Characterization of High Molecular Weight FK-506 Binding Activities Reveals a Novel FK-506-binding Protein as Well as a Protein Complex*

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The immunoregulant FK-506 potently inhibits particular calcium-associated signal transduction events that occur early during T-lymphocyte activation and during IgE receptor-mediated exocytosis in mast cells. FK-506 binds to a growing family of receptors termed FK-506-binding proteins (FKBPs), the most abundant being a 12-kDa cytosolic receptor, FKBP12. To date, there is no formal evidence proving that FKBP12 is the sole receptor mediating the immunosuppressive effects or toxic side effects of FK-506. Using gel filtration chromatography as an assay for novel FK-506-binding proteins, we identified FK-506 binding activities in extracts prepared from calf brain and from JURKAT cells. Both of these new activities comigrated with apparent molecular masses of 110 kDa. However, further characterization of both binding activities revealed that the two are not identical. The 110-kDa activity observed in brain extracts appears to be the FKBP12-FK-506-calcineurin (CaN) complex previously reported (Liu, J., Farmer, J., Lane, W., Friedmann, J., Weissman, I., and Schreiber, S. (1991) Cell 66, 807-815) while the 110 kDa activity observed in JURKAT cells is a novel FK-506-binding protein. Our characterization of the FKBP12-FK-506-CaN complex reveals a dependence upon calmodulin (CaM) for the peptidyl-prolyl cis-trans isomerase (PPIase) activity of FKBP12 is not required for binding of FKBP12-FK-506 to CaN or for inhibition of CaN phosphatase activity. The novel FK-506-binding protein in JURKAT cells has been purified to homogeneity, migrates with an apparent mass of 51 kDa on denaturing gels, and has been termed FKBP51. Like FKBP12, FKBP51 has PPIase activity, but, unlike FKBP12-FK-506, FKBP51-FK-506 does not complex with or inhibit the phosphatase activity of CaN. These results indicate that complex formation with CaN may not be a general property of the FKBPs. Peptide sequencing reveals that FKBP51 may be similar, if not identical, to hsp56, a component of non-transformed steroid receptors.

The macrolide FK-506 is a powerful immunosuppressant that, like the cyclic undecapeptide drug, cyclosporin A (CsA), inhibits specific calcium-dependent signal transduction events leading to T-lymphocyte activation, selectively blocking transcription of a set of coordinately expressed, early-phase genes crucial for lymphocyte growth and differentiation (2). Like CsA, FK-506 also selectively blocks calcium-dependent intracellular signaling events in other, possibly related, signal transduction pathways (3-5). FK-506 binds to cytosolic receptors (6) which are distinct from the major CaA receptor, cyclophilin A (7). A 12-kDa cytosolic FK-506-binding protein, FKBP12, was purified and characterized first (8, 9), and, like cyclophilin A, has since been shown to be a member of a growing family of receptors termed FKBPs (10-12). In addition to binding immunosuppressive ligands, another property shared by the FKBPs and the cyclophilins is that they are both peptidyl-prolyl isomerases (PPIases), enzymes that catalyze isomerization about peptidyl-prolyl bonds (8, 9, 12-14). Like cyclophilin A, FKBP12 is one of the most abundant cytosolic proteins in eukaryotes, is found in most tissues and cell types (15), and is extraordinarily well-conserved throughout phylogeny. These observations suggest that FKBP12 has a critical and central role in cellular physiology and may explain why FK-506 has a number of toxic side effects in animals and man (16). A structural relative of FK-506, rapamycin, is another immunomodulator which also binds to the FKBPs but whose immunosuppressive activity is a consequence of a block in the proliferative response of T-cells to growth-promoting lymphokines (17). The observations that FK-506 and rapamycin bind to a family of receptors and affect multiple signaling pathways are indicative that FKBPs may have multiple and diverse roles within cells.

Recently, it has been demonstrated that FKBP12-FK-506 binds specifically to the calcium and calmodulin (CaM)-dependent serine/threonine phosphatase, calcineurin (CaN), inhibiting its phosphatase activity in vitro (1). FKBP12, without drug present, will not bind to CaN. Furthermore, two members of the human cyclophilin family, cyclophilins A and C, will also bind to and inhibit CaN only when they are complexed with CsA. These results implicate CaN as a common downstream target of both FK-506 and CsA and help to explain the parallel effects of the two drugs. That one of the steps

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† The abbreviations used are: CsA, cyclosporin A; FKBP, FK-506-binding protein; PPIase, peptidyl-prolyl cis-trans isomerase; CaM, calmodulin; CaN, calcineurin; FMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.
involved in the early T-cell activation events is a calcium-
regulated dephosphorylation event and is also consistent with
these observations. CaNs have been implicated in controlling
signal transduction pathways emanating from the second
messenger, calcium (18), but prior to the discovery that immu-
nosuppressors such as FK-506 and rapamycin bind to and non-
lymphoid cells. In crude extracts prepared both from calf
brain and from the T-lymphocytic line, JURKAT, we discov-
ered two FK-506 binding activities that both migrate with
apparent molecular masses of about 110 kDa on gel filtration
columns. Subsequent to the report that FKBP12 binds to
CaN in the presence of FK-506 (1), we performed reconstitut-
ion experiments using purified proteins and found that the
FKBP12-FK-506-CaN complex comigrates with the 110-kDa
activities as observed in calf brain extracts. In this report we
confirm and extend the characterization of the FKBP12-FK-
506-CaN complex. We also characterize the 110-kDa FK-506
binding activity in JURKAT cells and show that it is a protein
similar in sequence to the hsp65 component of rabbit progres-
tin receptor complex (20).

