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Biochemistry

Yeast FKBP-13 is a membrane-associated FK506-binding protein encoded by the nonessential gene *FKB2*

(immunosuppressant/peptidylprolyl cis–trans isomerase/rotamase/rapamycin/*Saccharomyces cerevisiae*)

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ABSTRACT **The immunosuppressants FK506 and rapamycin prevent T-cell activation and also inhibit the growth of certain strains of the yeast *Saccharomyces cerevisiae*. It has previously been shown that yeast contains a 12-kDa cytosolic FK506-binding protein (yFKBP-12), which also possesses peptidylprolyl cis–trans isomerase activity, and that *fkb1* strains lacking yFKBP-12 are resistant to rapamycin and sensitive to FK506. The absence of yFKBP-12 permitted the detection and isolation of a second FK506- and rapamycin-binding protein, which is about 13 kDa in size (yFKBP-13) and membrane-associated. Purified yFKBP-13 binds FK506 with 15-fold lower affinity than yFKBP-12 and has peptidylprolyl cis–trans isomerase activity with a similar substrate profile. The sequence of the first 37 N-terminal amino acids was determined, and the yFKBP-13 gene (*FKB2*) was cloned and sequenced. A hydrophobic putative signal sequence precedes the N terminus of the mature protein. yFKBP-13 most closely resembles the membrane-associated human FKBP-13, which also possesses a signal peptide, whereas yFKBP-12 most closely resembles human FKBP-12. *fkb2* and *fkb1 fkb2* mutants are viable and unaltered in their sensitivity to FK506, suggesting that yeast possesses an additional target for this drug. Furthermore, *fkb2* null mutations confer no change in rapamycin sensitivity. These findings show that yFKBP-13 and yFKBP-12 have distinct functions within the cell.**

The macrolide FK506 is a potent immunosuppressant that inhibits an intermediate step in T-cell activation, blocking interleukin 2 production (reviewed in refs. 1 and 2). In addition, FK506 is an antibiotic that inhibits the growth of certain strains of yeast and fungi. The drug binds with high affinity to a family of highly conserved proteins referred to as FK506-binding proteins (FKBPs), which are found in many cell types and organisms. The FKBPs also possess peptidylprolyl cis–trans isomerase (PPIase) activity, which is thought to accelerate the folding of proteins and peptides into their native conformations. The cyclophilins, which bind the cyclic peptide immunosuppressant cyclosporin A, are also highly conserved and ubiquitous, and possess PPIase activity, but are unrelated to the FKBPs in either sequence or structure. Both cyclosporin and FK506 inhibit the PPIase activity of their respective binding proteins and interleukin 2 production in activated T cells.

PPIase activity is probably not the mechanism by which these drugs exert their immunosuppressive effects (1). The immunosuppressant rapamycin, a macrolide antifungal compound, is similar in structure to FK506, binds to FKBPs, and inhibits their PPIase activity but, instead of blocking interleukin 2 production, inhibits later events required for full T-cell activation. Rather, rapamycin and FK506 act as reciprocal antagonists in T-cell activation. The FK506–FKBP complex binds to and inhibits the calmodulin-regulated protein phosphatase calcineurin A, whereas the rapamycin–FKBP complex does not (3). The cyclosporin–cyclophilin complex also binds to and inhibits calcineurin A (3, 4). These intriguing results led to the suggestion that the inhibition of calcineurin A is the mechanism of immunosuppression.

Many tissues and cell types express FKBPs. DNA encoding several of these has been cloned and sequenced, including a 12-kDa cytosolic human FKBP (hFKBP-12) (5, 6), a 13-kDa membrane-associated human FKBP (hFKBP-13) (7), a 25-kDa bovine FKBP (8), a 59-kDa mammalian FKBP (9, 10), and a 12-kDa cytosolic yeast FKBP (yFKBP-12) (11–13). Many of these FKBPs are found in the same cell type and therefore presumably have different functions.

The existence of multiple FKBPs makes it difficult to determine which proteins mediate the various effects of FK506 and rapamycin. The antifungal properties of these drugs and the existence of FKBPs in yeast and fungi have prompted examinations of the mode of action of these compounds in these genetically tractable organisms. The antifungal activity may be related to immunosuppression or drug toxicity. Although much higher concentrations of FK506 are required to inhibit yeast growth than are needed to block T-cell activation, the observation that three FK506 analogs exhibit parallel antifungal and immunosuppressive potencies suggests that similar molecular mechanisms may be involved (14). In any case, yeast should be useful in detecting new FKBPs biochemically and genetically, as well as in clarifying their roles *in vivo*. Although disruption of *FKB1*, the *Saccharomyces cerevisiae* gene encoding yFKBP-12, eliminates sensitivity to rapamycin (12, 14), it reduces sensitivity to FK506 only slightly (14). Furthermore, mutations in three *FKR* genes conferring FK506 resistance have no effect on yFKBP-12 activity and map to loci distinct from *FKB1* (14). These results suggest the presence of at least one additional FKBP in this organism. Here we report the isolation and characterization of a second yeast FKBP from strains lacking yFKBP-12, as well as the cloning, sequencing, and disruption of its gene, *FKB2*.†

