FK506, an immunosuppressant that prolongs allograft survival, is a co-drug with its intracellular receptor, FKBP12. The FKBP12-FK506 complex inhibits calcineurin, a critical signaling molecule during T-cell activation. FKBP12 was, until recently, the sole FKBP known to mediate calcineurin inhibition at clinically relevant FK506 concentrations. The best characterized cellular function of FKBP12 is the modulation of ryanodine receptor isofrom-1, a component of the calcium release channel of skeletal muscle sarcoplasmic reticulum.

Recently, a novel protein, FKBP12.6, was found to inhibit calcineurin at clinically relevant FK506 concentrations. We have cloned the cDNA encoding human FKBP12.6 and characterized the protein. In transfected Jurkat cells, FKBP12.6 is equivalent to FKBP12 at mediating the inhibitory effects of FK506. Upon binding rapamycin, FKBP12.6 complexes with the 288-kDa mammalian target of rapamycin. In contrast to FKBP12, FKBP12.6 is not associated with ryanodine receptor isoform-1 but with the distinct ryanodine receptor isoform-2 in cardiac muscle sarcoplasmic reticulum. Our results suggest that FKBP12.6 has both a unique physiological role in excitation-contraction coupling in cardiac muscle and the potential to contribute to the immunosuppressive and toxic effects of FK506 and rapamycin.

FK506 (tacrolimus) is a powerful immunosuppressive drug for treating graft rejection and autoimmune disorders. Rapamycin (RAP, 1 sirolimus) is an immunosuppressant structural-....
FKBP12.6 Can Mediate FK506 Sensitivity of T-Cell Signaling

FKBP12.6 was cloned and expressed from human brain, and the protein was purified as described previously (15). The ORFs encoding hFKBP12.6, hFKBP13-His6, and hCFP25-His6 were subcloned into the EcoRI site of pUC19, and the expression vectors were used in a PCR reaction, generating, from the hFKBP12.6 cDNA, a BamHI-linked DNA fragment containing the ORF of hFKBP12.6. This fragment was digested with BamHI and subcloned into pGEX2T (Pharmacia) at the BamHI site for protein expression and purification. Purified hFKBP12.6 may be stored at -70 °C or at 4 °C.

Construction and Expression of a GST-hFKBP12.6 Fusion Gene—Ssense (AAGCTTGGATCCGGCGTGGAGATCGAGACC) and antisense (AAGCTTTTGGATCCCTACTCTAAACTGGAGCAG) oligonucleotides were used in a PCR reaction to generate, from the hFKBP12.6 cDNA, a BamHI-linked DNA fragment containing the ORF of hFKBP12.6. This fragment was digested with BamHI and subcloned into pGEX2T (Pharmacia) at the BamHI site for protein expression and purification. Purified hFKBP12.6 may be stored at -70 °C or at 4 °C.

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FKBP12.6 Can Mediate FK506 Sensitivity of T-Cell Signaling

The purified material was fully active as determined by its complete binding to RAP-Sepharose and by its peptidyl-prolyl isomerase activity, which agreed with published values (for review, see Ref. 17). Purified hFKBP25 was stored at -70°C.

The ORF encoding the processed form of human FKBP13 (hFKBP13) fused to 10 histidine residues was generated by PCR using the sense oligonucleotide 5’-CAATGCTACTGGCAGCAAGAATGTGGTGCTCACTC-3’ and the antisense oligonucleotide 5’-AACCTGGATCATCTACTGCTGGTACCTG-3’. The PCR product was digested with Ndel and BamHl and cloned into the Ncol and BamHl sites of pET15b (Novagen), a polyhistidine fusion vector. hFKBP25 was expressed and purified as described for hFKBP13 above. This recombinant hFKBP13 and hFKBP12 are fully active as confirmed by their complete binding to FK506-Sepharose and by their peptidyl-prolyl isomerase activities, which agree with published values (for review, see Ref. 17). lcPF4 was expressed and purified to homogeneity as described previously (18). hFKBP13 and hFKBP25 were stored at -70°C, and hCyPA was stored at 4°C.

Protein Determinations—Protein determinations were performed according to the method of Bradford (19) using bovine plasma albumin (Bio-Rad) as the standard. The protein reagent concentrate was purchased from Bio-Rad.

FK506 Binding. CaN Phosphatase, and Peptidyl-prolyl Isomerase Assays—The LH-20 binding assay was performed as described previously (20) with the modifications noted (21). The CaN phosphatase assay has been described previously (22). When immunoenzymometric titrations were performed (60 μl concentrations), 0.1 μl of [3H]ryanodine was added to the reaction mixture. When drugs were titrated, the reaction mixtures were identical except that 50 μM immunohue was substituted for 50 μM drug. The R1I peptide, PDLVPRGFRDHRVSAEE, was phosphorylated at the peptide residue as described previously (22). The CaN phosphatase reaction mixtures were incubated for 30 min at 30°C, and dephosphorylation was initiated by peptide addition and allowed to proceed for 10 min at 30°C. Termination of the reaction and separation of free phosphate from phosphorylated peptide were performed as described previously (22). The peptidyl-prolyl isomerase assay was performed as described previously (13) on peptide substrates obtained from Bachem.