MATERIALS AND METHODS

Purification of FKBP51 from JURKAT Cells—A cytosolic extract
prepared from 3 x 10^11 cells was prepared as described (5), dialyzed
in buffer containing 10 mM potassium phosphate (pH 7.2), 5 mM β-
mercaptoethanol, 1 mM EDTA, and 0.5 mM PMSF, and applied to a
5 x 20 cm Affi-Gel Blue column equilibrated with the same buffer.
After washing the column with 2 column volumes of equilibration buffer,
the column was washed with a linear gradient (total volume,
2 liters) of 100-500 mM potassium phosphate (in the equilibration
buffer). Fractions (12.5 ml each) were collected at a flow rate of 125
ml/h. FK-506 binding activity eluted between 200 and 325 mM
potassium phosphate. Active fractions were combined (total volume
124 ml) and applied to a 5 x 15 cm Phenyl-Superose HR 5/5 fast
protein liquid chromatography column equilibrated with buffer contain-
ing 25 mM HEPES (pH 7.5), 5 mM β-mercaptoethanol, 0.5 mM PMSF,
and 50 mM KC1. The column was washed with the equilibration buffer
containing 100 mM potassium phosphate and then with equilibration buffer
containing 250 mM KCI, and fractions (6.5 ml each) were collected at a flow rate of 120 ml/h. Active fractions were
concentrated by ultrafiltration to 8.6 ml and dialyzed against 4
liters of 25 mM Bis-Tris (pH 6.3). The concentrated material was
applied to a MonoP HR 5/20 fast protein liquid chromatography
chromatofocusing column equilibrated in the dialysis buffer. After
washing the column with 10 column volumes of the dialysis buffer,
the column was developed with 80 ml of polybuffer 74 (pH 4.0), and
fractions (1 ml) were collected at a flow rate of 1 ml/min. The active
binding fractions, which eluted between pH 4.6 and 4.1, were pooled
and dialyzed overnight against buffer containing 25 mM
HEPES (pH 7.5), 50 mM KCl, and 5 mM β-mercaptoethanol. The
dialyzed material was dialyzed again for 2 h against buffer containing
50 mM sodium phosphate (pH 7.0), 0.6 M (NH4)2SO4, and 5 mM
β-mercaptoethanol and applied to a phenyl-Superose HR 5/5 fast
protein liquid chromatography column equilibrated in the same
buffer. The column was developed with a linear gradient (total
volume, 5 ml) of 0.6-9.0 M (NH4)2SO4 in 50 mM sodium phosphate
(pH 7.0), 0.6 M (NH4)2SO4, and 5 mM β-mercaptoethanol were collected at a flow rate of 0.5 ml/min. Active fractions were combined,
dialyzed in 4 liters of the DEAE-Sepharose equilibration buffer,
and concentrated to 1.6 ml by ultrafiltration. Protein was assayed using
the Bio-Rad protein assay (Bio-Rad). Purified FKBP51 was stored
at -80°C.

To obtain peptides suitable for amino acid sequence analysis,
FKBP51 was treated with trypsin in a 50-μl reaction containing 50
mM ammonium bicarbonate, pH 9.0, and a trypsin/FKBP51 ratio of
1:100 (w/w). FKBP51 was digested at 37°C for 16 h after which time
the digest was neutralized with 5 μl of 10% trifluoroacetic acid.
Tryptic peptides were separated by reverse-phase HPLC using a 1.0
x 100-mm C18 column (ABI, Foster City, CA) and a buffer gradient
of 2% H2O/0.005% trifluoroacetic acid to 50% acetonitrile/H2O
containing 0.05% trifluoroacetic acid. Peptides were sequenced directly from PVDF membranes using an ABI 477 protein
sequencer (ABI).

Preparation of Calcitonin Genomic Assay—Calcitonin was homo-
ogenized in buffer (14, w/v) containing 10 mM Tris (pH 7.5), 100 mM KC1, 5 mM β-mercaptoethanol, 1 mM PMSF, and 1 mM EDTA. Homogenization
was performed for 1 min at 4°C in a Waring blender. The homogenate
was subjected to centrifugation (8,000 x g, 30 min) and further
centrifuged in ultracentrifugation (44,000 x g, 1 h) in a Ti-45 rotor.

