Comparative Analysis of Calcineurin Inhibition by Complexes of Immunosuppressive Drugs with Human FK506 Binding Proteins†

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ABSTRACT: Multiple intracellular receptors of the FK506 binding protein (FKBP) family of peptidylprolyl *cis/trans*-isomerases are potential targets for the immunosuppressive drug FK506. Inhibition of the protein phosphatase calcineurin (CaN), which has been implicated in the FK506-mediated blockade of T cell proliferation, was shown to involve a gain of function in the FKBP12/FK506 complex. We studied the potential of six human FKBPs to contribute to CaN inhibition by comparative examination of inhibition constants of the respective FK506/FKBP complexes. Interestingly, these FKBPs form tight complexes with FK506, exhibiting comparable dissociation constants, but the resulting FK506/FKBP complexes differ greatly in their affinity for CaN, with IC_{50} values in the range of 0.047−17 μM. The different capacities of FK506/FKBP complexes to affect CaN activity are partially caused by substitutions corresponding to the amino acid side chains K34 and I90 of FKBP12. Only the FK506 complexes of FKBP12, FKBP12.6, and FKBP51 showed high affinity to CaN; small interfering RNA against these FKBP allowed defining the contribution of individual FKBP in an NFAT reporter gene assay. Our results allow quantitative correlation between FK506-mediated CaN effects and the abundance of the different FKBPs in the cell.

Calcineurin (CaN,[1] PP2B) is a Ca^{2+}/calmodulin (CaM) dependent serine/threonine protein phosphatase that is known to regulate numerous cellular processes by dephosphorylation of transcription factors, such as the nuclear factor of activated T cells (NFAT), the myocyte-specific enhancer factor 2 (MEF2), and the Ets-like gene 1 (Elk-1), as well as regulatory proteins, such as NO synthase and cAMP-dependent protein kinase (*1*−*6*). CaN protein phosphatase activity regulates, for instance, the clonal expansion of T cells after stimulation by an antigen, leading to immune response (*7*). Application of FK506, a product of *Streptomyces tsukubaensis*, interferes with the CaN-mediated immune response and is therefore used in transplantation medicine to prevent allograft rejections. CaN protein phosphatase activity is inhibited by interaction with a complex formed by FK506 and its cellular receptors, the FK506 binding proteins (FKBPs). The initial formation of FK506/FKBP complexes is critical to CaN inhibition, because only the resulting composite surface binds CaN with high affinity, thus accounting for an inhibitory gain of function (*8*). CaN inhibition prevents dephosphorylation and thus nuclear translocation of the transcription factor NFAT, causing immunosuppression (*9*−*11*). In a similar manner, CaN protein phosphatase activity is inhibited by cyclosporine A (CsA)/cyclophilin complexes, leading to immunosuppression as well (*8*, *12*, *13*). Furthermore, the FKBP ligand rapamycin was identified as a potent immunosuppressant, antagonizing FK506-mediated immunosuppression under certain conditions (*14*, *15*). However, rapamycin was also described to act independently of CaN inhibition via gain of function inhibition of the serine/threonine protein kinase mammalian target of rapamycin (mTOR) (*14*).

The FKBP domain of human FKBPs is supposed to exhibit extensive structural plasticity (*16*). This property would principally allow them to function as a proline-directed folding helper enzyme as well as a presenter protein for FK506 and rapamycin. Although well-known for FKBP12, the possibilities for the interplay between both functions in other FKBPs are largely unknown. For example, FKBP12- and FKBP12.6-mediated suppression of EGF receptor signaling can be abolished without any CaN participation by inhibiting the PPIase activity using both FK506 and rapamycin (ref *17* and unpublished results).

Among the 16 different human FKBPs, only FK506 complexes of FKBP12 and FKBP12.6 were reported to inhibit CaN phosphatase activity in the low nanomolar concentration range (*8*, *18*). Although both FKBPs share significant sequence homology, tissue distribution, and affinity for FK506, there are data suggesting an involvement of FKBP12 only in immunosuppression (*19*). However, it would be surprising if only one member of the FKBP family would contribute to FK506-mediated immunosuppression on a cellular level, because FKBP13, FKBP38, FKBP51, and FKBP52 also exhibit PPIase activity and are highly abundant in human. Thus, FK506-mediated cellular effects on CaN

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[1] Abbreviations: FKBP, FK506 binding protein; PPIase, peptidylprolyl *cis*/*trans*-isomerase; CaN, calcineurin; CaM, calmodulin; pNPP, 4-nitrophenyl phosphate; pNA, 4-nitroanilide; NFAT, nuclear factor of activated T cells.

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inhibition are multifactorial because of the relative cellular abundance of FKBPs and the CaN inhibitory potency of the individual FK506/FKBP complexes.

Therefore, we were interested in evaluating (i) FK506 affinities to the six human FKBPs and (ii) CaN inhibition capacities of the different FK506/FKBP and rapamycin/FKBP complexes.

FKBPs are a structurally diverse family of the enzyme class of peptidylprolyl *cis/trans* isomerases (PPIases) differing in domain composition and protein sequences (*20*). However, the PPIase domains of the FKBP type share a common domain fold of at least five β strands and a spanning α helix that is referred to as half β barrel (*21*). The FK506 binding site is formed by a hydrophobic pocket of at least 12 amino acids between β strand 5 and the α helix, comprising numerous substitutions in the amino acid sequence of the FKBP domains that cause changes in the protein surface within or near the active site (*20*, *22*). Here we show that residues corresponding to K34 and I90 in β strand 5 and the loop between β strands 2 and 3 of FKBP12 determine the different affinities of FK506/FKBP complexes to CaN. In addition, our data show that only the complexes of FK506 with FKBP12, FKBP12.6, and FKBP51 among the tested FKBP can inhibit CaN under physiological conditions and that the CaN/FK506/FKBP51 complex also participates in FK506-mediated immunosuppression.