Construction of Expression and Reporter Constructs—The vector pcDL-SR was used for protein expression in Jurkat cells. A consensus ribosome binding site, CCACC (24), was inserted adjacent to the initiating codon of all ORFs. ORFs encoding the following were expressed, 20 μl of the supernatant from mock-transfected cells and from transfected cells were subjected to SDS-PAGE on a 16% denaturing gel (Novex) in parallel with concentration standards. Proteins were transferred to a membrane and analyzed by Western blotting as described previously (13). To detect hFKBP12, hFKBP12.6, or yFKBP12, the membrane was probed with a 1:10,000 dilution of an antipeptide antibody (R2935) directed against the sequence DVELLKLE. To detect hFKBP13 or hFKBP25, the membrane was probed with a 1:10,000 dilution of an antipeptide antibody (R2930) specific for the sequence LSNSSNIQ.

Preparation of Cardiac and Skeletal Muscle Sarco(pleo)plasmic Reticulum and Terminal Cisternae—The TC of skeletal muscle SR was isolated from dog or rabbit skeletal muscle as described previously (28). Cardiac microsomes were isolated from heart as described previously (29). Cardiac junctional SR was isolated from cardiac microsomes by sucrose density gradient centrifugation following Ca2+-phosphate loading as described previously (30).

Western Blot Analysis of Sarco(pleo)plasmic Reticulum Fractions—Muscle SR fractions were loaded onto SDS-PAGE gels separately or co-loaded with 30 ng of human recombinant FKBP12 or FKBP12.6. Proteins were separated by electrophoresis on a 12.5% polyacrylamide gel (12.5% acrylamide containing 2.6% cross-linker polymerized with 0.25% TEMED and 0.05% ammonium persulfate) at 90 V for 2 h. Western blotting
with a 1:10,000 dilution of a rabbit antipeptide antiserum to amino acids 3-16 of human FKBP12 (31) (recognizing FKBP12 and FKBP12.6) was performed as described previously (32).

Purification of FKBP12.6 from Cardiac RyR—The cardiac RyR (RyR-2) was purified as described previously (33). FKBP12.6 was isolated from RyR-2 by dissociation of the FKBP-drug complex from the RyR essentially as described for isolation of FKBP12 from the skeletal muscle RyR (32). Briefly, 100 μg of RyR-2 were incubated in Superose 6B column buffer (20 mM Tris-Cl (pH 7.4), 0.5 mM KCl, 0.5% CHAPS, 2 mM dithiothreitol, and 1 μM leupeptin) containing 12 μM [35S]Succinyl-Ala-Xaa-[N-γ-trityl]-Pro-Phe-[7-3H]Dihydro-FK506 (0.5 nM) as both purified bovine brain FKBP12.6 (13) and recombinant hFKBP12 (data not shown). The specific binding activities, measured using a modified LH-20 assay, of recombinant fFKBP12, hFKBP12, and hFKBP12.6 are approximately 30 ng of [3H]dihydro-FK506/μg of protein, a value in good agreement with that measured for FKBP12 (21). Because the Bradford assay used to measure protein concentration is known to overestimate protein concentration by 2.5-fold (21), the binding activities are close to the theoretical maximum expected for a 1:1 molar complex between FKBP and FK506. Despite a calculated molecular weight slightly less than that of hFKBP12, recombinant hFKBP12.6, like purified bovine brain FKBP12.6 (13), migrates more slowly on denaturing gels than hFKBP12 (Fig. 4).

The catalytic efficiency (kcat/Km) of hFKBP12 toward peptidyl-prolyl substrates correlates strongly with the hydrophobicity of the amino acid immediately preceding the proline (37). This contrasts with the promiscuous peptidyl-prolyl isomerase substrate specificity observed with CyPA, the binding protein for the structurally unrelated immunosuppressive drug, CsA. We compared the abilities of purified recombinant hFKBP12.6 and hFKBP12 to catalyze the isomerization to the trans form of tetratetrapeptides of the general structure N-succinyl-Ala-Xaa-dis-Pro-Phe-p-nitroanilide where Xaa is any one of 12 amino acids (Table I). hFKBP12.6 exhibits substrate preferences similar, but not identical, to those observed for hFKBP12. As with hFKBP12, substrates in which a hydrophobic amino acid precedes proline are greatly preferred by hFKBP12.6. For both FKBP12s, the most reactive substrates have Leu, Ile, Phe, or Nle at the Xaa position, while the least reactive substrate has Gly
suggesting that FKBP-C and FKBP12.6 are the same protein. To
grate slowly than hFKBP12 on SDS gels (32), sug-
both bovine (13) and human FKBP12.6 (Fig. 4), FKBP-C mi-
terized FKBP, termed FKBP-Cardiac (FKBP-C) (32). Like
RyR-1 in skeletal muscle and associates with a novel, unchar-
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grates slightly slower than hFKBP12 on SDS gels (32), sug-
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\begin{array}{|c|c|c|}
\hline
\text{Xaa} & \text{FKBP12.6} & \text{FKBP12} \\
\hline
\text{Nle} & 745 \pm 73 & 884 \pm 146 \\
\text{Ile} & 640 \pm 140 & 1,018 \pm 168 \\
\text{Leu} & 624 \pm 129 & 1,437 \pm 266 \\
\text{Phe} & 515 \pm 46 & 905 \pm 143 \\
\text{Val} & 313 \pm 16 & 448 \pm 53 \\
\text{Trp} & 88 \pm 1.8 & 180 \pm 42 \\
\text{Ala} & 70 \pm 15 & 197 \pm 21 \\
\text{His} & 58 \pm 2.6 & 35 \pm 7 \\
\text{Lys} & 57 \pm 6.4 & 107 \pm 53 \\
\text{Gin} & 38 \pm 3 & 3 \pm 2 \\
\text{Glu} & 1.2 \pm 1.1 & 2.0 \pm 1.3 \\
\text{Gly} & \text{No activity} & \text{No activity} \\
\hline
\end{array}
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confirm their identity. TC preparations from canine skeletal and
heart muscle SR fractions were analyzed by Western blot-
ing using an antibody that recognizes both FKBPs (Fig. 5).