P-3DG Assay for FKBP51—Forty-five μg of Bio-Gel P-3DG were reconstituted in 500 ml of TSK buffer containing 20 mM sodium phosphate
(pH 6.8), 50 mM Na2SO4, 5 mM β-mercaptoethanol, and 1 mM EDTA. Fractions, containing 0.1-10 μg of protein, were incubated with 50 nM [3H]dihydroFK-506 (50.4
mCi/ml) in TSK buffer containing 0.5% (w/v) BSA (Miles) in a volume of 100 μl. Bound and unbound [3H]dihydroFK-506 were separated by chromatography on individual 2-ml columns as
described for the LH20 assay (7).

Peptidyl-prolyl cis-trans Isomerase Assay—Peptidyl-prolyl isomer-
ization was assayed as previously described (5) with the following
changes: the peptide substrate used was N-succinyl-Ala-Leu-Pro-
Phenylalanine (BACHEM, Biberach, West Germany) and the
ammonium concentration of 66 mM. The release of p-nitroanilide by chymotrypsin
was quantitated by measuring the increase in absorbance at 405 nm using a Beckman DU68 spectrophotometer. After an initial rapid increase in absorbance due to hydrolysis of the trypsin peptide, the
slow secondary increase in absorbance, which reflects the conversion
of cis peptide to trans peptide, was measured at 3-s intervals.
The data were fit to a simple first-order rate law and the first-order
rate constant, k (s-1), calculated. Ks values for the inhibition of FKBP51
PPlase activity by FK-506 and rapamycin were determined at the first-order rate constant, k, on inhibitor concentr-
ation using a computer program written by Nancy Thornberry of
the Department of Enzymology, Merck Research Laboratories (28).

Calcineurin Binding Assay—Incubations (total volume, 500 μl)
were performed for 15 min at 30°C and contained various combina-
tions of the following components: 1.5 μg of purified bovine calcineu-
rin, 1 μg of recombinant human FKBP12, 1 μg of bovine calmodulin
(Sigma), and 100 nM [3H]dihydroFK-506. The incubation buffer
contained 20 mM Tris (pH 7.5), 100 mM NaCl, 6 mM MgCl2, 0.1 mM
CaCl2, 0.1 mg/ml BSA, and 0.5 mM dithiothreitol. The incubation
buffer, containing a chymotrypsinogen/A, C, T, G, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
C, A, G, C, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T, C, A, G, A, T,
the recommendations outlined by Martin and Castro (23). The oligonucleotide synthesized and the corresponding human FKBPI2 amino acid sequence from which it was derived is shown below.

HIS TYR THR GLY MET LEU GLU ASP GLY LYS LYS PHE ASP CAT TAT ACG GGI ATG TTT GAI GAT GGI AAI TTT GA C C C

The JURKAT cDNA library was plated on Escherichia coli strain C600 hflA. To isolate the human FKBPI2 cDNA, ten 150-mm plates containing approximately 30,000 plaques/plate of the JURKAT cDNA library were screened. Plate replicates were made on Nitran membranes (Schleicher & Schuell). Denaturation, renaturation, and baking of the phage DNA to the filters were performed according to the manufacturer’s instructions. The filters were prehybridized and then hybridized to the32P-labeled oligonucleotide probe according to the methods described by Itoh et al. (24). Briefly, the filters were prehybridized for 4 h at 45°C in a solution containing 5 × SSC, 10 × Denhardt’s, 50 mM sodium phosphate, and 0.1% SDS. The32P-labeled oligonucleotide probe (1 ng/ml, 4 × 106 cpm/ml) was added to the prehybridization solution and the filters hybridized at 45°C for 16 h. The filters were washed four times at 20 min/wash at room temperature in a washing solution containing 5 × SSC, 0.1% SDS, and 50 mM sodium phosphate. This was followed by an additional wash at 65°C for 1 min. The wet filters were wrapped in Saran Wrap and exposed to x-ray film (XAR-5, Eastman Kodak) for 36 h at −80°C using intensifying screens. Positive phage were plaque-purified, and the phage insert was subcloned into the EcoRI site of pUC19. Sequencing of both DNA strands by the dyeoxy method was performed directly from denatured plasmid miniprep DNA. The cDNA was in partial that the open reading frame was missing the nucleotides encoding the NH2-terminal 11 amino acids. The nucleotide sequence of this partial cDNA clone was in agreement with the sequence of the human FKBPI2 (from nucleotides 34 through 1454) that was published by Maki et al. (25) after we had obtained our own clone. Based upon the NH2-terminal sequence of human FKBPI2, the open reading frame of the FKBPI2 cDNA was completed using the synthetic oligonucleotide CATTGGGAGTGCAGGTGGAAACCATCTCCCCAGGA.

