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FKBP51, a Novel T-Cell-Specific Immunophilin Capable of Calcineurin Inhibition

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The immunosuppressive drugs FK506 and cyclosporin A block T-lymphocyte proliferation by inhibiting calcineurin, a critical signaling molecule for activation. Multiple intracellular receptors (immunophilins) for these drugs that specifically bind either FK506 and rapamycin (FK506-binding proteins [FKBPs]) or cyclosporin A (cyclophilins) have been identified. We report the cloning and characterization of a new 51-kDa member of the FKBP family from murine T cells. The novel immunophilin, FKBP51, is distinct from the previously isolated and sequenced 52-kDa murine FKBP, demonstrating 53% identity overall. Importantly, Western blot (immunoblot) analysis showed that unlike all other FKBPs characterized to date, FKBP51 expression was largely restricted to T cells. Drug binding to recombinant FKBP51 was demonstrated by inhibition of peptidyl prolyl isomerase activity. As judged from peptidyl prolyl isomerase activity, FKBP51 had a slightly higher affinity for rapamycin than for FK520, an FK506 analog. FKBP51, when complexed with FK520, was capable of inhibiting calcineurin phosphatase activity in an in vitro assay system. Inhibition of calcineurin phosphatase activity has been implicated both in the mechanism of immunosuppression and in the observed toxic side effects of FK506 in nonlymphoid cells. Identification of a new FKBP that can mediate calcineurin inhibition and is restricted in its expression to T cells suggests that new immunosuppressive drugs may be identified that, by virtue of their specific interaction with FKBP51, would be targeted in their site of action.

Cyclosporin A (CsA), FK506, and rapamycin are potent immunosuppressive drugs that inhibit T-lymphocyte proliferation. The action of these drugs is mediated by intracellular receptors, termed immunophilins, that bind either CsA (cyclophilins) or FK506 and rapamycin (FK506-binding proteins [FKBPs]) (for reviews, see references 43 and 47). Previous studies identified these receptors as abundant, cytosolic proteins possessing an inherent peptidyl prolyl *cis-trans* isomerase (PPIase; rotamase) activity that is inhibited by drug ligand binding (12, 16, 46).

The first FKBP to be described in detail at the protein (16, 46) and cDNA (25, 53) levels was FKBP12, a ubiquitous immunophilin of 11.8 kDa highly conserved in eukaryotes. More recently, additional members of this family have been identified on the basis of their ability to bind FK506 and rapamycin. Sequence analysis of the cloned genes corresponding to FKBP12.6, FKBP13, FKBP25, and FKBP52 (named to reflect their molecular weights) from mammalian sources reveals extensive conservation of amino acid sequence, in particular in the protein domain responsible for drug ligand binding and PPIase activity (for reviews, see references 15 and 58).

Although the actions of FK506 and rapamycin are mediated by the same family of intracellular receptors, these drugs achieve immunosuppression by different mechanisms (5). Rapamycin inhibits a calcium-independent event involved in the proliferative response of T cells to growth factors and progression through the cell cycle (1, 20, 28). In contrast, FK506 and CsA act earlier in the T-cell response during the calcium-dependent signal transduction pathway culminating in production of various growth factors, including interleukin-2 (47). Recent studies demonstrate that it is the direct interaction of the immunophilin-drug complex with calcineurin phosphatase, a key component in the pathway of interleukin-2 gene transcription, and the resultant inhibition of phosphatase activity that are pertinant to the mechanism of immunosuppression by both FK506 and CsA (10, 14, 24, 31). Of the identified FKBPs, only two, FKBP12 and FKBP12.6, have been shown in vitro to be potent inhibitors of calcineurin in the presence of drug (8, 45) and are therefore believed to be biologically relevant to immunosuppression. The ability of FKBP12 and FKBP12.6 to mediate the inhibitory effects of FK506 on interleukin-2 gene transcription has been confirmed in transfection experiments in which overproduction of FKBP12 or FKBP12.6 results in increased sensitivity to FK506 (8, 57a).

Although FK506 is a potent immunosuppressant, its clinical utility has been limited by significant neurological and nephrological adverse side effects (21, 30). Recent evidence suggests that the toxic effects of FK506 at these sites are related to inhibition of calcineurin (11). Thus, while FKBP expression in T lymphocytes mediates the immunosuppressive effects of FK506, the ubiquitous expression of calcineurin and members of the FKBP family that mediate calcineurin inhibition is directly responsible for the toxicity of these drugs. We report the cloning and characterization of a new, highly abundant member of the FKBP family, FKBP51, that mediates FK506 inhibition of calcineurin in vitro and is largely restricted in its expression to T lymphocytes. Identification of this new FKBP suggests a means to selectively target drug effects to T cells.

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MATERIALS AND METHODS

Chemical reagents. Reagents for Western blot (immunoblot) analyses, including alkaline phosphatase coupled goat anti-rabbit antibody, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine (BCIP), and nitroblue tetrazolium chloride, were obtained from Bio-Rad. Glutathione-Sepharose 4B used in affinity purification of the FKBP fusion protein was obtained from Pharmacia. Dexamethasone and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma. Forskolin was obtained from Calbiochem. Bisdiazotized benzidine used to couple synthetic peptide to keyhole limpet hemocyanin carrier protein (Pierce) was prepared as previously described (57), using benzidine purchased from Sigma. Radiolabeled [^{35}S]methionine (1,053 Ci/mmol) was obtained from Amersham Corporation. L-683,590 (FK520) and rapamycin were obtained from Merck Research Laboratories.