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\text{Fig. 3. Steady-state hFKBP12.6 and hFKBP12 mRNA levels in}
\text{various human tissues and regions of the human brain.}
\text{Northern blots (Clontech) containing, per lane, 2 \mu g of poly(A)+ RNA from various}
\text{tissues or anatomically distinct regions of the human brain were probed}
\text{with the ^32P-labeled, randomly primed cDNAs encoding hFKBP12.6}
\text{(panels A, B, and E), hFKBP12 (panel F), or \beta-actin (panels C, D, and G). Panels C}
\text{and D are the actin controls for panels A and B, respectively. Panel G is the actin control for panels E and F, the same blot}
\text{probed with hFKBP12.6 and hFKBP12, respectively. Arrows show the}
\text{locations of molecular weight markers in kilobase (kb) pairs. Hybrid-
izations were performed under high stringency conditions and were}
\text{washed according to the manufacturer’s conditions. The mRNA sources}
\text{are as follows: lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas; lane 9, spleen; lane 10, thymus; lane 11, prostate; lane 12, testis; lane 13, ovary; lane 14, intestine; lane 15, colon; lane 16, peripheral blood lymphocytes; lane 17, amygdala; lane 18, caudate nucleus; lane 19, corpus callosum; lane 20, hippocampus; lane 21, hypothalamus; lane 22, substantia nigra; lane 23, subthalamic nucleus; and lane 24, thalamus. The exposure times were as follows: panels A, B, and E, 4 days; panel F, 12 h; and panels C, D, and G, 2 h.}
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at the Xaa position. With most of the tetrapeptide substrates
tested, the catalytic efficiency of hFKBP12.6 is roughly 2-fold
tower than that observed with hFKBP12. When Xaa is Val or
Nle, the catalytic efficiencies of hFKBP12 and hFKBP12.6 are
about equal. Only with the His-Pro substrate does hFKBP12.6
exhibit more reactivity than hFKBP12.

Heart Muscle RyR Is Associated with FKBP12.6—It is well-
known that FKBP12 is associated with the skeletal muscle
RyR (RyR-1) (6, 31, 38), stabilizing calcium flux through the
CRC (6–8). RyR-2 in heart muscle is an isoform distinct from
RyR-1 in skeletal muscle and associates with a novel, unchar-
acterized FKBP, termed FKBP-Cardiac (FKBP-C) (32). Like
both bovine (13) and human FKBP12.6 (Fig. 4), FKBP-C mi-
grates slightly slower than hFKBP12 on SDS gels (32), sug-
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\text{Fig. 4. Purity of recombinant hFKBP12 and hFKBP12.6. Five}
\mu g of purified bacterially-expressed hFKBP12 (lane 2) and hFKBP12.6
(lane 3) were subjected to SDS-PAGE on a 16% gel (Novex). The follow-
ing proteins (and their molecular weights) were used as standards (lane 1):
phosphorylase b, 106,000; bovine serum albumin, 80,000; ovalbu-
min, 49,500; carbonic anhydrase, 32,500; soybean trypsin inhibitor,
27,500; and lysozyme, 18,500.}
\]
Confirmation that FKBP12.6 is associated with the cardiac muscle RyR was obtained by amino-terminal sequencing of FKBP-C obtained from purified canine cardiac RyR preparations. FKBP-C was stripped from purified cardiac RyR with FK506 and separated from the RyR by hydroxyapatite chromatography. Amino-terminal sequencing of the purified protein gave the eleven amino acid sequence GVEIETISXGD, identical to the amino-terminal sequence of both bovine and human FKBP12.6 and different in two amino acids from the 11 amino-terminal amino acids of both bovine and human FKBP12, GQVETISPGD. The observations that the RyR purified from canine heart is associated with a protein that co-migrates with FKBP12.6 on denaturing gels and has the same amino-terminal sequence as both bovine and human FKBP12.6 indicates that FKBP12.6 is specifically associated with the canine heart RyR. In the cytosol of dog heart and in canine skeletal muscle TC, only FKBP12 has been detected (32), indicating that the interaction between FKBP12.6 and the heart RyR is specific and not due to the absence of FKBP12 in heart muscle.