MATERIALS AND METHODS

Enzymes. Human FKBP12, FKBP12.6, FKBP13, FKBP38, FKBP51, and FKBP52 and human calmodulin were expressed by using pET28a (Novagen) in *Escherichia coli* Rosetta cells (*23*). Human calcineurin (His_6-CaNA/CaNB) was expressed and purified according to Mondragon et al. (*24*). Peptide substrates used were obtained from Bachem (Heidelberg, Germany). FK506 and rapamycin were purchased from Calbiochem (La Jolla, CA).

Calcineurin Activity Assay. (A) RII Phosphopeptide-Based CaN Activity Assay. CaN activity was measured using a scintillation proximity assay according to Baumgrass et al. (*25*). A ^{33}P-labeled biotinylated 19-residue peptide of a partial sequence of the RII subunit of the bovine PKA (RII phosphopeptide) was used as substrate. For determination of CaN inhibition by FK506/FKBP and rapamycin/FKBP complexes 3 nM CaN, 50 nM CaM, 30 μM FK506 or 20 μM rapamycin, and varying concentrations of FKBPs were preincubated in assay buffer [40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM DTT, 1 mM $CaCl_2$, 0.1 mg/mL bovine serum albumin (BSA)] at 30 °C for 15 min in a 96-well microtiter plate (Costar, Bodenheim, Germany). In the case of FKBP38 40 μM CaM was added to the assay in order to prevent competition for the CaN activating factor CaM. Then, 10 pmol of biotinylated ^{33}P-labeled RII phosphopeptide was added to each well in a total assay volume of 100 μL. After dephosphorylation for 30 min at 30 °C, 90 μL of the reaction mixture was transferred to a scintillation well coated with streptavidin (Perkin-Elmer, Germany). Biotinylated RII phosphopeptide was allowed to bind to streptavidin for 20 min. After washing, RII phosphopeptide-associated radioactivity was measured in a MicroBeta top counter (Wallac, Turku, Finland).

The active site concentrations of the different FKBPs and FKBP variants were determined by FK506 titration experiments, observing the tryptophan fluorescence of the FKBP according to Ramm et al. (*26*) (Supporting Information, Figure 3).

(B) CaN Activity toward 4-Nitrophenyl Phosphate (4-NPP). CaN activity was assayed in 40 mM Tris-HCl, pH 7.5, 1 μM CaM, 100 mM NaCl, 6 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM DTT, 1 mM $CaCl_2$, and 0.1 mg/mL BSA at 30 °C using 20 mM 4-NPP as substrate. CaN activity was measured by following the increase in absorbance at 410 nm. Initial rates were calculated from the slope of the experimental trace by linear regression.

Affinity Chromatography of FK506/FKBP Complexes. In order to analyze the formation of CaN/FK506/FKBP complexes, we incubated Jurkat cell lysates (400 μL, 1 mg/mL) with 500 pmol of CaN immobilized to Ni^{2+}NTA Sepharose in the presence and absence of 20 μM FK506. His_6-CaN was applied in excess over the total FKBP concentration (about 300 pmol) in the used cell lysates of Jurkat cells. After four washing steps, (i) 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 2 mM ATP, (ii) 20 mM PIPES, pH 7.0, 100 mM NaCl, (iii) 20 mM PIPES, pH 5.6, 100 mM NaCl, and (iv) 50 mM Tris-HCl, pH 7.5, bound protein was eluted with 200 mM imidazole, subjected to 15% SDS−PAGE, and analyzed by Western blot using polyclonal anti-FKBP13, anti-FKBP38 (Pab Production), polyclonal anti-FKBP12 (PA1-026, Dianova), anti-FKBP51 (PA1-20, Dianova), and anti-FKBP52 antibodies (SA-136, Biomol). The anti-FKBP12 antibody also recognizes FKBP12.6. Subsequently, Western blots were quantified using AIDA software (Raytest). Furthermore, we tested CaN/FK506/FKBP complex formation under physiological conditions. Therefore, we incubated Jurkat cells with 20 μM FK506 for 16 h and applied the lysates of harvested cells to the CaN affinity matrix.

RNA Interference Experiments. Synthetic small interfering dsRNAs (siRNAs) were from MWG Biotech. The siRNA sense sequences used in this study are as follows: FKBP51, 5′-GAAGACACUUCACGUAUUC-3′; FKBP12, 5′-GCACAAGUGGUAGGUUAAC-3′; FKBP12.6, 5′-GAACACAGAUCUCUUGUUC-3′. The siRNAs were transfected into Jurkat cells by electroporation (Amaxa). The transfection efficiency of the Jurkat cell line was approximately 50−60%, as confirmed by GFP fluorescence. The reduction of the cellular FKBPs by siRNAs was tested by Western blotting and compared to nontransfected cells.

NFAT Reporter Gene Assay. In order to test the effect of FKBP12, FKBP12.6, and FKBP51 on CaN phosphatase activity in the presence of FK506, Jurkat cells transfected with NFAT-luciferase reporter plasmid (Stratagene, The Netherlands) were transfected with FKBP12, FKBP12.6, and FKBP51 siRNAs. Then, cells were stimulated with 2 μM ionomycin and 100 nM PMA for 5 h. After cell lysis the protein content of each lysate was determined by the Bradford method. Equivalent amounts of protein were applied to determine the level of the extracted luciferase from the cells by bioluminescence measurement using the luciferase assay system (Promega, Mannheim, Germany). In addition, a β-galactosidase plasmid was cotransfected as the internal standard.