Cell culture. The WEHI-7TG cell line is a thioguanine-resistant derivative of the murine thymoma cell line WEHI-7 (6, 18). WEHI-7TG cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Cultures were maintained in humidified 87% air–13% CO_2.

cDNA library construction and screening. The construction of oligo(dT)-primed and randomly primed λZAPII cDNA libraries, using RNA from WEHI-7TG cells treated for 5 h with dexamethasone (1 μM) and forskolin (12 μM), and the subtractive hybridization procedure used to identify induced genes have been described elsewhere (17). The pBluescript SK(-) plasmid containing cDNA encoding FKBP51 (clone 213) was excised from the λZAPII vector by using an in vivo excision protocol (Stratagene). An approximately 500-nucleotide fragment from the 5′ end of the 2.2-kb cDNA insert of clone 213 was isolated by restriction enzyme digestion with *Eco*RI and *Xho*I, labeled to 10^9 cpm/μg with the Multiprime DNA-labeling kit (Amersham), and used as a hybridization probe for library screening as previously described (17). Two libraries, a murine thymus λZAP cDNA library (Stratagene) and a λ UniZAPII cDNA library prepared from poly(A)$^+$ RNA isolated from WEHI-7TG cells treated with dexamethasone (1 μM) and forskolin (12 μM) for various time intervals (0 min, 15 min, 30 min, 1 h, 2 h, or 5 h), were screened to obtain full-length cDNAs corresponding to clone 213. A combination of restriction enzyme digestion analysis and preliminary sequence determination using vector-encoded primers was used to identify the longest cDNA clones (approximately 3.7 kb) from each library. Clone 213-12A was obtained from the thymus library, while clone 213-19 was isolated from the WEHI-7TG λ UniZAPII library.

DNA sequencing and computer analysis. The nucleotide sequence of the cDNA corresponding to FKBP51 was determined by the dideoxy-chain termination method (41) with synthetic oligonucleotides as primers. Nested deletions of clone 213-12A were generated by the Erase-a-Base system (Promega) to determine the nucleotide sequence of the 5′ region containing the open reading frame (ORF) and approximately 700 bases of 3′ untranslated sequence. The sequence of an additional 59 nucleotides of 5′ untranslated region (UTR) was derived from analysis of clone 213-19. Comparisons of the nucleotide sequence with sequences in GenBank and the deduced amino acid sequence with sequences in SwissProt for FKBP51 were performed at the National Center for Biotechnology Information, using the BLAST network service (2). Alignment of the FKBP51 protein sequence with sequences of FKBP family members was performed by using the PILEUP progressive, pairwise alignment parameters contained in the Genetics Computer Group (University of Wisconsin) software package.

In vitro rabbit reticulocyte translation. The 3.7-kb *Eco*RI cDNA insert of clone 213-12A was inverted in orientation with respect to the pBluescript SK(-) vector to create clone 213-12A′, which contains the intact ORF for FKBP51 under the transcriptional control of the T7 viral promoter. DNA was linearized by digestion at the unique vector-encoded *Xba*I site at the 3′ end of the cDNA insert. Uncapped RNA synthesized in vitro by using a commercially available T7 polymerase transcription system (Promega) was translated by a rabbit reticulocyte lysate (Promega) in the presence of 7.5 μCi of [^{35}S]methionine (1,053 Ci/mmol). The radioactively labeled translation products were visualized by separation on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels followed by treatment with Fluorohance (Research Products International), drying, and autoradiography. Commercially available protein standards (Bio-Rad) were run as molecular weight markers.

Antiserum production. Synthetic peptide YGESQAMEEGKEKGHV was prepared on a Synergy peptide synthesizer (Applied Biosystems) and removed from the solid resin by trifluoroacetic acid cleavage as described by the manufacturer. The peptide was purified by methyl *t*-butyl ether extraction, solubilized in 20% acetonitrile, separated from the resin by filtration, and lyophilized. The purity of the resultant peptide was examined by mass spectroscopy (Anthony Craig, Salk Institute). The synthetic peptide was coupled to keyhole limpet hemocyanin carrier protein at a 3:1 weight excess of carrier to peptide, using bisdiazotized benzidine (57). The bisdiazotized benzidine reagent was removed following coupling by dialysis against 0.9% NaCl. New Zealand White rabbits were injected subcutaneously at 3-week intervals with 0.5 mg (injections 1, 2, and 3) or 0.25 mg (injections 4 and 5) of coupled peptide in Freund's complete (injection 1) or incomplete (injections 2 to 5) adjuvant. Bleeds were taken 2 weeks postinjection, the blood was allowed to clot, and the serum was collected by centrifugation and stored at −20°C. Preparation of the rabbit antipeptide antibody directed against the human FKPB12 amino acid sequence DVELLKLE has been previously described (45).

Purification of the GST-FKBP51 fusion protein. The ORF of clone 213-12A was amplified by PCR technology with the following primers and subcloned into the glutathione *S*-transferase (GST) fusion vector pGEM-3X (Pharmacia). The forward primer (CGGGATCCGGACAATGACTACTGATGAG, 28-mer) contains a flanking *Bam*HI site (underlined), five nucleotides preceding the translational initiation site (ATG) for FKBP51, and sequence corresponding to the first four amino acids of the protein. The reverse primer (CGGAATTCCGT CATACATGGCCTTTGGC, 28-mer) contains a flanking *Eco*RI site (underlined) and the sequence complementary to the region corresponding to the last 15 nucleotides of the ORF, the stop codon, and the 2 following adjacent nucleotides. The amplified fragment was digested with *Bam*HI and *Eco*RI, ligated into pGEM-3X, and transformed into *Escherichia coli* BL21 cells. Production of the GST-FKBP51 recombinant fusion protein was induced by the addition of 0.5 mM IPTG, and the overexpressed protein was purified by glutathione-Sepharose 4B affinity chromotography as previously described (48). The GST-FKBP51 protein was stored at −105°C in the presence of 10% glycerol.

Western analysis. Murine tissues (brain, heart, kidney, liver, lung, spleen, and thymus) were excised from 4-week-old BALB/c mice, frozen in liquid nitrogen, and disrupted by homogenization in a lysis buffer as described by Ruff et al. (39). The protein extracts were clarified by centrifugation at 20,000 × *g* for 30 min at 4°C and stored at −105°C. WEHI-7TG cells were collected by centrifugation, washed in phosphate-buffered saline (PBS), and resuspended in lysis buffer. The cells were disrupted by freeze-thawing, and protein extracts were prepared as described above. Protein concentrations of the tissue and WEHI-7TG extracts and of purified GST-FKBP51 protein were determined by Coomasie blue dye binding (7). Proteins were separated on an SDS–10% (for analysis of FKBP51) or SDS–15% (for analysis of FKBP12) polyacrylamide gel and subsequently transferred to a BA85 nitrocellulose membrane (Schleicher & Schuell) at 1 mA/cm for 1 h, using a semidry transfer apparatus (Novablot; LKB). The membrane was blocked for 16 h at 4°C in PBS containing 5% powdered milk. The membrane was washed for 10 min in two changes of PBS containing 0.05% Tween 20 (PBST) and then incubated for 2 h at room temperature in PBS containing 1% powdered milk and an antibody against the FKBP51 peptide (1:2,000 dilution) or against the FKBP12 peptide (1:2,500 dilution). Unbound antibody was removed by washing at room temperature with three changes of PBST for a total of 30 min. The membrane was incubated in PBS containing 1% powdered milk and alkaline phosphate-coupled goat anti-rabbit antibody (1:8,000 dilution) at room temperature for 90 min. The membrane was washed at room temperature with three changes of PBST for a total of 30 min and then washed twice for 5 min each time in PBS. Antibody complexes were detected with BCIP and nitroblue tetrazolium chloride color development reagents.