There Are Four FKBP12.6 Molecules per Heart Muscle RyR—

The binding isotherm of [3H]dihydro-FK506 to canine cardiac muscle TC is a simple hyperbola with Scatchard analysis yielding a straight line indicative of a single class of FK506 binding sites (data not shown). The binding parameters, obtained from five different TC preparations, gave a dissociation constant (K_d) of 13.2 ± 4.8 nM and a B_max of 25.1 ± 5.5 pmol/mg of protein (Table II). The affinity of the interaction between FKBP12 and FK506 is lower than reported (21) (0.4–0.8 nM) due to the presence of 0.5% CHAPS in the assay (6). The CRC contains a single high affinity ryanodine binding site/homotetramer (33, 39). Therefore, the ratio of [3H]dihydro-FK506 binding to ryanodine binding is a measure of the FKBP12.6:RyR-2 protomer stoichiometry. Table II compares the number of [3H]dihydro-FK506 binding sites to ryanodine binding sites in several SR and TC preparations from rabbit and dog. The stoichiometry is approximately 4 mol of FKBP12.6/mol of canine cardiac muscle RyR homotetramer. This ratio is equivalent to the FKBP12:RyR-1 ratio observed in skeletal muscle (Table II) (6). Thus, the structure of the native CRC in canine heart muscle SR can be represented as (FKBP12.6)_n(RyR-2 protomer)_{n-2}.

The observation that FKBP12.6 associates specifically with the cardiac CRC makes it likely that FKBP12.6 modulates channel gating of the cardiac isoform in a manner similar to that observed for modulation of the skeletal muscle RyR-1 by FKBP12. Thus, the few amino acid differences between FKBP12 and FKBP12.6 have important consequences for channel binding specificity. We have expanded our characterization of hFKBP12.6 and have performed a pharmacological comparison of hFKBP12.6 and hFKB12 both in vitro and in Jurkat cells in an effort to uncover differences between the two molecules that might help to explain their apparently different physiological roles.

The hFKBP12.6–FK506 Complex is a Potent CaN Inhibitor In Vitro—We have compared the abilities of all known human FKBP (hFKBPs), yeast FKBP12 (yFKBP12), and human CyPA (hCyPA) to mediate inhibition of CaN phosphatase activity by FK506 in vitro. All FKBPs and hCyPA were expressed in E. coli and purified to homogeneity. hFKBP13 and hFKBP52 were histidine-tagged to aid in their purification while all other FKBPs, as well as hCyPA, contained only native sequences. The phosphatase assays were designed to measure CaN inhibition by the various immunophilin–drug complexes and to minimize any effect made by the equilibrium between the complex and the free immunophilin and drug molecules. Therefore, immunophilin–drug complex formation at a particular immunophilin or drug concentration was maximized by having an excess of one component. In one set of assays (Fig. 6A), drugs (FK506 or CsA) were titrated in the presence of a constant high concentration (50 μM) of immunophilin to insure that most of the added drug would be bound. In a second set of assays (Fig. 6B) the immunophils were titrated in the presence of a high concentration (50 μM) of drug to insure saturation of added binding protein. As expected, both types of assays gave similar results. Irrespective of which component is titrated, the IC_{SO} of FKBP12.6 is associated with the RyR of canine heart SR.

Samples of canine skeletal muscle terminal cisternae of SR (lanes 1–3) or cardiac SR (lanes 4–6) were analyzed by Western blot analysis. Samples were loaded in the absence (−) or presence (+) of either 30 ng of hFKBP12 (lanes 2 and 5) or 30 ng of hFKBP12.6 (lanes 3 and 6). Lanes 7 and 8 were loaded with 30 ng of hFKBP12 and 30 ng of FKBP12.6, respectively. The position of molecular weight standards, the bromphenol blue dye front (D) and the top of the resolving gel (T) are indicated to the left of the figure. The positions of bands corresponding to hFKBP12 and hFKBP12.6 are indicated at right. All other immunoreactive bands in lanes 1–6 are nonspecific because they are also observed in the absence of primary antibody. Cardiac SR was isolated in the presence of 0.6 M KCl, which is responsible for the band broadening (toward the bottom of the gel) and for the slightly slower mobility of both hFKBP12 and hFKBP12.6 in lanes 4–6.