**Bacterial Expression of Human FKBPI2**—A 5′ sense primer containing a NcoI site and a 3′ antisense primer containing a BamHI site were used in a polymerase chain reaction reaction to generate a 329-bp NcoI-BamHI fragment encoding the entire open reading frame for FKBPI2. This fragment was ligated into NcoI-BamHI-digested pET3d (26) and transformed into BL21 (DE3) cells. Plasmid-containing cells were grown in M9 media containing 50 pg/ml ampicillin (final concentration) and isopropyl-1-thio-(3-D-galactopyranoside (0.1 mM) to induce expression of the fusion protein. The cells were allowed to grow until the OD600 reached 0.8 OD units. Isopropyl-1-thio-(3-D-galactopyranoside and a YM-5 membrane. FKBPI2 was purified from the concentrated cell extract (2 mg), and the supernatant collected, and proline and sialic acid were removed by centrifugation, 0.5 mg PMSF. Yields varied between 300 and 200 mg of FKBP12/liter of bacterial culture. The purified protein appeared homogeneous by Coomassie staining of SDS-polyacrylamide gels and had nearly identical FKBPI2 binding and PPIase activities compared to FKBPI2 isolated from JURKAT cells. Amino acid sequencing of the recombinant protein demonstrated that the first 38 amino acids matched those encoded by the cDNA except that the NH2-terminal methionine was missing.

**Expression and Purification of Radiolabeled FKBPI2**—An overnight culture of the FKBPI2-producing bacterial strain described above was used to inoculate 50 ml of M9 media containing 50 µg/ml ampicillin such that the OD600 was 0.1 OD units. When the OD600 reached 0.4 OD units, 3 mCi of [35S]methionine (1233 Ci/mmol, Amersham Corp.), 3 mCi of [35S]cysteine (1198 Ci/mmol, Amersham Corp.), and isopropyl-1-thio-β-D-galactopyranoside (0.5 mM final concentration) were added, and the culture was incubated overnight at 37°C. The cells were lysed and FKBPI2 purified to radiochemical homogeneity as described above. The specific activity of the radiolabeled protein was 1.3 Ci/mol.

**Construction of the F36Y FKBPI2 Mutant**—The TTT (Phe) codon at position 36 in human FKBPI2 was changed to TAC (Tyr) by a polymerase chain reaction using overlapping mutant primers using a procedure described by Ho et al. (27). Oligomers flanking the ATG and TGA codons were used in a subsequent polymerase chain reaction to incorporate the F36Y alteration into the entire open reading frame. The resulting fragment was digested with NcoI and BamHI and ligated to NcoI- and BamHI-digested pET3d vector DNA. After transformation into BL21(DE3) cells, the 327-base pair DNA fragment encoding the mutant FKBPI2 of one recombinant was sequenced to verify the presence of the mutation. The F36Y isolate used in these studies has an insertion of 5 residues (TAC) at the NcoI site, and as a result cannot be cut with this enzyme. The presence of these additional residues did not significantly alter expression levels of this protein in E. coli compared with the wild-type recombinant FKBPI2 gene that has an intact NcoI site.

Cells expressing FKBPI2 F36Y were grown in LB media containing 100 µg/ml ampicillin supplemented with 0.4% glucose until the OD600 reached 1.0–1.5 OD units. Then the cells were induced with 0.1 mM (final concentration) isopropyl-1-thio-β-D-galactopyranoside and grown for an additional 15 h. The induced cells were subjected to centrifugation for 10 min at 5000 × g, resuspended in 1/50 of the original culture volume with 20 mM Tris (pH 7.4) and stored at −70°C until purified as described above.

**RESULTS**

**Purification of a Novel FK-506 Binding Activity from JURKAT Cells**—We prepared extracts from JURKAT cells and from a non-lymphoid tissue, calf brain, incubated the extracts with [3H]dihydroFK-506 and fractionated the incubation mixture by HPLC gel filtration. In addition to the major peak of radioactivity associated with FKBP12 (Fig. 1, peak B), we observed in both extracts, a smaller peak of activity corresponding to a protein with a relative mass of 110 kDa (Fig. 1, peak A). Attempts to purify the 110-kDa FK-506 binding activity from extracts of calf brain proved unsuccessful. In contrast, the 110 kDa binding activity from JURKAT cells was more amenable to purification. Table I presents a summary of the purification of the activity from extracts of nonactivated JURKAT cells. Like all other FK-506-binding

**Fig. 1.** [3H]dihydroFK-506 associates with a high molecular weight factor(s) in both JURKAT cells and bovine brain. Extracts (S-100) were prepared from JURKAT cells and bovine brain as described under "Materials and Methods." Binding reactions (500 µl) contained: 200 nM [3H]dihydroFK-506 and either JURKAT extract (2 mg), or calf brain extract (0.5 mg). Mixtures were incubated for 30 min at 37°C and fractionated by HPLC gel filtration using a 0.3 × 600-mm TSK-250 column (Bio-Rad). 0.4 ml fractions were collected and 0.35 ml counted in 3 ml of Aquasol II (Amersham Corp.). A denotes the position of the high molecular weight [3H]dihydroFK-506 complex and B, the position of the FKBP12-FK-506 complex.