PPIase assay. PPIase assays were performed as described previously (12) except for the following changes: the peptide substrate used was *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (BACHEM) at a final concentration of 72 μM, and chymotrypsin (Sigma) was used at a concentration of 6 μM. Protein concentrations (200 nM GST-FKBP51 or 83 nM recombinant human FKBP12) that were determined to be in the linear response range for isomerization of the peptide were used in the assay. Reactions (in a 1.5-ml mixture) were initiated by addition of the peptide. The data were fitted to a simple first-order rate equation, and the first-order rate constant, k (s^{-1}), was calculated. Results are expressed as a percentage of the activity in the absence of drug. Each datum point represents the average of triplicate assays with a standard error of less than 10%.

Calcineurin phosphatase inhibition assay. The calcineurin phosphatase reaction is performed as follows. The final concentrations of each of the components of the reaction are as follows: 40 mM Tris (pH 8.0), 100 mM NaCl, 6 mM magnesium acetate, 100 μM $CaCl_2$, 100 μg of bovine serum albumin (fraction V) per ml, 500 μM dithiothreitol, 40 μM [^{33}P]RII peptide (600 cpm/pmol; Peptides International), 190 nM bovine calmodulin, 3 nM bovine calcineurin, 50 μM drug (rapamycin or L-683,590), and either GST-FKBP51 or recombinant human FKBP12 at the indicated concentrations. The RII peptide has the sequence DLDVPIPGRFDRRVSVAAE, and phosphorylation at the serine residue was carried out as described previously (24, 26). Reaction mixtures (60 μl) were incubated at 30°C for 30 min in the absence of labeled RII peptide. Reactions were initiated by addition of the peptide, and the dephosphorylation reaction was 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 (24, 26). Calcineurin inhibition results are expressed as a percentage of the dephosphorylation that occurs in the absence of any drug. Each datum point represents the average of triplicate assays with a standard error of less than 10%.

Nucleotide sequence accession number. The GenBank accession number for the murine FKBP51 cDNA sequence is U16959.

RESULTS

Isolation and sequence analysis of cDNA clones corresponding to FKBP51. We have previously described the isolation and initial characterization of 13 genes induced in the murine thymoma cell line WEHI-7TG during glucocorticoid-induced ap-

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ggggcgggacgacggcgggcgctgcccggcgcactagccggcttgcgggcgctgccagtCTCCGGCGGCGGTGTCCGGCGCGCGGCTGAG      90
CGACCGAGCGTGCGGACGGAGCGGCGGCCTGCTGGGCGGGCTGAGCGGCGCGCGCGGCGGCGGAGAGACGCGGAGCGAGGGACGCGGCGG     180
CGGCGGACGCGGCGACAGGTCTTCTACTTACAAAGGACAATG ACT ACT GAT GAG GGC ACC AGT AAC AAT GGA GAG AAC     258
                                          Met Thr Thr Asp Glu Gly Thr Ser Asn Asn Gly Glu Asn      13

CCA GCA GCC ACC ATG ACT GAG CAG GGT GAA GAT ATC ACT ACG AAG AAA GAC AGA GGA GTA TTA AAG ATT    327
Pro Ala Ala Thr Met Thr Glu Gln Gly Glu Asp Ile Thr Thr Lys Lys Asp Arg Gly Val Leu Lys Ile     36


GTC AAA AGA GTG GGG ACT AGT GAC GAG GCC CCA ATG TTT GGT GAC AAA GTT TAT GTC CAC TAC AAA GGG    396
Val Lys Arg Val Gly Thr Ser Asp Glu Ala Pro Met Phe Gly Asp Lys Val Tyr Val His Tyr Lys Gly     59

ATG TTG TCA GAT GGA AAG AAG TTT GAT TCC AGT CAT GAC AGA AAG AAG CCA TTT GCC TTT AGC CTT GGC    465
Met Leu Ser Asp Gly Lys Lys Phe Asp Ser Ser His Asp Arg Lys Lys Pro Phe Ala Phe Ser Leu Gly     82

CAA GGC CAG GTT ATC AAA GCC TGG GAC ATT GGG GTG TCT ACT ATG AAG AAA GGC GAG ATC TGC CAT TTA    534
Gln Gly Gln Val Ile Lys Ala Trp Asp Ile Gly Val Ser Thr Met Lys Lys Gly Glu Ile Cys His Leu    105

TTA TGT AAA CCA GAA TAT GCT TAT GGC TCG GCT GGC CAC CTC CAA AAA ATT CCA TCA AAT GCA ACT CTC    603
Leu Cys Lys Pro Glu Tyr Ala Tyr Gly Ser Ala Gly His Leu Gln Lys Ile Pro Ser Asn Ala Thr Leu    128

TTT TTT GAG ATT GAG CTC CTT GAT TTC AAA GGT GAG GAT TTA TTT GAA GAT TCA GGC GTT ATC CGT AGA    672
Phe Phe Glu Ile Glu Leu Leu Asp Phe Lys Gly Glu Asp Leu Phe Glu Asp Ser Gly Val Ile Arg Arg    151

ATC AAA CGG AAA GGC GAG GGA TAC TCA AAC CCA AAC GAA GGA GCA ACG GTA AAA GTC CAC CTG GAA GGC    741
Ile Lys Arg Lys Gly Glu Gly Tyr Ser Asn Pro Asn Glu Gly Ala Thr Val Lys Val His Leu Glu Gly    174