MATERIALS AND METHODS

Strains, Media, and Microbiological Methods. Strains YFK005 (*MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52*), YFK187 (*MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52 fkb1-Δ2::URA3*), and YFK016-28D (*MAT***a** *ade2-101*

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Abbreviations: FKBP, FK506-binding protein; h, human; y, yeast; PPIase, peptidylprolyl cis–trans isomerase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M90767).

his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52) have been described (14). YFK187 was mated to YFK016-28D to generate the diploid strain YFK319. Media and procedures for mating, sporulation, tetrad analysis, and determination of antibiotic sensitivities [minimal inhibitory concentration (MIC) assays] have been described (14).

Enzyme Assays. Binding of [^{3}H]dihydro-FK506 (21 nM) to yFKBP-12 was assayed chromatographically on Sephadex LH20 (14). Binding to yFKBP-13 was assayed on Superose. [^{3}H]Dihydro-FK506 was diluted in 50% methanol and added to cell extract in Superose buffer (20 mM potassium phosphate, pH 7.0/1 mM EDTA/0.2 mM 2-mercaptoethanol) at a final concentration of 52 nM. After 10 min at room temperature, the reaction mixtures were injected onto a Superose 12 HR 10/30 column (Pharmacia) and eluted at 0.5 ml/min at 4°C. A rapid assay using Bio-Gel P6-DG resin (Bio-Rad) (with 0.1% Triton X-100 in the elution buffer) was performed essentially as described for LH20 resin (14). yFKBP-12 and yFKBP-13 were assayed for PPIase activity as described (15).

Purification of yFKBP-13. Strain YFK187 (*fkb1-Δ2*) was grown in YPAD medium, harvested, and resuspended in 1 vol (vol/wt) of breakage buffer [20 mM potassium phosphate, pH 7/10% (wt/vol) glycerol/5 mM 2-mercaptoethanol/1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride with leupeptin at 10 μg/ml] and broken by prolonged shaking with glass beads (0.5 mm) at 4°C. All subsequent operations were carried out at 4°C. The cell extract was centrifuged at 100,000 × *g* for 3 hr, and the supernatant (S100), containing 25–30 mg of protein per gram of starting wet weight of cells, was heated at 54°C for 20 min, clarified by centrifugation, and brought to 65% saturation with solid ammonium sulfate. The precipitate was dissolved in 10 mM Hepes, pH 7.3/0.2 mM 2-mercaptoethanol/1 mM EDTA, dialyzed against this buffer, and fractionated by chromatography on a column of DE52 (Whatman; 250 ml/100 g of cells). yFKBP-13 was eluted between 5 and 12 mS conductivity with a linear gradient of 0–0.25 M KCl in loading buffer. Active fractions were pooled, concentrated, and desalted in an Amicon cell with a YM1 membrane. The concentrate was brought to 2.5 mM in potassium phosphate (pH 7.0) and applied to a column of Blue Sepharose CL-6B (Pharmacia; 250 ml/100 g of cells) equilibrated in the latter buffer. yFKBP-13 was eluted in the loading buffer. Active fractions were chromatofocused on a Mono P column (HR 5/20; Pharmacia) with a pH gradient of 6 to 5 (Polybuffer 74). yFKBP-13 was eluted in three 1.5-ml fractions at pH 5.3–5.7. These fractions were applied to an FK520-Sepharose column (15) equilibrated in 10 mM potassium phosphate, pH 7.0/1 mM EDTA/0.2 mM 2-mercaptoethanol. The column was washed with loading buffer and bound material was eluted with buffer containing 0.25 M KCl. Fractions were collected and monitored by SDS/PAGE and silver staining. After the purification reported in Table 1, we discovered that addition of 0.1% (vol/vol) Triton X-100 to the cell extract before high-speed centrifugation increases yFKBP-13 yield in the S100 by 25–50% without altering its behavior in subsequent steps. A 2-μg sample of the active fraction from FK520-Sepharose was concentrated in a Millipore Ultrafree 10-kDa-cutoff microconcentrator lined with Trans-blot paper. Sequencing was performed on a gas-phase automated Edman sequencer (Porton Industries, Tarzana, CA).