![Fig. 5. FKBP12.6 is associated with the RyR of canine heart SR.](image)

The binding parameters for [3H]FK506 and [3H]ryanodine binding to skeletal (SKM) or cardiac muscle SR fractions were determined by Scatchard analysis. The ratio of B_max values for [3H]FK506 to [3H]ryanodine binding provides an estimate of the stoichiometry of FKBP per CRC or foot structure. The binding parameters were determined from five rabbit SKM–TC, two canine SKM–TC, five canine heart TC, and four canine heart SR preparations.

| Membrane fraction | FK506 binding | Ryanoide binding | Stoichiometry |
|-------------------|---------------|------------------|----------------|
|                   | K_d (nM)      | B_max (pmol/mg)  | K_d (nM)      | B_max (pmol/mg) | (FKBP/foot structure) |
| Rabbit SKM TC     | 6.8 ± 1.4     | 124 ± 25         | 8.5 ± 1.9     | 28 ± 5            | 4.4 ± 0.3             |
| Canine SKM TC     | 7.5 ± 5.0     | 84 ± 20          | 7.5 ± 0.5     | 16 ± 3.5          | 5.3 ± 0.3             |
| Canine heart TC   | 13.2 ± 4.8    | 25.1 ± 5.5       | 2.9 ± 0.8     | 7.8 ± 1.8         | 3.4 ± 0.7             |
| Canine heart SR   | 20.3 ± 4.4    | 15 ± 4.0         | 3.6 ± 0.4     | 3.7 ± 1.0         | 4.2 ± 0.7             |

TABLE II

Stoichiometry of FKBP12.6 and FKBP12 per CRC in cardiac and skeletal muscle sarcoplasmic reticulum, respectively

| Membrane fraction | FK506 binding | Ryanoide binding | Stoichiometry |
|-------------------|---------------|------------------|----------------|
|                   | K_d (nM)      | B_max (pmol/mg)  | K_d (nM)      | B_max (pmol/mg) | (FKBP/foot structure) |
| Rabbit SKM TC     | 6.8 ± 1.4     | 124 ± 25         | 8.5 ± 1.9     | 28 ± 5            | 4.4 ± 0.3             |
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| Canine heart TC   | 13.2 ± 4.8    | 25.1 ± 5.5       | 2.9 ± 0.8     | 7.8 ± 1.8         | 3.4 ± 0.7             |
| Canine heart SR   | 20.3 ± 4.4    | 15 ± 4.0         | 3.6 ± 0.4     | 3.7 ± 1.0         | 4.2 ± 0.7             |
FKBP12.6*818 is a powerful pharmacological probe for helping to identify CaN-dependent immunosuppression or toxicity. 818 binds tightly to hFKBP12, displacing FK506 (K<sub>i</sub> values are 0.7 and 0.4 nM for 818 and FK506, respectively), but the resulting hFKBP12*818 complex is a poor CaN inhibitor (4, 5). These observations suggest that the C-18 position of FK506, on the solvent-exposed face of the FKBP12-drug complex (41), contacts CaN, since the added hydroxyl group in 818 abolishes CaN binding, either by forcing the hydrophilic hydroxyl group into an unfavorable hydrophobic environment or through steric hindrance.

In noteworthy contrast to hFKBP12, the yFKBP12 complex with 818 is a potent CaN inhibitor (5). Surrounding FK506 on the solvent-exposed surface of the complex are approximately 26 amino acids that are likely to be close to CaN. Throughout these 26 residues, there are 10 differences between yeast and human FKBP12. Because the three-dimensional structures of the yeast and human FKBP12*818 complexes are almost identical, among the 10 changes in yFKBP12 there must be amino acids that directly interact with CaN, thereby compensating for 818 (42). These amino acids in yFKBP12 in some way neutralize the effects of the hydroxyl group at C-18 (5). In the absence of a crystal structure for the FKBP12-FK506-CaN complex, 818 is a useful pharmacological probe for helping to identify FKBP residues that might interact with CaN.

Of the 18 amino acid differences between hFKBP12.6 and hFKBP12, two residue changes in hFKBP12.6 (Arg<sup>90</sup> and Val<sup>91</sup> in Fig. 2), are among those 26 surface residues that surround the drug on the face of the complex. Because 818 uncovered differences between yeast and human FKBP12 not observed
FKBP12.6 Can Mediate FK506 Sensitivity in a T-Cell Line—CaN inhibition in vitro does not always correspond to inhibition of CaN-dependent signaling pathways in vivo. For example, although the cyclophilin C/CsA complex inhibits CaN in vitro, cyclophilin C does not mediate CsA-sensitivity in J urkat cells (12). The ability of hFKBP12.6 to mediate FK506-sensitivity in T-cells was therefore examined. Previously, the effects of overexpressing hFKBP12, hFKBP13, hFKBP25, and three of the human cyclophilins on the IC50 of FK506 and CsA were measured (12). Overexpression of an immunophilin will result, at equilibrium, in a greater concentration of the immunophilin—drug/CaN complex at a particular drug concentration. The previous experiments showed that overexpression of hFKBP12 rendered Jurkat cells 2–3-fold more sensitive to FK506, whereas overexpression of hFKBP13 and of hFKBP25 had no effect (12). This indicated that, among the three FKBP’s tested, only hFKBP12 can mediate the inhibitory effects of FK506 in cells.