Measurement of PPIase Activity. (A) Protease-Coupled Assay. PPIase activity to proline-containing peptide substrates was tested as described (*27*). In a competition assay designed to detect putative FK506 binding, human FKBP12 was used

Table 1: Inhibition Constants of FK506 and Corresponding FK506/FKBP Complexes for Inhibition of PPIase Activity of FKBPs and Phosphatase Activity of CaN, Respectively[a]

	inhibition of	
FKBP	PPIase activity by FK506 IC_{50} (nM)	CaN activity by FK506/FKBP IC_{50} (nM)
FKBP12	0.88 ± 0.2	47 ± 3
FKBP12.6	2.9 ± 0.4	147 ± 17
FKBP13	1.5 ± 0.3	4990 ± 590
FKBP38	51.0 ± 6.2	17000 ± 2500
FKBP51	14.6 ± 1.8	744 ± 98
FKBP52	12.0 ± 1.4	15000 ± 1600

[a] PPIase activities of FKBP12, FKBP12.6, and FKBP13 were measured in a protease-coupled assay. FKBP38 activity was measured in a competition assay (*32*), and the activities of FKBP51 and FKBP52 were determined in a protease-free assay. Inhibition constants were determined by measurement of the residual PPIase activity of FKBPs in the presence of various FK506 concentrations. Usually, FKBP concentrations ranged from 10 to 50 nM. Inhibition of CaN protein phosphatase activity was determined in a scintillation proximity assay with RII phosphopeptide as substrate using 30 μM FK506 and various concentrations of FKBPs.

in a concentration that accelerated the isomerization of the peptide Suc-Ala-Phe-Pro-Phe-pNA 3-fold (three acceleration units). With the addition of FK506, this acceleration was inhibited to one acceleration unit (*32*).

(B) Protease-Free PPIase Assay. To overcome possible degradation of PPIases by proteases in the proteolytic assay, a protease-free test was performed as previously described (*28*). The disturbance of *cis/trans* equilibrium was achieved by a solvent jump of the peptide from LiCl/trifluorethanol to 35 mM HEPES/NaOH buffer (pH 7.8). Reequilibration, which is accelerated by PPIases, was measured.

RESULTS

Human FKBPs Bind Tightly to FK506. To investigate the interaction of FK506 with the active sites of FKBP12, FKBP12.6, FKBP13, FKBP38, FKBP51, and FKBP52, dose-dependent inhibition of these FKBPs by the inhibitor was determined by monitoring the resulting PPIase activity. Interestingly, the inhibition of all tested FKBPs by FK506 was in the nanomolar range following the tight binding model (Table 1). The single domain FKBP were inhibited by FK506 in a range between 0.9 and 2.9 nM, which is comparable to published data for FKBP12 and FKBP12.6 (*18*). However, our analyses using direct measurements and PPIase competition measurements revealed a K_i value for FKBP13 inhibition by FK506 that differed from the previously published IC_{50} value of about 50 nM (*29*). In a protease-free PPIase assay, FKBP51 and FKBP52 were inhibited with IC_{50} values in the range of 10 nM, which is in agreement with published data (*30*, *31*). As an exception, the active FKBP38/Ca^{2+}/calmodulin complex is inhibited with a K_i value as high as 48 nM (*32*). These findings show that despite 57% dissimilarity in the corresponding active site residues, all members of the heterologous group of FKBPs bind FK506 tightly.

FK506 Complexes of FKBP12, FKBP12.6, and FKBP51 Inhibit CaN with High Affinity. As previously described, the FK506/FKBP12 complex inhibits CaN phosphatase activity (*8*). However, members of the FKBP family differ 67% in the amino acid composition of the β5 strand and the loop between β strands 2 and 3 in the FKBP domains that likely form the binding moiety for CaN. The heterogeneity in this part of the FKBP domains indicates differences in the FK506/FKBP binding properties to CaN among the different human FKBPs.

We assayed CaN activity against RII phosphopeptide in the presence of preformed FK506/FKBP complexes. The FK506 concentration (30 μM) was applied to ensure that the measured FKBP was completely bound to FK506. Under these conditions the concentration of the FK506/FKBP complex equals the amount of FKBP added. We then calculated IC_{50} values from measured dose-dependent inhibition of CaN protein phosphatase activity by various FK506/FKBP complexes. Our results revealed that the FK506/FKBP12.6 complex inhibits CaN phosphatase activity in a similar range as the FK506/FKBP12 complex (Figure 1A, Table 1). The FK506/FKBP38/CaM/Ca^{2+} complex exhibits the highest IC_{50} value of the FK506/FKBP complexes tested for CaN inhibition. Interestingly, the FK506/FKBP13 complex inhibits CaN with a 100-fold higher IC_{50} value than the FK506/FKBP12 complex. This observation is of special interest, because FKBP12 and FKBP13 have 62% sequence homology and interact with FK506 with similar binding affinities, indicating similar structure and amino acid composition of the PPIase site of both proteins. Another surprising fact is that the FK506/FKBP52 complex inhibits CaN activity with an IC_{50} value of 15 μM, which is about 20-fold lower binding affinity than observed with the 77% sequence homologous FKBP51.

Since it was shown that the CsA/Cyp18 complex inhibits the phosphatase activity of CaN toward the RII phosphopeptide but stimulates the CaN activity toward the small artificial substrate 4-nitrophenyl phosphate (4-NPP), we investigated the effect of the various FK506/FKBP complexes on the initial rate of the CaN-catalyzed dephosphorylation of 4-NPP (*33*, *34*). As shown in Figure 1B, differences in CaN inhibition by FK506/FKBP complexes found in the RII phosphopeptide assay can be observed with the 4-NPP substrate as well. The FK506 complexes of FKBP12 and FKBP52 activate CaN in a dose-dependent manner, whereas the FKBP12-related FKBP12.6 inhibits phosphatase activity of CaN by 20% in complex with FK506 (Figure 1B). However, the observed activation of CaN by the FK506/FKBP52 complex in the 4-NPP assay is 10-fold lower than the activation by the FK506/FKBP12 complex and occurs in the micromolar range. Additional measurements of CaN activity with the 4-NPP substrate investigating FK506 complexes of FKBP13, FKBP38, and FKBP51 showed no significant effect on CaN activity (data not shown).