TGC TGT GGT GGA AGG ACA TTT GAT TGC CGA GAT GTG GTG TTC GTT GTT GGG GAA GGA GAA GAC CAC GAC    810
Cys Cys Gly Gly Arg Thr Phe Asp Cys Arg Asp Val Val Phe Val Val Gly Glu Gly Glu Asp His Asp    197

ATT CCG ATT GGG ATC GAC AAA GCC CTG GTG AAG ATG CAG AGA GAA GAA CAG TGT ATT CTA TAT CTT GGA    879
Ile Pro Ile Gly Ile Asp Lys Ala Leu Val Lys Met Gln Arg Glu Glu Gln Cys Ile Leu Tyr Leu Gly    220

CCA CGC TAT GGT TTT GGA GAA GCC GGG AAG CCT AAG TTT GGC ATT GAC CCC AAT GCT GAG CTT ATG TAC    948
Pro Arg Tyr Gly Phe Gly Glu Ala Gly Lys Pro Lys Phe Gly Ile Asp Pro Asn Ala Glu Leu Met Tyr    243

GAG GTC ACC CTT AAG AGC TTC GAG AAG GCC AAA GAA TCT TGG GAG ATG GAC ACC AAA GAA AAG CTG ACG   1017
Glu Val Thr Leu Lys Ser Phe Glu Lys Ala Lys Glu Ser Trp Glu Met Asp Thr Lys Glu Lys Leu Thr    266

CAG GCT GCC ATC GTG AAA GAG AAG GGA ACT GTG TAC TTC AAG GGA GGC AAG TAC ACG CAG GCC GTG ATT   1086
Gln Ala Ala Ile Val Lys Glu Lys Gly Thr Val Tyr Phe Lys Gly Gly Lys Tyr Thr Gln Ala Val Ile    289

CAG TAC AGG AAG ATA GTG TCC TGG CTG GAG ATG GAA TAC GGC CTG TCA GAG AAG GAG TCC AAA GCC TCA   1155
Gln Tyr Arg Lys Ile Val Ser Trp Leu Glu Met Glu Tyr Gly Leu Ser Glu Lys Glu Ser Lys Ala Ser    312

GAG TCG TTC CTC CTC GCA GCC TTC CTG AAC CTG GCC ATG TGC TAC CTG AAG CTC CGA GAG TAC AAC AAA   1224
Glu Ser Phe Leu Leu Ala Ala Phe Leu Asn Leu Ala Met Cys Tyr Leu Lys Leu Arg Glu Tyr Asn Lys    335

GCC GTG GAG TGC TGC GAC AAG GCC CTT GGA CTG GAC AGT GCC AAT GAG AAA GGC TTG TAC AGA AGG GGC   1293
Ala Val Glu Cys Cys Asp Lys Ala Leu Gly Leu Asp Ser Ala Asn Glu Lys Gly Leu Tyr Arg Arg Gly    358

GAG GCC CAG CTG CTC ATG AAT GAC TTT GAG TCG GCC AAG GGC GAC TTC GAG AAG GTG TTG GCA GTC AAT   1362
Glu Ala Gln Leu Leu Met Asn Asp Phe Glu Ser Ala Lys Gly Asp Phe Glu Lys Val Leu Ala Val Asn    381

CCT CAG AAC AGG GCC GCT CGC CTG CAG ATC TCC ATG TGC CAG AGG AAG GCG AAG GAG CAC AAC GAG CGG   1431
Pro Gln Asn Arg Ala Ala Arg Leu Gln Ile Ser Met Cys Gln Arg Lys Ala Lys Glu His Asn Glu Arg    404

GAC CGC AGG GTG TAC GCC AAC ATG TTC AAG AAG TTC GCA GAG CGG GAC GCA AAG GAG GAA GCC AGC AAA   1500
Asp Arg Arg Val Tyr Ala Asn Met Phe Lys Lys Phe Ala Glu Arg Asp Ala Lys Glu Glu Ala Ser Lys    427

GCT GGG AGC AAG AAG GCT GTA GAA GGA GCC GCT GGC AAA CAA CAC GAG AGT CAG GCC ATG GAA GAA GGA   1569
Ala Gly Ser Lys Lys Ala Val Glu Gly Ala Ala Gly Lys Gln His Glu Ser Gln Ala Met Glu Glu Gly    450

AAG GCC AAA GGC CAT GTA TGACGCTGCGCCACGGAGGGAAGAGAGTCCTAATGAACTCGGCCCTCCTCGCTGGGCTCGCCTCCA   1653
Lys Ala Lys Gly His Val                                                                      456

ACTCAGGACTGAACAGTGTTTAGTGTAAGGTTTGTTACAGTCTCTGTGATTCTGGAAGCAAATGGCATACCAGTAGCTTCCCAAATGACC   1743
ACCTGCTGCTGCGGGGGGGTGGGGGTGGGGGACATGCCAGGAAACAGCAGAGAAGGCCGCTGGTGTGAAGAGACCAGGCCAGCAGCTCAG   1833
TCCAGCCCATTTCAGTTTGTCACCTTTCAGTGTCCAGCACAGCATCCCTGTGAACCTAGGGCCCAGCTGCTGTGGGTTCTACATCGGCAC   1923
TAGGGTCACACTGCAGAAACCGTTGATAAAACAAACTCAGTGATCTCTGCTTTCCTATTGGTGGGCATGGCAGGGGCGGGTGATGAGATT   2013
TGCTTAGCACTGACTGACTGGCCTGCTAAGAACACAAGCCCACAGCCAGGGGCTCCCTGGTCCACAGCTGGGTCTCAGGCCCCTTACCTG   2103
CCTTCCAAGTCCTTTCGCAGACTCTTGAGTGTGGCTTTCTGTCCTAGCCAGCATGTCCCACAGACTCTGTTGTTCCTCCAACGCCCGTCA   2193
TTAGTGACAGCTTTCTCTCTGAGTTTCTGTGGTGTGGAGAGTGGGTAGAAGTAGGTTTATCTTTCCCGCTGTCTGCCCCACTCAAGGACG   2283
AT                                                                                           2285
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FIG. 1. Nucleotide and predicted amino acid sequences of murine FKBP51 cDNA. The 2,285-nucleotide sequence corresponding to the 5′ region of the complete 3.4-kb cDNA of FKBP51 is shown. Nucleotide sequence shown in uppercase (positions 60 to 2285) was derived from clone 213-12A, while sequence shown in lowercase (positions 1 to 59) is present only in clone 213-19. Nucleotide (top line) and amino acid residue (bottom line) numbers are listed to the right, starting at the 5′ end of the cDNA and the initiating methionine, respectively.