We performed an assay similar to the one described (12), but to increase sensitivity, we incorporated an important modification. Overexpression of the catalytic subunit of CaN (CaNα) is known to render activated J urkat cells 4–5-fold less sensitive to the effects of FK506 and CsA (3, 43). Our modification was to overexpress the cDNAs encoding both the catalytic (CaNα) and regulatory (CaNβ) subunits of CaN by transient transfection in J urkat cells using the mammalian expression vector pCDL-SRα296 (SRα) (23). Protein overexpression was confirmed by Western blot analysis comparing extracts from transfected and nontransfected cells (Fig. 7), and drug sensitivity was quantitated by measuring β-galactosidase production from a co-transfected reporter plasmid, pL2.Gal, containing the IL-2 promoter fused to the β-galactosidase reporter gene. Overexpression of both CaNα and CaNβ rendered the cells insensitive to the effects of up to 10 nM FK506, almost 1000 times the normal IC50, 0.012 nM (Fig. 8A). There are two possible explanations for the drug insensitivity generated by CaN overexpression. Either the CaN is ectopically expressed in a subcellular compartment, separating it from the hFKBP12/FK506 complex, or the CaN levels have exceeded the hFKBP12 levels such that, even at the highest drug concentrations, there is sufficient free CaN available to participate in the signaling pathway. If the second explanation is correct, then co-expression of hFKBP12 will revert the cells to drug sensitivity and we will have generated an assay with a greatly amplified readout relative to the 2–3-fold shift in IC50 observed in the previous assay (12). To test between these alternatives, J urkat cells overexpressing CaNα and CaNβ were co-transfected with expression constructs encoding the FKBP’s tested in the previous assay (hFKBP12, hFKBP13, and hFKBP25) (12). Overexpression of hFKBP12, confirmed by Western blotting (Fig. 7), reestablishes the FK506-sensitivity of the cells (Fig. 8A). Reflecting the greatly increased amount of CaN that must be inhibited, the IC50 (0.26 nM) has shifted 20-fold relative to nontransfected cells (0.012 nM). This result indicates that CaN-overexpression is cytosolic and that hFKBP12 mediates FK506-sensitivity in a T-cell line, thereby validating our assay. In contrast, sensitivity to FK506 is not recovered upon hFKBP25 overexpression (Fig. 8A), confirming the results of Baram et al. (12) and our result that the hFKBP25/FK506 complex cannot inhibit CaN in vitro. We find that hFKBP13 has some ability to mediate the inhibitory effects of FK506, consistent with our observation (see Fig. 6) and with the observations of others (44, 45) that the hFKBP13/FK506 complex can inhibit, albeit weakly, CaN phosphatase activity.

Finally, the ability of hFKBP12.6 to restore FK506 sensitivity in CaN-overexpressing J urkat cells was examined. We also tested hFKBP52 and yFKBP12 since the FK506 complexes with these FKBP’s have been characterized for CaN inhibition, in vitro (5, 46), but have not been tested in J urkat cells. Transfection of the CaN-overexpressing J urkat cells with the cDNA encoding hFKBP12.6 restored FK506-sensitivity (Fig. 8B). The IC50 of FK506 in the cells overexpressing hFKBP12.6 is 0.18 nM, demonstrating that hFKBP12.6 is equipotent to hFKBP12 at mediating the inhibitory effects of FK506 upon CaN-dependent signaling events in J urkat cells and corroborating our result that the hFKBP12.6 and hFKBP12 complexes with FK506 are equipotent CaN inhibitors in vitro. Also in agreement with the CaN phosphatase assay, yFKBP12 is equipotent to both hFKBP12 and hFKBP12.6 at mediating the inhibitory effects of FK506 in J urkat cells (Fig. 8B). In contrast, the FK506 complex with hFKBP52 is only a weak inhibitor of signaling since, even at 10 μM drug concentrations, it is unable to completely block IL-2 promoter activation (Fig. 8B). T-Cells Can Be Made Sensitive to ‘818 by Overexpression of
FKBP12.6 Can Mediate FK506 Sensitivity of T-Cell Signaling

FKBP12.6—To confirm the efficacy of the various FKBs at mediating the inhibitory effects of FK506, we used the antagonist and closest FK506 relative, '818, which cannot block IL-2 promoter stimulation in activated Jurkat cells containing wild-type levels of CaN (Fig. 8C). We have previously demonstrated (Fig. 6, C and D) that, at high hFKBP12 or hFKBP12.6 concentrations, '818 can inhibit CaN in vitro. Therefore, we reasoned that by overexpressing FKBs relevant to the inhibitory effects of FK506, the IL-2 promoter could be made sensitive to '818, thereby converting it from an antagonist to an agonist. Because '818 is such a weak agonist, its use in this assay provides a more stringent test of the abilities of the various FKBs to mediate inhibition of FK506-sensitive signaling pathways in the cell. Moreover, it allows distinctions to be made between FKBs, such as between yFKBP12 and hFKBP12, that cannot be observed when FK506 is used. Therefore, expression plasmids encoding each of the known human FKs (as well as yFKBP12) were co-transfected into Jurkat cells with the pL2.Gal reporter plasmid activated in the presence of FK506, the amount of β-galactosidase was measured, and the IC50 values were determined.