However, in contrast to measurements using the large RII phosphopeptide as substrate, FK506/FKBP12-mediated effects on CaN activity toward the 4-NPP substrate were achieved even in the absence of the regulator CaM (Figure 1C). When compared with the CaN/CaM/Ca^{2+} complex, the activation of CaN occurred at higher concentrations of the FK506/FKBP12 complex, but with the same final k_{cat}/K_M, implying a similar binding mode of FK506/FKBP complexes underlying the observed effect.

Identification of CaN-Interacting FK506/FKBP Complexes. In an attempt to further delineate the CaN inhibitory role of FK506/FKBP complexes in the cell, we examined

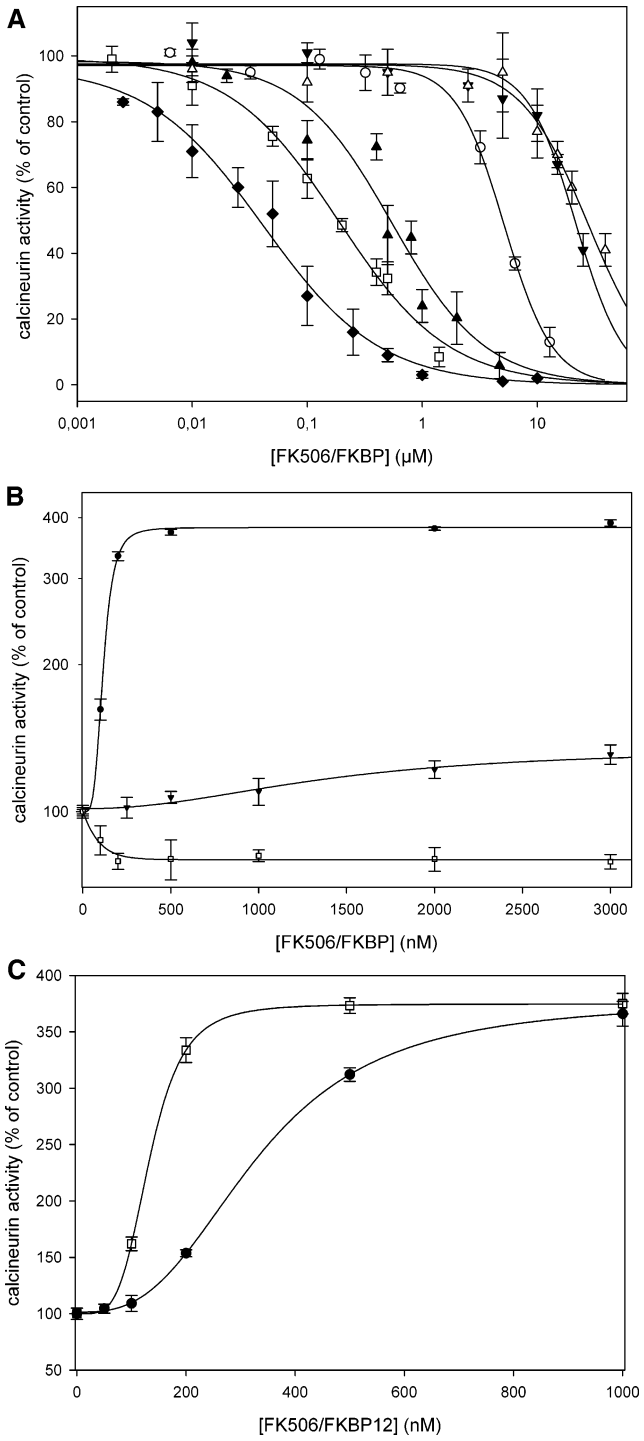

FIGURE 1: Effect of FK506/FKBP complexes on CaN activity. (A) CaN protein phosphatase activity was measured using scintillation proximity assay with RII phosphopeptide as substrate. For determination of CaN inhibition by FK506/FKBP complexes 3 nM CaN, 50 nM CaM, 30 μM FK506, and various concentrations of FKBP12 (◆), FKBP12.6 (□), FKBP13 (○), FKBP38 (△), FKBP51 (▲), and FKBP52 (▼) were applied in the assay. Exceptionally, in the case of the CaM/Ca^{2+}-dependent FKBP38, measurements were carried out in the presence of 40 μM CaM in order to avoid competition effects. Inhibition constants were calculated and are depicted in Table 1. (B) Effect of FK506/FKBP complexes on 4-NPP activity of CaN. The assay usually contained 45 nM CaN, 1 μM calmodulin, 30 μM FK506, and various concentrations of FKBP12 (●), FKBP12.6 (□), and FKBP52 (▼), respectively. (C) Activation of CaN 4-NPP activity by the FK506/FKBP12 complex in the absence (●) or presence (□) of 1 μM CaM. For measurements 45 nM CaN, 30 μM FK506, and various concentrations of FKBP12 were used.