optosis (3, 17). One of these genes, represented by cDNA clone 213, expresses a 3.7-kb mRNA that is induced by dexamethasone in WEHI-7TG cells and murine thymocytes and is expressed essentially only in thymus tissue, compared with brain, heart, kidney, liver, lung, and spleen tissues. Preliminary sequence analysis of the original clone 213 cDNA, consisting of 2.2 kb of primarily 3′ UTR, did not allow identification of this gene. Full-length cDNA clones (3.7 kb) were subsequently isolated from WEHI-7TG and murine thymus cDNA libraries as described in Materials and Methods, and the nucleotide sequence of one full-length isolate, clone 213-12A, was determined (Fig. 1). Analysis of the sequence of clone 213-12A revealed a favorable initiation consensus sequence for translation of an ORF consisting of 456 amino acids with a predicted M_r of 50,969. The ORF is followed by a relatively long (approximately 2.1-kb) 3′ UTR possessing a canonical AATAAA polyadenylation signal and a poly(A) tail (data not shown). Additional full-length cDNA clones were analyzed to determine if clone 213-12A represented the entire upstream region of the 3.7-kb mRNA corresponding to this gene. The result of the analysis of one clone, clone 213-19, revealed the existence of 59 additional nucleotides (Fig. 1) within the 5′ UTR encoded by this gene, thus bringing the length of the 5′ UTR to 219 nucleotides. No upstream, in-frame translation termination codon exists within the 5′ UTR preceding the ORF depicted in Fig. 1. Therefore, assignment of the initiating methionine for this ORF cannot be made unequivocally. While it is formally possible that additional 5′ UTR sequence exists, primer extension analysis of poly(A)$^+$ RNA obtained from murine thymus by using a 33-nucleotide oligodeoxynucleotide primer (positions 104 to 136; Fig. 1) produced a predominant product of 130 nucleotides, in agreement with the sequence depicted in Fig. 1 (data not shown). Furthermore, the translation initiation codon located at position 220 (Fig. 1) functions to direct protein synthesis. RNA synthesized from clone 213-12A and translated in vitro produced a protein of approximately 50 to 52 kDa (Fig. 2), in excellent agreement with the assignment of the ORF.

Clone 213-12A encodes a putative FKBP. The nucleotide and derived amino acid sequences shown in Fig. 1 were compared with sequences in the GenBank and SwissProt databases. The results of these searches revealed sequence similarity between clone 213-12A and the family of genes encoding FKBPs. At the nucleotide level, the sequence of clone 213-12A had highest identity (63%) to the murine sequence for a 52-kDa FKBP (42), hereafter referred to as FKBP52. This particular FKBP has been referred to previously by numerous names, including FKBP56, hsp56 or p56 (29, 33, 39, 40, 61), FKBP59, p59, or p59/HBI (9, 35, 36, 42, 54, 55), FKBP51 (59), and p50 (50, 52). High scores of similarity at the nucleotide level were also obtained for the sequence of our gene compared with other mammalian sequences for FKBP52 (22, 32) and for other members of the FKBP gene family that encode 12-, 13-, and 25-kDa proteins. The protein encoded by clone 213-12A, hereafter called FKBP51 to reflect its predicted molecular weight, and the murine FKBP52 share 53% identity over the entire protein at the amino acid level. The similarity between murine FKBP51 and other closely related mammalian FKBPs is depicted in Fig. 3. All of the characterized FKBPs have a protein domain involved in drug binding (residues 32 to 138; Fig. 3). Residues determined by solution and crystal structure studies (27, 56) to be involved in FK506 binding to FKBP12 (indicated by diamonds in Fig. 3) are strongly conserved in the murine FKBP51 sequence.

FKBP51 is expressed preferentially in T cells. An antiserum directed against a unique C-terminal peptide (residues 443 to 456; Fig. 3) of the cloned FKBP51 was generated as described in Materials and Methods and used to examine the expression of FKBP51 in WEHI-7TG cells and murine tissues by Western analysis (Fig. 4A). An approximately 51-kDa protein is observed in the WEHI-7TG cell line and in thymus tissue extracts. This observation is in excellent agreement with the mRNA expression previously observed for this gene (3). Expression of a 51-kDa protein is observed in the lung at levels that are four- to fivefold lower than those in the thymus and may reflect either contamination of the protein extract by circulating T lymphocytes or a cross-reacting antibody epitope in lung extracts. Previously identified FKBPs, including FKBP12, FKBP12.6, FKBP13, FKBP25, and FKBP52, have been found in diverse cell lines and organs (reviewed in reference 15) and are generally considered to be ubiquitously expressed. Analysis of the expression of FKBP12 by Western hybridization (Fig. 4B) demonstrates that FKBP12 is expressed in all murine tissues tested, with highest levels detected in the brain and thymus. Purified GST-FKBP51 fusion protein was prepared as described in Materials and Methods and used to estimate the abundance of FKBP51 in the various protein extracts. A known quantity (25 ng) of GST-FKBP51 was mixed with an aliquot of thymus extract and analyzed by Western blotting (Fig. 4A, lane 9). Comparison of the signal intensities between the known quantity of GST-FKBP51 fusion protein and the endogenous FKBP51 signal indicated that FKBP51 is abundantly expressed (at least 0.05% of total protein) in the thymus.

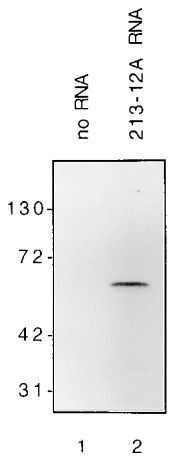

FIG. 2. In vitro translation of cDNA for FKBP51. The rabbit reticulocyte translation products in the absence of exogenous RNA (lane 1) or in the presence of RNA corresponding to clone 213-12A cDNA (lane 2) are shown. Migrations of molecular weight standards, with sizes in kilodaltons, are shown at the left.