D. Boulton, unpublished results
proteins purified to date, this new FKBP does not bind CsA and the FK-506 binding is competitively inhibited by rapamycin, another PPIase, in earlier purification steps. Furthermore, the standard LH-20 assay used to measure PPIase activity because the activity was completely retained by the LH-20 resin. Therefore, a binding assay using Bio-Gel P-6DG gel filtration as described in Table I and using the PPIase assay, FKBP51 appears to be fully active when compared to both FKBP12 and FKBP25. These discrepancies may be due to an underestimation of FK-506 binding using Bio-Gel P-6DG due to the presence of cyclophilin A, also a PPIase, in earlier purification steps. Furthermore, the standard LH-20 assay used to measure PPIase activity (Fig. 4) allowing kinetic data to be used to assess the affinity of these ligands for FKBP51. We have calculated a $K_i$ of $116 \pm 39$ nM for the inhibition of FKBP51 PPIase by FK-506 and a $K_i$ of $345 \pm 185$ for rapamycin. These values indicate that FKBP51 binds FK-506 and rapamycin with approximately a 100-300-fold lower affinity than FKBP12 ($K_i$ values approximately 1 nM (28)).

Peptide sequencing of the protein revealed a single NH$_2$ terminus, a further indication that FKBP51 was homogenous (Table II). Shown below six of the seven peptide sequences derived from FKBP51 is an alignment with related sequences.

**FIG. 2.** Panel A, SDS-polyacrylamide gel electrophoresis of purified FKBP51. Protein from each step of the purification of FKBP51 was subjected to SDS-polyacrylamide gel electrophoresis and stained with combined Coomassie Brilliant Blue and silver as previously described (8). Lane 1, protein standards; lanes 2–6, protein (100 ng) from steps I–V (Table I), respectively. Panel B, reverse-phase HPLC of step V-purified FKBP51. 50 µl (11 µg of protein) of step V-purified FKBP51 was applied to a 1.0 × 100-mm, ABI C4 reverse-phase column. FKBP51 was eluted using a buffer gradient of 15% H$_2$O, 0.6% trifluoroacetic acid to 75% 90/10 acetonitrile, H$_2$O containing 0.55% trifluoroacetic acid. Chromatography was performed using an ABI 130 separation system.

**FIG. 3.** HPLC gel filtration of FKBP51. 0.5 mg of step III-purified FKBP51 was incubated with 100 nM [H]dihydroFK-506 for 30 min at 37°C and subjected to HPLC gel filtration as described in the legend for Fig 1. 8 µg of recombinant human FKBP12 was assayed in a similar manner. The inset indicates the position of FKBP51 relative to molecular weight standards. ○, FKBP51; □—□, FKBP12.
FK-506 Binding Proteins

FKBP51

**Alignment between amino acid sequences derived from sequencing human FKBP51 and the corresponding sequences from the open reading frame of cloned rabbit uterine hsp56**

The first alignment (A) is between the NH₂-terminal sequence of FKBP51 with an internal sequence of rabbit hsp56. All other alignments are between sequences of tryptic peptides from human FKBP56 with internal sequences of the cloned rabbit gene. FKBP51 peptide fragments were generated by trypsin treatment as described under "Materials and Methods."

| A. FKBP51 | APPLMEGVVD15PKQDEG |
| Rabhsp56 | APPLMEGVVD15PKQDEG |
| B. FKBP51 | ATESIAYLAPYYAFR |
| Rabhsp56 | GEHSLYLKPSYAFG |
| C. FKBP51 | VLQLYPNNK |
| Rabhsp56 | VLQLYPNNK |
| D. FKBP51 | MALLIYK |
| Rabhsp56 | QALLQYK |
| E. FKBP51 | LETGVVS |
| F. FKBP51 | YELHVK |
| Rabhsp56 | YELHVK |
| G. FKBP51 | YWD1A |
| Rabhsp56 | YWD1A |

found in the open reading frame predicted by the DNA sequence of the cDNA encoding rabbit uterine hsp56 (20). Hsp56 is a heat shock protein found in nontransformed steroid receptor complexes and contains a domain having 55% identity to FKBP12. It is not known if rabbit uterine hsp56 binds FK-506 or if it has PPⅠase activity. The similarity of six of the seven FKBP51 peptide sequences to sequences in rabbit hsp56 as well as the similarity in size of the two proteins suggests that FKBP51 is either the human homolog of rabbit uterine hsp56 or another closely related protein.

**FK-506-FKBP12 Complex Formation with Calcineurin**

As specified previously, we were unable to purify the 110 kDa activity from bovine brain even though an FK-506 binding activity, similar to that observed in JURKAT extracts, was clearly present in crude extracts. Recently, it has been shown that FKBP12-FK-506 complexes bind to the Ca²⁺ and CaM-dependent protein phosphatase, CaN (1). In view of the fact that CaN is extraordinarily abundant in brain, we reasoned that the 110-kDa [³H]dihydroFK-506 complex observed in brain extracts might be such a complex. In order to test this hypothesis, we first determined the requirements for complex formation between FKBP12 and CaN.