Activities are plotted as the percent of β-galactosidase produced in the absence of drug (No-Drug Controls) varied by less than 15% among the various transfectants, thereby demonstrating that transfection efficiencies were equivalent and uniform. Each data point represents the mean of three experiments with a standard error of less than 10%. Panel A, FK506-insensitivity caused by overexpression of the catalytic (CaNa) and regulatory (CaNβ) subunits of CaN is reversed by overexpression of hFKBP12. Jurkat cells transfected with pL2.Gal were mock-transfected or co-transfected with the SRα expression vector containing the indicated cDNAs and activated in the presence of FK506, the amount of β-galactosidase was measured, and the IC50 values (in parentheses) for FK506 were determined:

- SRα vector only (no inhibition); ○, CaNa and CaNβ (no inhibition); ●, hFKBP12, CaNa, and CaNβ (0.26 nM); ■, hFKBP13, CaNa, and CaNβ (partial inhibition); O, hFKBP25, CaNa, and CaNβ (no inhibition). Panel B, FK506-insensitivity caused by CaN overexpression is reversed by overexpression of hFKBP12.6. The expressed proteins and the IC50 values (in parentheses) for FK506 are as follows:
  - SRα vector only (replotted from panel A; 0.012 nM); ○, CaNa and CaNβ (replotted from panel A; no inhibition); ◮, hFKBP12.6, CaNa, and CaNβ (0.18 nM); ◯, hFKBP52, CaNa, and CaNβ (partial inhibition); x, yFKBP12, CaNa, and CaNβ (0.37 nM). Panel C, effect of FKBP overexpression on the IC50 of '818. Jurkat cells transfected with pL2.Gal were mock-transfected or co-transfected with the SRα expression vector containing the indicated cDNAs and activated in the presence of '818, the amount of β-galactosidase was measured, and the IC50 values (in parentheses) of '818 were determined:
  - '818 mock transfection (no inhibition); ◮, yFKBP12 (2.7 µM); ○, hFKBP12 (2.7 µM); □, hFKBP12.6 (2.3 µM); ■, hFKBP13 (no inhibition); ○, hFKBP25 (no inhibition); x, yFKBP52 (no inhibition). Panel D, CaSα insensitivity caused by CaN-overexpression is reversed by overexpression of hCyPA. Jurkat cells transfected with pL2.Gal were mock-transfected or co-transfected with the SRα expression vector containing the indicated cDNAs and activated in the presence of CsA, the amount of β-galactosidase was measured, and the IC50 (in parentheses) of CsA determined:
  - CsA mock transfection (0.57 µM); ○, SRα vector only (0.45 µM); ○, hCyPA, CaNa, and CaNβ (27.5 µM); ●, hFKBP12, CaNa, and CaNβ (24.2 µM); ◯, hFKBP12.6, CaNa, and CaNβ (19.5 µM); ■, hFKBP13, CaNa, and CaNβ (24.3 µM); ○, hFKBP25, CaNa, and CaNβ (25.9 µM); x, hCyPA, CaNa, and CaNβ (3.0 µM).

hFKBP12.6 is equipotent to hFKBP12 at mediating the FK506-sensitivity of the IL-2 promoter in transfected Jurkat cells. Activities are plotted as the percent of β-galactosidase produced in the absence of drug (No-Drug Controls) varied by less than 15% among the various transfectants, thereby demonstrating that transfection efficiencies were equivalent and uniform. Each data point represents the mean of three experiments with a standard error of less than 10%. Panel A, FK506-insensitivity caused by overexpression of the catalytic (CaNa) and regulatory (CaNβ) subunits of CaN is reversed by overexpression of hFKBP12. Jurkat cells transfected with pL2.Gal were mock-transfected or co-transfected with the SRα expression vector containing the indicated cDNAs and activated in the presence of FK506, the amount of β-galactosidase was measured, and the IC50 values (in parentheses) for FK506 were determined:

- SRα vector only (no inhibition); ○, CaNa and CaNβ (no inhibition); ●, hFKBP12, CaNa, and CaNβ (0.26 nM); ■, hFKBP13, CaNa, and CaNβ (partial inhibition); O, hFKBP25, CaNa, and CaNβ (no inhibition). Panel B, FK506-insensitivity caused by CaN overexpression is reversed by overexpression of hFKBP12.6. The expressed proteins and the IC50 values (in parentheses) for FK506 are as follows:
  - SRα vector only (replotted from panel A; 0.012 nM); ○, CaNa and CaNβ (replotted from panel A; no inhibition); ◮, hFKBP12.6, CaNa, and CaNβ (0.18 nM); ◯, hFKBP52, CaNa, and CaNβ (partial inhibition); x, yFKBP12, CaNa, and CaNβ (0.37 nM). Panel C, effect of FKBP overexpression on the IC50 of '818. Jurkat cells transfected with pL2.Gal were mock-transfected or co-transfected with the SRα expression vector containing the indicated cDNAs and activated in the presence of '818, the amount of β-galactosidase was measured, and the IC50 values (in parentheses) of '818 were determined:
  - '818 mock transfection (no inhibition); ◮, yFKBP12 (2.7 µM); ○, hFKBP12 (2.7 µM); □, hFKBP12.6 (2.3 µM); ■, hFKBP13 (no inhibition); ○, hFKBP25 (no inhibition); x, yFKBP52 (no inhibition). Panel D, CaSα insensitivity caused by CaN-overexpression is reversed by overexpression of hCyPA. Jurkat cells transfected with pL2.Gal were mock-transfected or co-transfected with the SRα expression vector containing the indicated cDNAs and activated in the presence of CsA, the amount of β-galactosidase was measured, and the IC50 (in parentheses) of CsA determined:
  - CsA mock transfection (0.57 µM); ○, SRα vector only (0.45 µM); ○, hCyPA, CaNa, and CaNβ (27.5 µM); ●, hFKBP12, CaNa, and CaNβ (24.2 µM); ◯, hFKBP12.6, CaNa, and CaNβ (19.5 µM); ■, hFKBP13, CaNa, and CaNβ (24.3 µM); ○, hFKBP25, CaNa, and CaNβ (25.9 µM); x, hCyPA, CaNa, and CaNβ (3.0 µM).
We have cloned the cDNA encoding human FKBP12.6 and have characterized the expressed protein pharmacologically and physiologically. Physiologically, FKBP12.6 has a role distinct from that of FKBP12. FKBP12 is associated with RyR-1 of skeletal muscle SR, whereas FKBP12.6 is specifically associated with RyR-2 of cardiac muscle SR. Pharmacologically, FKBP12.6 is almost indistinguishable from FKBP12. FKBP12.6 is the only other FKBP family member equipotent to FKBP12 at inhibiting CaN in vitro and at mediating the FK506-sensitivity of a CaN-dependent signal transduction pathway. Moreover, when complexed with RAP, FKBP12.6, like FKBP12, binds mTOR.

The cardiac CRC (RyR-2) is a 565-kDa protomer 64% identical to RyR-1 (50, 51). The hydrophathy profiles and predicted secondary structures of the cardiac and skeletal isoforms are virtually identical (51). Both are activated by Ca2+ and caffeine; both are inactivated by Mg2+ and ruthenium red; and both contain one high affinity and several low affinity ryanodine binding sites (52). Although morphologically and functionally similar, the channels are not identical (52). We have shown that FKBP-C (32), isolated from the canine cardiac RyR, co-migrates with hFKBP12.6 on SDS-PAGE gels and has the same 11-amino acid amino-terminal sequence as both bovine and human FKBP12. Our finding that there are four FK506 binding sites per high affinity ryanodine binding site in cardiac SR suggests that the structure of the cardiac CRC can be represented as (RyR-2)(FKBP12.6)x, analogous to the structure of the skeletal muscle CRC, (RyR-1)(FKBP12). Thus, the native CRC isoforms in heart and skeletal muscle SR are further distinguished from one another in that different FKBP isoforms comprise a portion of their structures. The structural and functional similarities between FKBP12.6 and FKBP12 and between the cardiac and skeletal muscle RyR isoforms, suggests that the role of FKBP12.6 in the native cardiac CRC is similar to the role of FKBP12 in the skeletal muscle CRC.

The stoichiometry of four molecules of FKBP12.6 per native tetrameric CRC obtained by [3H]dihydro-FK506 binding isotherms relies on the assumption that the ryanodine receptor is the predominant or only SR protein that binds FKBP12.6. Recent studies confirm that this is the case. Endogenous FKBP of cardiac SR was exchanged with the GST-FKBP12.6 fusion protein using exchange methodology developed for the skeletal muscle RyR (9). The TC was then solubilized with CHAPS, and protein complexes with the GST-FKBP12.6 fusion protein were affinity purified on a GST-Sepharose affinity column. RyR-2 was the predominant protein in the SR that was tightly bound to GST-FKBP12.6.

In the presence of drug, the abilities of several human FK-BPs to inhibit CaN in vitro and a CaN-dependent signaling pathway in cells have been compared. The abilities of the immunophilin-drug complexes to inhibit CaN and their abilities to block IL-2 transcription correlate precisely. The FK506 complexes with FKBP13 and FKBP52, weak CaN inhibitors in vitro, are weak inhibitors of IL-2 promoter activity when the proteins are overexpressed in Jurkat cells. This correlation extends to the yFKBP12-818 complex, a more potent CaN inhibitor and a more potent inhibitor of IL-2 promoter stimulation than the 818 complexes with hFKBP12 or hFKBP12.6. Thus, our results support the proposed mechanism of action of FK506 in which CaN inhibition blocks IL-2 transcription, thereby preventing T-cell activation (54). The RAP complexes with hFKBP12.6 and hFKBP12 exhibit similar but not identi-

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FKBP12.6 and FKBP12 are physiologically associated with distinct RyR isoforms may be unrelated to peptidyl-prolyl isomerase activity. Both in the presence and absence of drug, the biochemical and cellular read-outs used in our study have demonstrated that FKBP12.6 is highly similar to FKBP12 and that, where it is abundant, FKBP12.6 will be an important mediator of the inhibitory effects of FK506 and RAP. Our inability to uncover any significant biochemical or pharmacological differences between the two immunophils that might account for their overlapping, and yet distinct, physiological roles suggests further complexities among the FKBP12s remaining to be understood.

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