Determination of the PPIase and calcineurin inhibition activities of FKBP51 in vitro. Immunophilins are characterized by an inherent PPIase activity that is inhibited upon drug ligand binding. Sequence comparison of FKBP51 with other FKBPs (Fig. 3) suggested that FKBP51 would possess PPIase activity and that this activity would be inhibited by FK506 (or FK506 analogs) and rapamycin. The PPIase activity of purified recombinant GST-FKBP51 fusion protein compared with that of FKBP12 in the presence and absence of rapamycin or FK520, a structural analog that is virtually identical to FK506 except that it has a twofold-reduced binding affinity to all tested FKBPs (57a), is shown in Fig. 5. In the absence of a drug inhibitor, FKBP51 demonstrated PPIase activity. Inhibition of this activity by rapamycin or FK520 was measured as a function of inhibitor concentration. Fifty percent inhibitory concentra-

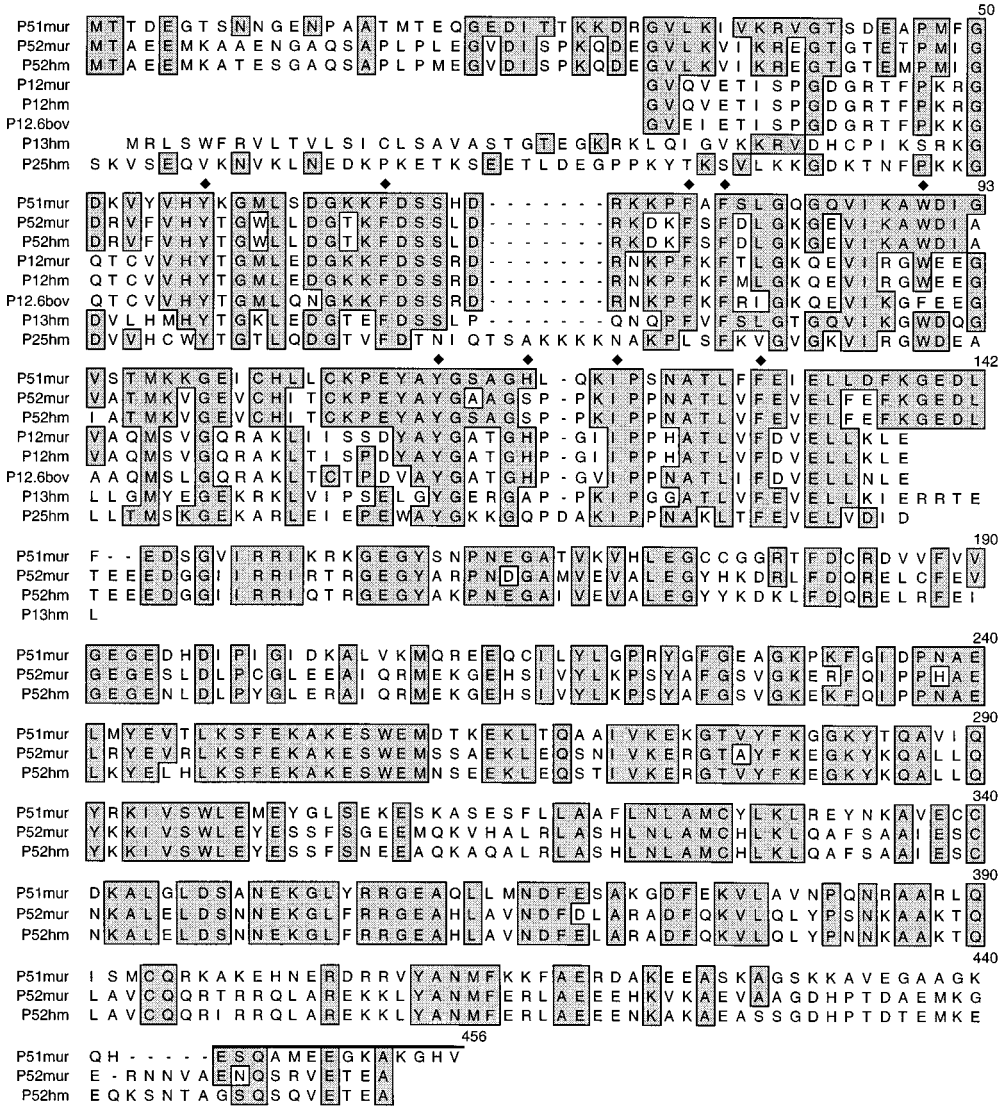

FIG. 3. Alignment of murine FKBP51 with related family members. The sequence of FKBP51 is aligned with sequences of the following related FKBPs, given with their GenBank accession numbers (in parentheses): the human (suffix "hm") sequences for p52 (M88279), p12 (A35780), p13 (A39602), and p25 (M90309), the murine (suffix "mur") sequences for p52 (X70887) and p12 (X60203), and the bovine (suffix "bov") sequence for p12.6 (see reference 45). All sequences are aligned in their entirety except that of p25, which is depicted beginning at amino acid residue 72. Residue numbers listed at the right are with respect to FKBP51. Amino acid residues that have identity with FKBP51 are boxed. Diamonds indicate amino acids implicated in FK506 drug binding to FKBP12. The 14-amino-acid C-terminal peptide sequence used to generate an FKBP52-specific antiserum is overlined.

tion (IC_{50}) values of 29 and 166 nM were obtained for FKBP51 (compared with values of 14 and 15 nM for FKBP12) with rapamycin and FK520, respectively. These results indicated that FKBP51 has higher affinity for rapamycin than for FK520. In addition, while the IC_{50} values for rapamycin inhibition of FKBP51 and FKBP12 PPIase activities are comparable, the IC_{50} value of FK520 inhibition of FKBP51 PPIase activity is higher than that of FKBP12 inhibition.

