcl-2 displays its anti-apoptotic activity by forming heterodimers with its pro-apoptotic binding partners, blocking their activity and subsequently the transduction of the signal of the programmed cell death. CaN, calcineurin, protein phosphatase 2.
REFERENCES

1. Fischer, G., and Aumuller, T. (2003) Rev. Physiol. Biochem. Pharmacol. 148, 105–150.
2. Harrar, Y., Bellini, C., and Faure, J. D. (2001) Trends Plant Sci. 6, 426–431.
3. Edlich, F., Weiwad, M., Erdmann, F., Fanghanel, J., Jarczowski, F., Rahfeld, J. U., and Fischer, G. (2005) EMBO J. 24, 2688–2699.
4. Weiwad, M., Edlich, F., Erdmann, F., Jarczowski, F., Kilka, S., Dorn, M., Peichstein, A., and Fischer, G. (2005) FEBS Lett. 579, 1591–1596.
5. Edlich, F., Weiwad, M., Wildemann, D., Jarczowski, F., Kilka, S., Moutty, M.-C., Jaehres, G., Lucke, C., Schmidt, W., Striggow, F., and Fischer, G. (2006) J. Biol. Chem. 281, 14961–14970.
6. Lam, E., Martin, M., and Wiederrecht, G. (1995) Gene (Amst.) 160, 297–302.
7. Wang, J. D., Tong, W. Y., Zhang, X. N., Chen, L., Yi, Z. G., Tan, T. T., Hu, Y. W., Xiang, L., and Yuan, Z. H. (2006) FEBS Lett. 580, 4392–4400.
8. Okamoto, T., Nishimura, Y., Ichimura, T., Suzuki, K., Miyamura, T., Suzuki, T., Morishita, K. M., and Matsuura, Y. (2006) EMBO J. 25, 5015–5025.
9. Bulgakov, O. V., Eggenschwiler, J. T., Hong, D. H., Anderson, K. V., and Li, T. (2004) Development (Cambridge, U.K.) 131, 2149–2159.
10. Fong, S., Mounkes, L., Liu, Y., Maibaum, M., Alonzo, E., Desprez, P. Y., Thor, A. D., Kashani-Sabet, M., and Debs, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14253–14258.
11. Rosner, M., Hofer, K., Kubista, M., and Hengstschläger, M. (2003) Oncogene 22, 4786–4798.
12. Crackower, M. A., Kolas, N. K., Noguchi, I., Sarao, R., Kikuchi, K., Kaneko, H., Kobayashi, E., Kawai, Y., Koziardzki, I, Landers, R., Mo, R., Hui, C. C., Nieves, E., Cohen, P. E., Osborne, L. R., Wada, T., Kunieda, T., Moens, P. B., and Penninger, J. M. (2003) Science 300, 1291–1295.
13. Silverstein, A. M., Galgiani, M. D., Kanelakis, K. C., Radanyi, C., Renoir, J. M., and Pratt, W. B. (1999) J. Biol. Chem. 274, 36980–36986.
14. Pratt, W. B., Silverstein, A. M., and Galgiani, G. M. (1999) Cell. Signal. 11, 839–851.
15. Pratt, W. B., Galgiani, M. D., Harrell, J. M., and DeFranco, D. B. (2004) Cell. Signal. 16, 857–872.
16. Petrlis, I. R., and Peredw, G. H. (2002) Chem. Biol. Interact. 141, 25–40.
17. Kazlauskas, A., Poellinger, L., and Pongratz, I. (2002) J. Biol. Chem. 277, 11795–11801.
18. Young, J. C., Obermann, W. M. J., and Hartz, F. U. (1998) J. Biol. Chem. 273, 18007–18010.
19. Owings-Grillo, J. K., Hoffmann, K., Hutchinson, K. A., Ye, A. W., Deibel, M. R., Jr., Handschumacher, R. E., and Pratt, W. B. (1995) J. Biol. Chem. 270, 20479–20484.
20. Radanyi, C., Renoir, J. M., Sabbah, M., and Baudieu, E. E. (1989) J. Biol. Chem. 264, 2568–2573.
21. Mimami, Y., Kawasaki, H., Miyata, Y., Suzuki, K., and Yahara, I. (1991) J. Biol. Chem. 266, 10999–11003.
22. Perdew, G. H., Hord, N., Hollenback, C. E., and Welsh, M. J. (1993) Exp. Cell Res. 209, 350–356.
23. Maruya, M., Sameshima, M., Nemoto, T., and Yahara, I. (1999) J. Mol. Biol. 285, 903–907.
24. Richter, K., Muschler, P., Hainzl, O., and Buchner, J. (2001) J. Biol. Chem. 276, 33689–33696.
25. Cambiasso, V., Gonzalez, M., Isamit, C., and Maccioni, R. B. (1999) FEBS Lett. 457, 343–347.
26. Mimami, Y., Kawasaki, H., Suzuki, K., and Yahara, I. (1993) J. Biol. Chem. 268, 9604–9610.
27. Rhoads, A. R., and Friedberg, F. (1997) FASEB J. 11, 331–340.
28. Hoeflich, K. P., and Ikura, M. (2002) Cell 108, 739–742.
29. Fontana, J., Fulton, D., Chen, Y., Fairchild, T. A., McCabe, T. J., Fujita, N., Tsuruo, T., and Sessa, W. C. (2002) Circ. Res. 90, 866–873.
30. Song, Y., Zweier, J. L., and Xia, Y. (2001) Biochem. J. 355, 357–360.
31. Takahashi, S., and Mendelsohn, M. E. (2003) J. Biol. Chem. 278, 9339–9344.
32. Brouet, A., Sonveaux, P., Desvy, C., Balligand, J. L., and Feron, O. (2001) J. Biol. Chem. 276, 32663–32669.
33. Christner, C., Herdegen, T., and Fischer, G. (2001) Mini Rev. Med. Chem. 1, 377–397.
34. Kofron, J. L., Kuzmic, P., Kishore, V., Colon-Bonilla, E., and Rich, D. H. (1991) Biochemistry 30, 6127–6134.
35. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F. X. (1989) Nature 337, 476–478.
36. Deleted in proof.
37. Deleted in proof.
38. Deleted in proof.
39. Deleted in proof.
40. Deleted in proof.
41. Deleted in proof.
42. Deleted in proof.
43. Deleted in proof.
44. Deleted in proof.
45. Deleted in proof.
46. Deleted in proof.
47. Deleted in proof.
48. Deleted in proof.
49. Deleted in proof.
50. Deleted in proof.