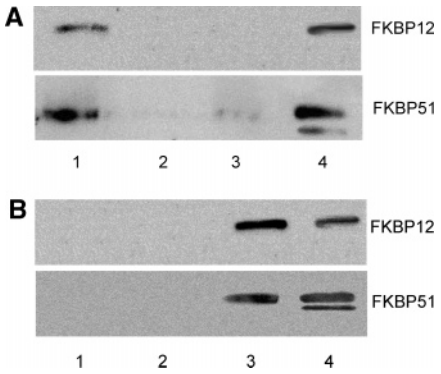

FIGURE 2: FK506 complexes of FKBP12 and FKBP51 interact with CaN in Jurkat cell lysates. (A) In order to identify FK506/FKBP complexes of Jurkat cell lysates interacting with CaN, we incubated 400 μL cell extracts (1 mg/mL) with 500 pmol of CaN immobilized on a Ni^{2+}NTA affinity matrix in the presence of 20 μM FK506. After four washing steps, bound protein was eluted with imidazole, subjected to SDS−PAGE, and analyzed by Western blotting with antibodies against human FKBPs (lane 1). As controls, Jurkat cell lysates were incubated with the Ni^{2+}NTA beads in the absence of CaN (lane 2) or with CaN affinity matrix in the absence of FK506 (lane 3). In lane 4, 10 μL of Jurkat cell lysate (1 mg/mL) was applied. (B) To evaluate CaN interaction with FK506/FKBP complexes under in vivo conditions, Jurkat cells were grown in the absence or presence of 20 μM FK506 for 16 h, harvested, and lysed. Lysates of FK506-treated cells (lane 3) or untreated cells (lane 2) were then applied to the CaN affinity matrix. Bound proteins were eluted with imidazole, subjected to SDS−PAGE, and analyzed by Western blot. Additionally, lysates of Jurkat cells grown in the presence of 20 μM FK506 were applied to a Ni^{2+}NTA affinity matrix in the absence of CaN. In lane 4, 10 μL of Jurkat cell lysate (1 mg/mL) was applied.

the physical interaction patterns of CaN by using an affinity matrix with CaN bound to Ni^{2+}NTA Sepharose in the presence and absence of FK506. After four washing steps bound protein was eluted, separated by SDS−PAGE, and analyzed by Western blot. Figure 2A shows that FKBP51 was identified in addition to FKBP12 in complex with CaN in the presence of FK506, whereas no other endogenous FKBP was found in the eluate. CaN was applied in 60% excess over the total FKBP concentration in the used cell lysates of Jurkat cells. The detection limit was ≥0.1 pmol of endogenous FKBP in the Jurkat cell lysate.

Furthermore, we incubated Jurkat cells with 20 μM FK506 for 16 h and applied the lysates of harvested cells to the CaN affinity matrix. Similar interaction patterns were found and may reflect the exclusive role of FKBP51 and FKBP12 in CaN-mediated biological effects (Figure 2B). FKBP12.6, in contrast, was not identified using this method due to its lower abundance in Jurkat cells compared to FKBP12. A quantitative analysis of the Western blot results revealed that 19.0 ± 0.7% of CaN-associated FK506/FKBP complexes contained FKBP51, indicating a significant contribution of FKBP51 to FK506-mediated CaN inhibition in Jurkat cells.

Contribution of Different FK506/FKBP Complexes to Cellular CaN Inhibition. Affinity chromatography revealed that FKBP12 and FKBP51 physically interacted with CaN in Jurkat cells when cells were treated with FK506. Based on its strong structural and sequence similarity to FKBP12, the FKBP12.6 protein must also be considered for mediating FK506 effects on CaN activity in the cell. In order to dissect the contribution of the three FKBPs to FK506-mediated CaN

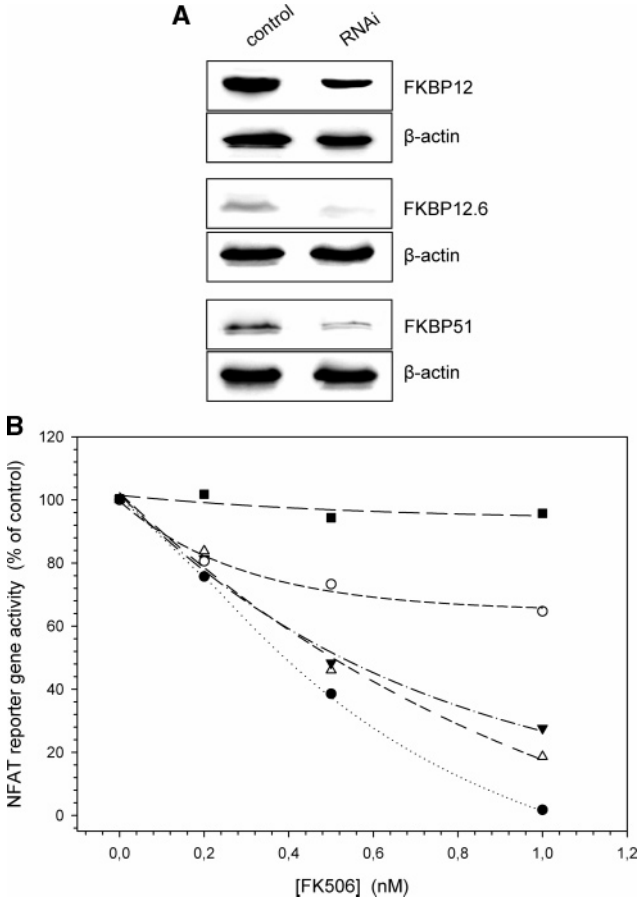

FIGURE 3: Effect of FKBP12, FKBP12.6, and FKBP51 siRNA on NFAT reporter gene activity. (A) FKBP siRNAs were transfected into Jurkat cells by electroporation. The expression pattern of the FKBPs was tested 10 h after transfection by Western blotting and compared to nontransfected cells. β-Actin was used as the loading control. (B) In order to study the effect of FKBP siRNAs on CaN phosphatase activity, we measured NFAT reporter gene activity using a luciferase assay system (Promega). Cells were transfected with FKBP12 (○), FKBP12.6 (▼), and FKBP51 (△) siRNA or with a combination of the three siRNAs (■). Nontransfected cells were used as the control (●). To stimulate CaN activity, cells were treated with 2 μM ionomycin for 5 h. Then, cells were subjected to various FK506 concentrations for 15 min. After cell lysis, the level of extracted luciferase from the cells was determined by bioluminescence measurement using the luciferase assay system. Results are expressed as the activity relative to reference values without inhibitor (SD < 10%).