The ability of FKBP51 to inhibit calcineurin phosphatase activity when complexed to FK520 was tested in an in vitro assay (Fig. 6). Side-by-side comparison of the FK520 complexes with GST-FKBP51 or FKBP12 indicated that FKBP51 is capable of inhibiting calcineurin activity, although it is much less potent than FKBP12. The IC_{50} values for inhibition of calcineurin are 19 nM and 5 μM for FKBP12 and FKBP51, respectively. FKBP51 has significant sequence homology with FKBP52 (Fig. 3) over its entire sequence, including a region that contains a putative calmodulin binding site (22). Assays performed in the presence of excess calmodulin indicated that although FKBP51 is less potent than FKBP12, the observed FKBP51-drug complex inhibition of calcineurin phosphatase activity is not the result of sequestration of calmodulin by FKBP51. The lower activity of FKBP51 than of FKBP12 in the calcineurin phosphatase inhibition assay most likely reflects a combination of a lower affinity of the FKBP51-FK520 complex for calcineurin and a lower affinity of the drug for FKBP51.

DISCUSSION

We have identified a new FKBP of approximately 51 kDa, termed FKBP51, that is expressed in murine thymus and WEHI-7TG cells. The existence of one or more 50- to 59-kDa

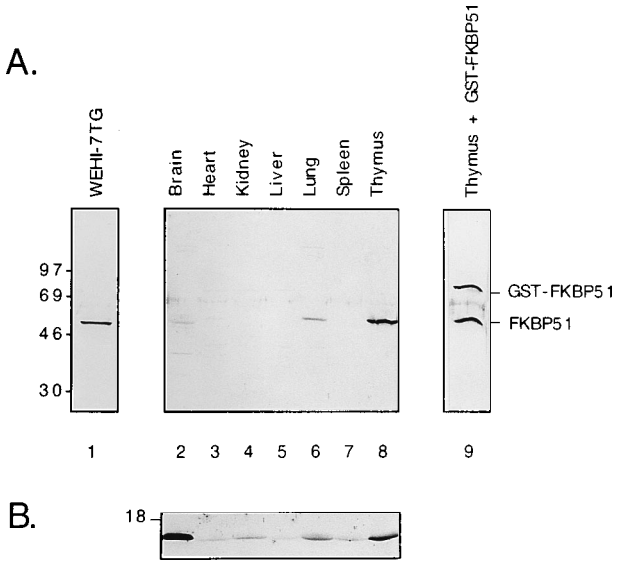

FIG. 4. Expression of FKBPs in the WEHI-7TG cell line and various murine tissue extracts. (A) Proteins (25 μg) from WEHI-7TG cells (lane 1) or proteins (50 μg) prepared from the indicated murine tissues (lanes 2 to 9) were separated in an SDS–10% polyacrylamide gel and analyzed by Western hybridization with an FKBP51-specific antiserum as described in Materials and Methods. Lane 9 contains 25 ng of purified GST-FKBP51 fusion protein in combination with 50 μg of thymus tissue extract. Relative migrations of protein molecular weight standards, with sizes in kilodaltons, are indicated to the left. The positions of FKBP51 and the GST-FKBP51 fusion protein are indicated to the right. (B) Protein extracts from murine tissues were prepared as described above (panel A, lanes 2 to 8), separated in an SDS–15% polyacrylamide gel, and analyzed by Western hybridization with an FKBP12 peptide-specific antiserum. The relative migration of an 18-kDa protein standard is indicated to the left.

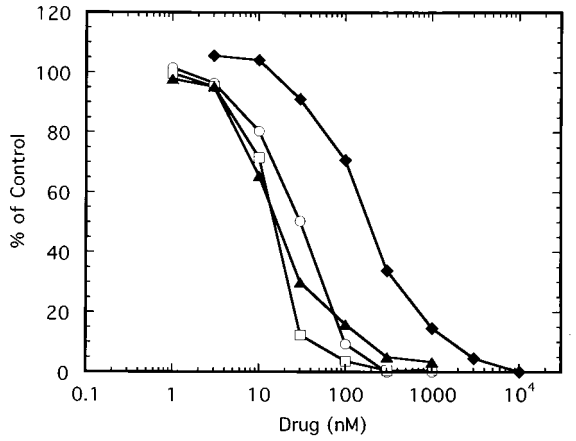

FIG. 5. Drug inhibition of FKBP51 and FKBP12 PPIase activity. The PPIase activity of FKBP51 in the presence of rapamycin (○) or FK520, an FK506 analog (◆), is shown as a function of drug concentration. For comparison, the PPIase activity of FKBP12 in the presence of increasing rapamycin (□) or FK520 (▲) is shown. Assays were performed and plotted as described in Materials and Methods, using protein levels (200 nM FKBP51 and 83.3 nM FKBP12) determined to be in the linear response range.

FKBPs (13, 50) and the sequence of one, FKBP52 (22, 32, 42), have been previously reported. Although FKBP51 is related to FKBP52, having 53% identity at the amino acid level, FKBP51 is restricted in its pattern of tissue expression to T cells at both the mRNA (3) and protein (Fig. 4) levels. This pattern of expression is in direct contrast to the observed ubiquitous expression of other FKBPs (14, 16, 32, 39, 44, 45, 54), including FKBP12, which is highly expressed in the brain and thymus yet significantly expressed in all other tissues tested (Fig. 4). Our experiments demonstrate that FKBP51 comprises at least 0.05% of the total protein of thymus tissue. Thus, FKBP51 corresponds to a new immunophilin that not only is restricted in its expression to T cells but by virtue of its abundance must represent a major immunophilin in these cells.