We find that incubation of bovine CaN, bovine CaM, recombinant human FKBP12, [³H]dihydroFK-506, Ca²⁺, and Mg²⁺ results in the formation of a protein complex which has an apparent mass of about 110 kDa as determined by HPLC gel filtration (Fig. 5A). The larger peak of radioactivity migrating with an apparent mass of 12 kDa is due to free FKBP12-³H]dihydroFK-506 complexes. In the absence of CaN, no complex is detected and all of the [³H]dihydroFK-506 is bound to FKBP12 alone (Fig. 5A). Furthermore, we find that in the absence of CaM, the 110-kDa complex is virtually undetectable (Fig. 5A). A residual amount of complex is occasionally observed in the absence of CaM but may be the result of CaM contamination of CaN. CaM binds with very high affinity to CaN (29) and is difficult to remove completely. The potent CaM antagonist, calmidazolium (30), was used to verify our observation that formation of the FKBP12-FK-506-CaN complex is dependent upon CaM. Addition of calmidazolium to an incubation mixture containing all of the components required for complex formation prevented formation of the complex (Fig. 5B). Calmidazolium had no inhibitory effect on FK-506 binding by FKBP12. These results differ from those reported by Liu et al. (1) who did not observe a strict CaM requirement for FKBP12-CaN complex formation. Using labeled FKBP12, we also confirmed that FKBP12-calcineurin complex formation is ligand-specific. Rapamycin, which binds to FKBP12 with an affinity similar to that of FK-506, but which is inactive as an inhibitor of interleukin-2 synthesis (2), fails to promote complex formation with CaN (Fig. 5C).

**Nature of the High Molecular Weight FK-506 Complex in Bovine Brain**

Our finding that complex formation between FKBP12-FK-506 and CaN is dependent upon CaM provided us with the means to directly determine the makeup of the high molecular weight FK-506 complex observed in crude bovine brain extracts. Addition of calmidazolium to brain extracts almost totally abolishes formation of the 110-kDa peak (Fig. 6A). Addition of calmidazolium to the same amount of protein from JURKAT extracts also partially reduces the 110-kDa peak (Fig. 6B). These results suggest that virtually all of the 110 kDa binding activity in brain is due to FKBP12-FK-506-CaN-CaM complex while, in JURKAT extracts, a majority of the 110-kDa FK-506 binding activity is due to FKBP12. Interestingly, when FKBP51 was substituted for FKBP12 in either the CaN binding assay or the CaN phosphatase assay, no complex formation or inhibition of phosphatase activity was observed (data not shown). These observations suggest that CaN binding, in the presence of FK-506, is not a general property of FK-506-binding proteins.

**PPⅠase Activity of FKBP12 Is Not Required for CaN Complex Formation**

The conformation of FK-506 when bound to FKBP12 is known to be dramatically different from the conformations existing in solution. The amide bond of FK-506 exists in both cis and trans conformations in solution while only the trans form is associated with FKBP12. In addition, the pyranose ring is on the outside of the macrocycle when FK-506 is in solution but is on the inside of the macrocycle when it is bound to FKBP12 (31–33). It is not known if the enzymatic activity of FKBP12 is responsible for the
observed alterations in the conformation of FK-506 upon binding. By site-directed mutagenesis, we have constructed a number of single amino acid changes in residues that are most conserved throughout phylogeny in FKBP12. One mutant, termed F36Y (a Tyr for Phe substitution at position 36), has 85–90% of the FK-506 binding activity relative to wild-type FKBP12 (data not shown) but has less than 0.1% of the wild-type's PPIase activity (Fig. 7A). The requirements for functional PPIase activity in the FK-506-dependent inhibition of CaN phosphatase activity were examined using the PPIase-deficient human FKBP12 mutant. Increasing amounts of wild-type recombinant FKBP12 or PPIase-deficient FKBP12 were added to a cocktail containing all of the components necessary for complex formation and for measurement of CaN phosphatase activity (Fig. 7B). The wild-type and the PPIase-deficient FKBP12 proteins exhibited nearly identical IC_{50} values in the CaN phosphatase assay. These results indicate that the PPIase activity of FKBP12 is not required for the inhibition of CaN phosphatase activity by FKBP12-FK-506 complexes.