inhibition in human cells, Jurkat cells were transfected with FKBP12, FKBP12.6, and FKBP51 siRNA or with a combination of these siRNAs. CaN activity in Jurkat cells transfected with FKBP siRNAs was measured using an NFAT reporter gene assay at various FK506 concentrations. The depletion of the three FKBPs was tested by Western blot and compared to nontransfected cells (Figure 3A). Noteworthy, the siRNA experiments did not affect the protein levels of other FKBPs. As shown in Figure 3B, the FK506-mediated inhibition of CaN activity was completely abolished when cells were transfected with a combination of the three siRNAs. Moreover, our results reveal a major contribution of FKBP12 to FK506 effects on CaN activity in Jurkat cells. Cells transfected with FKBP12 siRNA exhibited up to 60% decreased responsiveness to FK506 compared to nontransfected cells. The siRNAs of each FKBP12.6 and FKBP51 elevated NFAT reporter gene activity up to 20%, indicating

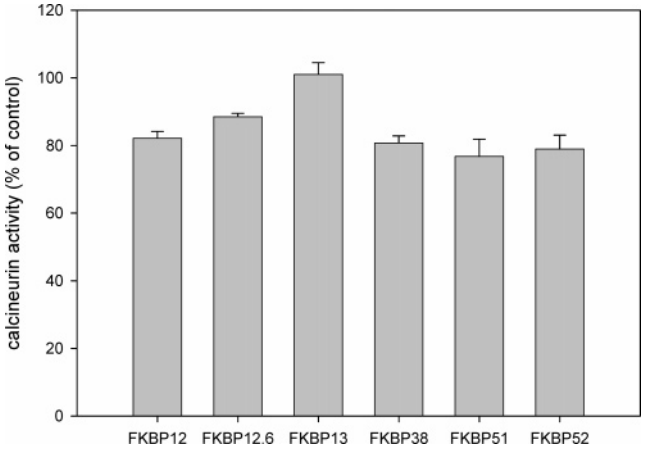

FIGURE 4: Influence of rapamycin/FKBP complexes on CaN activity. CaN activity was measured using RII phosphopeptide as substrate. For determination of CaN inhibition by rapamycin/FKBP complexes 20 μM rapamycin and 5 μM FKBPs were applied in the assay under standard conditions.

that both FKBPs participate in FK506-mediated CaN inhibition as well.

Rapamycin/FKBP Complexes Do Not Inhibit CaN Activity. On the basis of fundamental differences in the CaN inhibition by various FK506/FKBP complexes, we were interested in whether rapamycin/FKBP complexes may influence CaN activity. So far, it has only been reported that the complex of FKBP12 with the immunosuppressive drug rapamycin does not inhibit CaN activity (*8*); the influence of other rapamycin/FKBP complexes has to date not been investigated. Similar to FK506/FKBP complexes, rapamycin interacts with FKBPs with high affinity, exhibiting inhibition constants in the low nanomolar range (*23*, *30*, *31*), with the exception of FKBP38, which is inhibited by rapamycin with a K_i value of 499 nM (*32*). Therefore, we performed CaN activity measurements as outlined for FK506/FKBP complexes in the presence of 20 μM rapamycin.

In contrast to FK506/FKBP complexes, the corresponding rapamycin/FKBP complexes had no significant influence on CaN protein phosphatase activity (Figure 4). Concentration-dependent effects on CaN activity were not detected in both assays, using either the RII phosphopeptide or 4-NPP as substrate.

Contribution of FKBP12 Residues K34 and I90 for CaN Binding. To address whether the formation of the inhibitory CaN/FK506/FKBP complexes relies on single amino acid substitutions in the FKBP12-like domain of the FKBPs, we identified residues located in the CaN contact region of the FK506/FKBP12/CaN complex (1TCO) published by Griffith et al. (*35*) and performed a sequence alignment of the corresponding regions for the different FKBPs (Figure 5). The β5 strand and the loop between β strands 2 and 3 of the FKBP12 molecule were found to be crucial for CaN binding. The amino acid sequences of the FKBPs contain substitutions in these regions, including K34, H87, and I90, which were tested by site-directed mutagenesis of FKBP12 for their influences on FK506-mediated CaN inhibition. To introduce the corresponding residues of FKBP38, the FKBP with the lowest affinity in the FK506/FKBP complex for CaN, into the sequence of FKBP12 that exhibited the highest affinity for CaN in complex with FK506, the variants FKBP12 K34T, FKBP12 H87R, and FKBP12 I90Y were analyzed. In

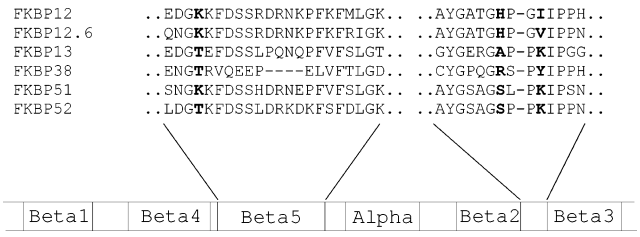

FIGURE 5: Sequence alignment of the amino acid sequences of the β5 strand and loop between β strands 2 and 3 of the FKBP domain of FKBP12, FKBP12.6, FKBP13, FKBP38, FKBP51, and FKBP52 potentially contributing to the contact surface to CaN. The secondary structure arrangement of the FKBP domain is displayed below. Amino acids corresponding to K34, H87, and I90 in FKBP12 are marked in bold.