The cDNA clone corresponding to FKBP51 was originally isolated during a screen for genes involved in glucocorticoid-induced apoptosis of murine T cells (3, 17). Glucocorticoid treatment of murine thymocytes in vitro results in a significant and rapid induction of the level of FKBP51 mRNA (3). To the best of our knowledge, no data exist on the transcriptional regulation of other FKBPs by glucocorticoids or on the potential involvement of FKBPs in hormone-mediated apoptosis of T cells. FKBP52 has been found in association with hsp90 (33, 40) and various untransformed steroid hormone receptor complexes, including those for glucocorticoid, progesterone, androgen, and estradiol (22, 32, 33, 50, 54, 61). In the case of the human and avian progesterone hormone complex, at least two distinct FKBPs of approximately 50 to 60 kDa have been detected (49, 50), only one of which corresponds to the previously cloned FKBP52. Further experiments are necessary to determine if FKBP51 corresponds to the other identified protein in this complex. The function of FKBPs in the untransformed steroid complex remains unidentified. Although FK506 has been reported to potentiate glucocorticoid and progesterone receptor-mediated gene transcription (29, 55), FK506 does not effect receptor folding or function in vitro (19, 36). Pratt and colleagues (34) propose that FKBP52 may function as a nuclear localization signal-binding protein. They have identified an acidic region in the FKBP52 protein (EDLTEEED, residues 140 to 147 of the human sequence) that may play a role in the interaction between FKBP52 and the basic nuclear localization signal of the glucocorticoid receptor. The FKBP51 sequence diverges significantly from the sequence of FKBP52 in this region. In particular, two acidic residues implicated in this charged interaction are absent in the FKPB51 sequence (Fig. 3). Thus, if this region is critical for association with, and

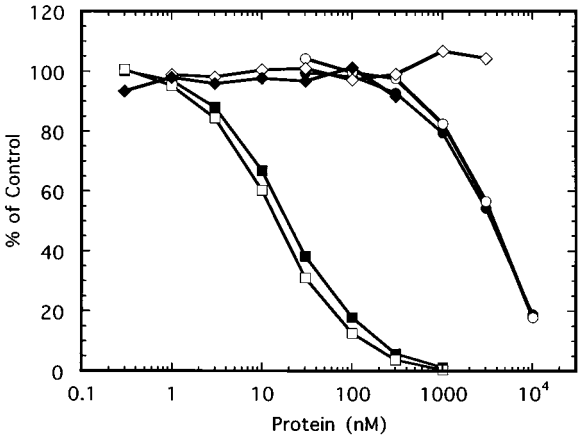

FIG. 6. Inhibition of calcineurin phosphatase activity by FKBP-drug complexes. Calcineurin phosphatase activity inhibition assays in the presence of calcineurin (3 nM), FK520 (50 μM), $MgCl_2$, and standard (190 nM) or excess (30 μM) levels of calmodulin were performed as described in Materials and Methods. Calcineurin phosphatase activity as a function of increasing FKBP51 protein concentrations in the presence (○) or absence (●) of excess calmodulin is shown. Similar assays are shown as a function of FKBP12 protein levels in the presence (□) or absence (■) of excess calmodulin. For comparison, calcineurin phosphatase as a function of increasing FKBP51 (◇) or FKBP12 (◆) in the absence of drug is shown.

nuclear localization of, steroid receptor, FKPB51 would not be expected to function in this capacity.

FKBP51 demonstrates PPIase activity that is inhibited by FK520 or rapamycin binding as would be predicted on the basis of its sequence homology to other FKBPs. Of additional interest, however, is the fact that FKBP51 when complexed with FK520 is capable of inhibiting calcineurin phosphatase, the relevant cellular target of FK506 in immunosuppression. High activity has previously been observed for only two of the characterized FKBPs, FKBP12 (8, 10, 14, 24, 31) and FKBP12.6 (45). The observed inhibition of calcineurin by FKBP51 is somewhat unexpected. FKBP51 has significant sequence homology with FKBP12; however, it possesses even greater homology with FKBP52, a protein previously reported to be inactive with respect to calcineurin inhibition (23, 59). The current assay (see Materials and Methods) has been modified to allow the formation of micromolar concentrations of immunophilin-drug complex. Use of this assay detects a weak yet definitive inhibition activity for FKBP51 and FKBP52 (57a). FKBP51 is approximately 400-fold weaker than FKBP12 in inhibiting calcineurin when complexed to FK520 (Fig. 6), and this behavior most likely reflects a combination of a lower affinity of the FKBP51-FK520 complex for calcineurin and a decreased affinity of the drug for FKBP51. While inhibition of calcineurin by the FKBP51-drug complex suggests that FKBP51 could mediate immunosuppression, other factors influence the ability of a given immunophilin to mediate immunosuppression in vivo. These factors include the relative abundance of the protein in specified tissues, the subcellular localization of the protein, and the existence and influence of any associated proteins. For example, while cyclophilin C can inhibit calcineurin in vitro, its subcellular localization in the endoplasmic reticulum makes it unavailable to mediate immunosuppression in vivo (8). FKBP51 does not appear to contain an obvious endoplasmic reticulum targeting signal; however, future studies are necessary to assess the subcellular localization and ability of FKBP51 to inhibit calcineurin in vivo.

The inhibition of calcineurin phosphatase requires components from both the drug ligand and the immunophilin protein. Neither individual component binds calcineurin independently; rather, it is the composite surface formed by the FKBP-drug complex that recognizes and interacts with the calcineurin molecule (60). Alterations in either the protein (37, 60) or the drug ligand (4, 51) can affect the ability of the complex to inhibit calcineurin without compromising drug binding to the immunophilin. For example, FKBP13 when complexed with FK506 is a poor inhibitor of calcineurin phosphatase. However, minor alterations in the amino acid sequence of FKBP13 that do not affect the affinity of FKBP13 for FK506 activate the inhibitory action of the complex for calcineurin (37, 60). On the other hand, while the FKBP12-FK506 complex from both human and yeast cells inhibits calcineurin, complex formed with L-685,818, a modified drug that does not affect binding affinity, renders the human, but not the yeast, complex inactive in calcineurin inhibition (38). We suggest that it should be possible to identify novel FK506 analogs that either bind specifically to FKBP51 or specifically increase the affinity of the FKBP51-drug complex for calcineurin. In this regard, we have already observed an altered specificity of FKBP51 for rapamycin compared with that of FK520 in the PPIase activity assay (Fig. 5). On the basis of the unique tissue distribution of FKBP51 and its abundance in T cells, and of the fact that FKBP51 can inhibit calcineurin, such newly identified drugs would be targeted in their site of action to T cells. Current evidence suggests that it is the inhibition of calcineurin in nonlymphoid cells that is responsible for the observed toxic side effects of FK506 when it is used as an immunosuppressant (11). Thus, it is the ubiquitous expression of FKBPs mediating inhibition of calcineurin phosphatase activity that is directly responsible for the toxicity of FK506. New drugs specific for FKBP51 would be expected to have limited adverse side effects while still functioning as immunosuppressants.

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