**DISCUSSION**

Using gel filtration as a method to identify novel FK-506 binding activities and/or high molecular weight complexes, we observed peaks of activity in crude extracts prepared from lymphoid and non-lymphoid sources that were well-separated from the previously identified peak corresponding to FKBP12. Although both of the new activities migrated with identical apparent molecular masses of approximately 110 kDa, further characterization of the activities revealed important differences. We were unable to further purify the activity from JURKAT cells, while purification of the activity from calf brain, when compared with the activity from JURKAT cells, yielded a homogenous protein with an apparent molecular mass on SDS-polyacrylamide gel electrophoresis gels of 51

**FIG. 5. Complex formation between FKBP12-[\textsuperscript{3}H]dihydroFK-506 and CaN.** Panel A, requirements for FKBP12-CaN complex formation. Complex formation was assayed by HPLC gel filtration as described in the legend to Fig. 1. Assays (500 \mu l) contained: 1 \mu g FKBP12, 100 nM [\textsuperscript{3}H]dihydroFK-506, 5 \mu g of bovine brain CaN and 1 \mu g of bovine brain CaM. Complete reaction, ●—●; no CaN, ▲—▲; no CaM, O—O, and no FKBP12, Δ—Δ. Panel B, inhibition of FKBP12-CaN complex formation by calmidazolium. Assay conditions were identical to those used in panel A. All components present, ●—●; plus 36 \mu M calmidazolium, O—O. Panel C, rapamycin-FKBP12 complex fails to bind CaN. Assay conditions were essentially identical to panel A with the following changes; assays contained, 600 \mu g of \textsuperscript{35}S-labeled FKBP12 (specific activity 1.6 \times 10^5 cpm/\mu g), 20 \mu g of CaN, 5 \mu g of CaM with 500 nM FK-506 (●—●), rapamycin (▲—▲), or no drug (O—O).

**FIG. 6. Effect of calmidazolium on the high molecular weight [\textsuperscript{3}H]dihydroFK-506 complex in bovine brain and JURKAT cells.** Assay conditions are as described in the legend for Fig. 1. Panel A, [\textsuperscript{3}H]dihydroFK-506 + bovine brain extract, ▲—▲; with calmidazolium, ▲—▲. Panel B, [\textsuperscript{3}H]dihydroFK-506 + JURKAT extract, ●—●; with calmidazolium O—O.

**FIG. 7. Panel A, PPIase of recombinant human FKBP12 and the FKBP12 mutant F36Y.** PPIase activity was assayed as described under "Materials and Methods." Wild type FKBP12, □—□; F36Y mutant, ■—■. Panel B, FKBP12 and F36Y inhibition of calcineurin phosphatase activity in the presence of FK-506. Calcineurin phosphatase activity was assayed as described under "Materials and Methods." Wild-type FKBP12, ●—●; F36Y mutant, O—O.
kDa. This suggests that the new protein, which we have labeled FKBP51, either forms a dimer in its native state or, due to an unusual tertiary shape, migrates anomalously on sizing columns.

Peptide sequence analysis of FKBP51 suggests that it will be similar, if not identical, to hsp56, a heat shock protein found complexed with hsp70 and hsp90 in nontransformed pituitary, glanulosa, androblast, and estradiol receptor complexes isolated from the human lymphocytic line, IM-9. Purified human hsp56 has been subjected to Edman degradation and the last six amino acids of the 20 obtained from NH2-terminal sequencing (34) match the first six amino acids that we obtained from NH2-terminal sequencing of FKBP51. Although the cDNA encoding human hsp56 has not been isolated, the cDNA encoding rabbit hsp56 has been cloned and sequenced. Five of six sequenced tryptic peptides from FKBP51 are similar to amino acid sequences found in the translation product of the rabbit cDNA sequence. The open reading frame of rabbit hsp56 contains regions highly homologous to known FK-506-binding proteins indicating that it is a member of the FKBP family. However, experiments demonstrating that human or rabbit hsp56 bind to FK-506 in solution or that they have PPlase activity have not been reported. Recently, a 60-kDa human protein purified by affinity to immobilized FK-506 has been described (35). It is not known if this 60-kDa protein binds FK-506 in solution or if it has PPlase activity. Nevertheless, one might speculate that if the protein revealed that it has the same NH2-terminal sequence as human hsp56 (34). Presently, there is no enough sequence information from FKBP51, human hsp56, or from the 60-kDa protein isolated on the FK-506 affinity column to determine whether or not all three proteins are identical. Although it is possible that the difference we observe at the NH2 terminus is due to proteolysis of FKBP51 during purification, isolation and sequencing of the corresponding cDNA must be performed in order to firmly establish the degree of relatedness between FKBP51 and hsp56.

If FKBP51 is, in fact, the human homolog of rabbit hsp56, it will be interesting to determine what effect FK-506 binding to FKBP51 has upon integrity of protein-protein interactions in glucocorticoid receptor complexes or if FK-506 can compete with glucocorticoids for binding. The latter result might suggest that mammalian cells have an endogenous small molecule ligand that is mimicked by FK-506. Like FKBP12, we have found that FKBP51 isomerizes peptide-prolyl bonds in peptide substrates; however, no cellular substrates for FKBP12 or FKBP51 have been identified to date. It is not known if hsp56 has PPlase activity but if FKBP51 and hsp56 are identical, then hsp70, hsp90, or the glucocorticoid receptor itself would be obvious candidates for an endogenous substrate. One could imagine that subsequent to steroid binding by the glucocorticoid receptor, FKBP51-catalyzed isomerization about a particular peptide-prolyl bond helps promote disassociation of the proteins contained in the complex.