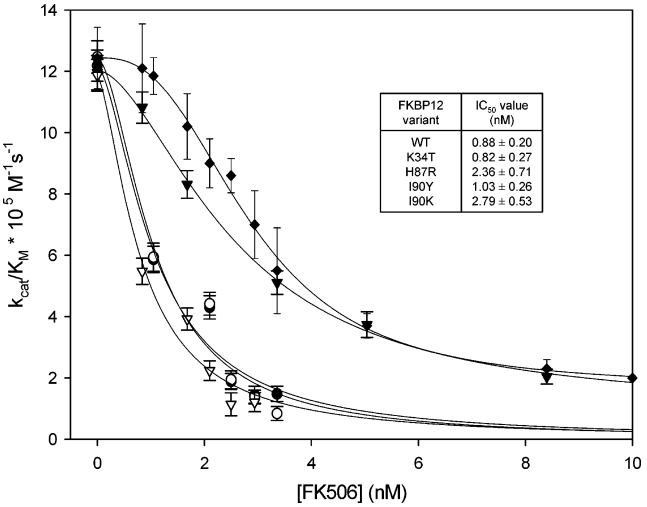

FIGURE 6: Inhibition of the PPIase activity of FKBP12 WT (●) and the FKBP12 variants K34T (○), H87R (▼), I90Y (▽), and I90K (◆) by FK506. Inhibition was determined by measurement of the residual PPIase activity of FKBP12 in the presence of various FK506 concentrations in a protease-coupled assay. The IC_{50} values were calculated and are displayed in the inset.

addition, the FKBP12 I90K variant was tested to examine the influence of the amino acid substitution in this position present in the FKBPs that form FK506/FKBP complexes with lower CaN affinity. IC_{50} values were determined to exclude the influence of differential FK506 binding of the FKBP12 variants on the CaN binding pattern (Figure 6). FKBP12 K34T and FKBP12 I90Y were inhibited by FK506 with IC_{50} values of about 1 nM, which is in the same range as in the wild-type protein. The IC_{50} values of FKBP12 H87R and FKBP12 I90K are slightly higher, but in the range of the constant for the wild-type protein. Residual CaN activity against RII phosphopeptide was measured using preformed FKBP complexes at 30 μM FK506 in dependence on the concentrations of the FKBP12 variants (Figure 7). The FK506/FKBP12 complexes of all four variants were able to inhibit CaN, even though their affinities differed greatly. FKBP12 K34T, FKBP12 I90Y, and FKBP12 I90K inhibited CaN activity with significantly higher IC_{50} values that are 15-fold, 30-fold, and 150-fold higher, respectively, than the wild type (Figure 7). In contrast, the H87R variant inhibited CaN in complex with FK506 with an IC_{50} value comparable to the value found in the wild type. These results indicate that the amino acid side chains in positions 34 and 90 are able to modify the composite surface of the FK506/FKBP12

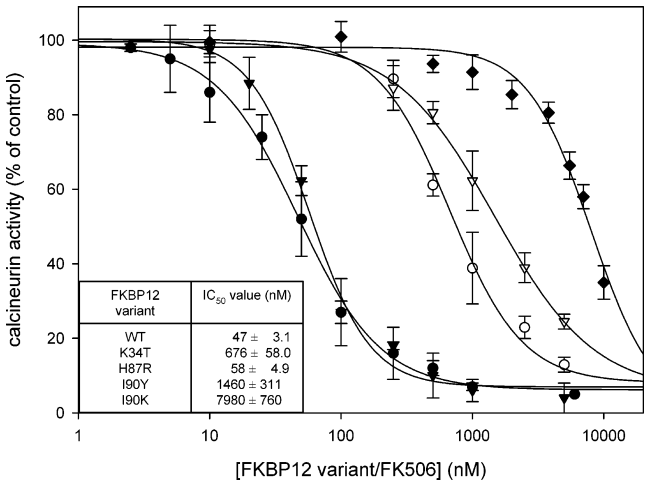

FIGURE 7: CaN phosphatase inhibition by FK506 complexes of FKBP12 WT (●) and the FKBP12 variants K34T (○), H87R (▼), I90Y (▽), and I90K (◆), respectively. Inhibition was determined by measurement of the residual CaN phosphatase activity using RII phosphopeptide substrate. Inhibition constants were determined and are depicted in the inset.

complex that mediates interactions to CaN and thus contribute to the reduced affinity of FK506/FKBP complexes for CaN.

DISCUSSION

Our comparative study demonstrates that CaN inhibition by FK506 in Jurkat cells is mediated by at least three different FK506/FKBP complexes. The FK506/FKBP12 complex, which was previously thought to represent the sole source of CaN inhibitory effects, generates about 60% of FK506-mediated CaN inhibition in an NFAT reporter gene assay (Figure 3B). The FK506/FKBP51 and FK506/FKBP12.6 complexes contribute to the remaining decrease in CaN activity. Due to their up to 350-fold higher IC_{50} values FKBP13, FKBP38, and FKBP52 unlikely contribute to FK506-mediated CaN inhibition in the presence of sufficient protein levels of FKBP12, FKBP12.6, and FKBP51 (Table 1). Affinity chromatography studies on CaN beads confirmed the functional patterns obtained from the siRNA experiments (Figure 2). Generally, the capability of the various FKBPs to inhibit CaN in the presence of FK506 is not limited by the formation of the respective FK506/FKBP complexes because comparable dissociation constants were found ranging from 0.8 to 48 nM (*18*, *29*−*32*, *36*, *37*).

The relatively large contribution of the FK506/FKBP51 complex in T cells might be caused by high expression levels of FKBP51 in this cell type (*38*, *39*). In other tissues the CaN inhibition pattern might differ greatly, because of different cellular FKBP concentrations. In spleen, for instance, where FKBP12 is the most abundant FKBP with 3-fold higher concentrations than FKBP51 (*18*, *39*), large quantities of CaN are probably inhibited by FK506/FKBP12 complexes. In brain tissue, where FKBP12 and FKBP12.6 are highly abundant and FKBP51 is expressed in very low levels, both FK506/FKBP12 and FK506/FKBP12.6 are likely to mediate CaN inhibition. In contrast, in heart tissue FKBP51 is expressed in high cellular concentrations, and low FKBP12 and FKBP12.6 levels were found, pointing to FK506/FKBP51-mediated CaN inhibition. Previous studies reported that CaN inhibition by FK506/FKBP12.6 and

FK506/FKBP12 complexes were equipotent and thus might mediate FK506 effects in immunosuppression (*18*). Our data revealed a slight decrease in the inhibitory potency when FKBP12.6 replaces FKBP12 in its FK506 complex (Table 1). Even though previous experiments using FKBP12.6-deficient T cells from mouse indicate that the FKBP12.6 contribution to CaN inhibition is small (*19*), our results show a significant involvement of this protein in FK506-mediated CaN inhibition in human Jurkat cells. The contribution of FK506/FKBP51 in the NFAT response can be explained by its relatively strong interaction with CaN, revealing an inhibition constant of 744 nM. Previous reports indicated a significant higher IC_{50} value of about 5 μM (*38*, *39*). The discrepancies might be due to usage of GST protein fusion that likely interferes with affine CaN interactions and therefore results in a 7-fold higher inhibition constant.