Two of our results demonstrated that the 110 kDa binding activity discovered in calf brain was different from the activity we purified from JURKAT cells. First, the activity in the brain extract was labile and could not be purified. Once the cDNA encoding FKBP51 is obtained, multiple tissue Northern blots will be performed in order to determine if the low level of FKBP51 activity we have observed in brain is due to weak expression of the corresponding mRNA in that tissue. Second, subsequent to the report that FKBP12-FK-506 binds to CaN (1), we added the CaM inhibitor calmidazolium to crude extracts prepared from both sources and were unable to detect any 110 kDa binding activity in the brain extract. In contrast, activity was retained in the crude extract prepared from JURKAT cells when calmidazolium was added. We have confirmed the observation that FKBP12-FK-506 binds specifically to the phosphoprotein phosphatase, CaN, inhibiting its phosphatase activity and the observation that FKBP12-rapamycin does not complex with CaN. The latter observation correlates well with the experimental evidence that, in vivo, FK-506 and rapamycin are potent reciprocal antagonists of one another (36, 37). Another potent competitive antagonist of FK-506, L-685,818, also binds with equivalent affinity to FKBP12 but fails to promote CaN complex formation.

In contrast to previous findings (1), we observe a dependence upon CaM for the binding of FKBP12-FK-506 to CaN. In a reconstituted system using purified components, almost no complex was detected unless CaM was present in the incubation mixture. Further corroboration of the CaM requirement was made using the CaM antagonist, calmidazolium. Addition of calmidazolium either to the complete, reconstituted system or to the crude brain extract completely abolished FKBP12-FK-506-CaN complex formation. The residual complex formation observed in our reconstituted system when CaM was omitted from the reaction mixture might be due to the presence of small amounts of CaM in the CaN preparation. CaN has been identified as the major binding protein for the calcium receptor, CaM (39). CaM binding may evoke a conformational change in CaN which neutralizes a COOH-terminal CaN inhibitory domain (40). Our results are consistent with this model which proposes that in vivo, native CaN (in the absence of CaM) exists in a conformational state unsuitable for FKBP12-FK-506 binding. Interaction with CaM would reveal a site for FKBP12-FK-506 binding. Previous reports of FKBP12-FK-506 interaction in the absence of CaM (1) might be explained by the investigators use of partially purified CaN which may be contaminated with CaM or by the presence of proteolytically fragmented CaN. The reported interaction of FKBP12-FK-506 complex with the 43-kDa CaN A fragment lacking the CaM binding and autoinhibitory domains (41) would be consistent with our model since the catalytic and FKBP12-FK-506 binding domains would be expected to be exposed. Further clarification of whether there is a strict CaM requirement for FKBP12-FK-506-CaN complex formation or whether CaM enhances complex formation might be made using recombinant bacterially produced CaN once it becomes available.

CaN has a heterogeneous tissue distribution and is extremely abundant in the brain, comprising nearly 1% of total brain protein (42). CaN is much less abundant in peripheral tissues although it has also been identified as the major CaM binding protein in T-lymphocytes (43). The FKBP12-FK-506 interaction with CaN was originally discovered in extracts prepared from brain and CaN's tissue distribution explains why we observed so much of the complex in the extracts prepared from brain. The FKBP12-FK-506-CaN interaction has not yet been reported using extracts prepared from T-lymphocytes although the partial calmidazolium sensitivity of our 110-kDa peak in JURKAT extracts is strongly suggestive that the same interaction does, in fact, occur in drug-treated T-cells.

We have identified an FKBP12 mutant, F36Y, that has near-normal affinity for FK-506 but which has less than 1/1000th the PPlase activity of FKBP12. Furthermore, the wild-type and F36Y mutant proteins are equivalent in their abilities to inhibit CaN phosphatase activity in the presence of FK-506. Our observations indicate that the PPlase activity

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of FKBP12 is unrelated to its affinity for FK-506 and that the conformation of FK-506 required for interaction with CaN is not induced by the PPIase activity of FKBP12. These results also correlate well with the observation that inhibition of PPIase activity is not germane to the immunosuppressive activity of FK-506 (36, 37). CaA, like FK-506, is markedly hydrophobic and, again like FK-506, has strikingly different activities of their respective binding proteins but that the CaN is not induced by the PPIase activity of FKBP12. These results also correlate well with the observation that inhibition of PPIase activity is not germane to the immunosuppressive activity of FK-506 (36, 37). CaA, like FK-506, is markedly hydrophobic and, again like FK-506, has strikingly different conformations when free in organic solvents and when bound to its cognate immunophilin (44, 45). However, recent results strongly suggest that cyclophilin does not induce the bound conformation of CaA but binds a conformation preexisting in aqueous solution (38). Collectively, all of these results suggest that FK-506 and CaA are not substrates for the PPIase activities of their respective binding proteins but that the bound conformations are the preferred ones in the hydrophobic binding pockets of FKBP12 and cyclophilin, respectively.

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