The FKBP ligand rapamycin was also shown to exhibit immunosuppressive properties via binding to the mammalian target of rapamycin (mTOR), inducing cell cycle arrest (*14*). The switch of the FKBP/drug complex between cellular targets is controlled, at least in part, by FKBP residues contributing to the composite interaction surface (*40*). Thus, it is possible that a change of the FKBP component in the FKBP/rapamycin complex can result in a composite surface with affinity for CaN, leading to allosteric CaN inhibition. Although all tested FKBP, except FKBP38/CaM/Ca^{2+}, bind tightly to rapamycin (*30*−*32*), which is a precondition for gain of function, our comparative analysis revealed that the rapamycin complexes do not affect CaN activity. The low affinity of rapamycin/FKBP complexes to CaN might be due to the part of rapamycin between C19 and C30 of its ring system that extends the space of the CaN contact surface in complex with FKBPs when compared to FK506, thus interfering with the occupied space of L115, M118, and V119, the backbone of CaNB, and the side chains of W352, S353, and F356 of CaNA (Supporting Information, Figure 4).

To determine the impact of amino acid substitutions in the CaN interacting surface of the tight-binding FK506/FKBP12 complex, we introduced four point mutations in the sequence of the high affine FKBP12, changing the positions K34, H87, and I90 to the corresponding residues of FKBPs that exhibited low CaN affinity in complex with FK506. These three residues represent the most variable positions within the CaN contact surface of the FKBP domain. For instance, the lysine residue corresponding to FKBP12 K34 is found in FK506/FKBP with high CaN affinity, whereas FKBPs exhibiting lower affinity for CaN in complex with FK506 contain in the corresponding position a threonine residue lacking the positive charge and occupying significantly less space. The special importance of the lysine residue in this position might be caused by its interactions with Y159 and F160 residues in the CaN A-chain in the FK506/FKBP12/CaN complex (Supporting Information, Figure 2). Indeed, the K34T substitution in FKBP12 has a dramatic impact on the affinity of FK506/FKBP complexes to CaN, reducing the affinity of the FK506 complex of the variant 14-fold compared with the wild type.

Another variable key position for tight CaN interaction corresponds to I90 in FKBP12, which is replaced in any other FKBP. FKBP12 I90 likely interacts with the CaN A-chain residues P344 and M347 in the supramolecular complex and is therefore crucial for the formation of the contacting surface. Accordingly, the I90Y and I90K substitutions caused at least a 30-fold higher inhibition constant of the variant compared with FKBP12 WT. The bulky tyrosine and the charged lysine in this position likely influence the position of the loop between β strands 2 and 3 and thereby interfere with several crucial interactions, such as those between FKBP12 P88 and T351 and P355 in the CaN A-chain and L159 in the B-chain. In contrast to the FKBP12 variants K34T and I90Y, the FKBP12 variant H87R inhibited CaN activity with an affinity comparable to that of the wild type, even though slightly lower FK506 affinity was measured. This example indicates that this position corresponding to FKBP12 has only a minor contribution to interactions with CaN.

However, 4-NPP measurements demonstrate that FK506/FKBP complexes interact with CaN in the absence of CaM. This fact is important for the understanding of FK506-mediated immunosuppression by CaN inhibition because the inactive form of CaN is blocked by FK506/FKBP complexes prior to possible CaN activation by CaM/Ca^{2+}, excluding the risk of short-term CaN activity in the presence of sufficient FK506. Differences in the binding pattern of FK506/FKBP complexes to CaN in the presence or absence of CaM are likely caused by an interference of FK506/FKBP complexes with the autoinhibitory domain of CaN that is released from the active site in the presence of CaM/Ca^{2+}. Therefore, allosteric binding of the FK506/FKBP complex removes the autoinhibitory domain of CaN from the active site, improving the chance of small substrates to enter the active site of the phosphatase.

Intriguingly, CaN activation was not a unique property among the FK506/FKBP complexes because the FK506/FKBP52 complex slightly activates CaN, whereas the FK506/FKBP12.6 complex inhibits the phosphatase to 75% residual activity. Interestingly, binding constants of FK506/FKBP complexes calculated from 4-NPP measurements are in the same range as determined using RII phosphopeptide, even though CaN is completely inactive in the RII phosphopeptide assay. The differential effects of FK506/FKBP complexes in the 4-NPP assay can be explained by structural differences of the loop preceding the β5 strand, which is about 8 Å away from active site, implying potential hydrogen bonds of the loop with the NO_2 moiety of 4-NPP (Supporting Information, Figure 1).

Summarizing, FKBP12, FKBP12.6, and FKBP51 can mediate FK506 effects on CaN activity in human cells, whereas other FK506/FKBP and all rapamycin/FKBP complexes do not contribute to the inhibition of CaN protein phosphatase activity. On the basis of our results FKBP12, FKBP12.6, and FKBP51 mediate CaN inhibition in complex with FK506 in Jurkat cells and can therefore be referred to as immunophilins. The three FK506/FKBP complexes can interact with CaN molecules without prior activation by CaM/Ca^{2+}.

SUPPORTING INFORMATION AVAILABLE

Secondary structure elements of FKBP forming the FK506 binding site and the composite surface interacting with CaN and an active site titration of FKBP. This material is available free of charge via the Internet at http://pubs.acs.org.

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