Determination of Subcellular Distribution. Protoplasts of strains YFK005 and YFK187 were prepared and washed through Ficoll (16) and then broken by 30 sec of agitation with 0.5-mm glass beads in a Bead Beater (Biospec Products, Bartlesville, OK) at low speed. Extracts were centrifuged at 100,000 × *g* for 1.5 hr, yielding a supernatant (soluble) fraction and a pellet (membrane) fraction, which was dissolved in lysis buffer containing 0.5% Triton X-100. Detergent-solubilized material was clarified at 15,000 × *g*. yFKBP-12 was assayed in the YFK005 extract with LH20, and yFKBP-13 was assayed in the YFK187 extract with Bio-Gel P6-DG.

Cloning and Sequencing. Degenerate PCR primers, designed by reverse translation of the N-terminal amino acid sequence [5′-GA(A/G)AT(A/T/C)GGIAT(A/T/C)AT(A/T/C)AA(A/G)(A/C)GIAT(A/T/C)CC-3′ and 5′-AC(C/T)-TTIAC(C/T)TT(A/G)TCICCIGGC-3′, where I is deoxyinosine] were used at a concentration of 4 μM to amplify a 74-base-pair fragment from 200 ng of YFK005 genomic DNA in a thermal cycler (Perkin–Elmer) with *Taq* polymerase (BRL). The PCR product was subcloned and used to screen a yeast genomic DNA library of strain S288C in λDASH (Stratagene; catalogue no. 943901). Double-stranded DNA was sequenced with Sequenase (United States Biochemical), and sequence analysis was performed with the computer programs provided by the Genetics Computer Group of the University of Wisconsin (17).

RESULTS

Purification of yFKBP-13. Although mutants lacking yFKBP-12 possessed extremely low levels of FK506-binding activity in LH20 assays (11, 14), this activity increased linearly with increasing amounts of protein, suggesting the presence of an additional FKBP. Cell extracts incubated with [^{3}H]dihydro-FK506 showed a discrete peak of FK506-binding activity on Superose chromatography that was eluted slightly earlier than a yFKBP-12 standard. The magnitude of this peak was at least 50 times more than predicted from LH20 assays (data not shown). This activity was purified using the Superose 12 assay to monitor binding of FK506 (Table 1). N-terminal sequence analysis (see below) and SDS/PAGE (data not shown) indicated that the final preparation was homogeneous. The protein possessed PPIase activity as well as [^{3}H]dihydro-FK506-binding activity (Table 1). The specific activity in the binding assay, 9400 ng of FK506 per mg of protein, corresponds to 0.15 mol of ligand per mol of protein. The abundance of yFKBP-13 in detergent-solubilized extracts was about one-fifth that of yFKBP-12 as estimated by Coomassie staining of two-dimensional polyacrylamide gels (results not shown).

yFKBP-13 Is a PPIase That Binds FK506 and Rapamycin. The equilibrium dissociation constant (K_d) for FK506 measured by competitive displacement of [^{3}H]dihydro-FK506 was 18 nM, while that for rapamycin was 11 nM. For comparison, the K_d values of yFKBP-12 for FK506 and rapamycin determined by competitive displacement were 0.9 and 0.5 nM, respectively.

FKBPs and cyclophilins possess an enzymatic activity that catalyzes the cis–trans isomerization of peptidylprolyl bonds in peptides and proteins (1, 2). hFKBP-12 exhibits more stringent substrate requirements than cyclophilin A (18). yFKBP-13 has PPIase activity comparable to that of yFKBP-12 and shows a marked preference for leucine or

Table 1. Purification of yFKBP-13

Fraction	Protein, mg	FK506 binding ng/mg	FK506 binding % yield	PPIase, $sec^{-1}·\mu g^{-1}$
S100 (heated)	1736	0.7	100	
0–65% $(NH_4)_2SO_4$	504	2.1	89	
DE52	50	13.8	57	
Blue Sepharose	1.2	65	28	0.04
Mono P	0.52	314	11	
FK520-Sepharose	0.01	9400	8.7	2.04

yFKBP-13 was purified to homogeneity and assayed for [^{3}H]dihydro-FK506-binding activity as described in *Materials and Methods*. PPIase activity was measured using the substrate *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide as described previously (15).

phenylalanine preceding proline in the model substrate (Fig. 1*A*, Table 2). FK506 and rapamycin inhibit this activity with K_i values of 8.3 ± 2.1 nM and 0.7 ± 0.2 nM, respectively (Fig. 1*B*). These values probably more accurately reflect the affinity of yFKBP-13 for FK506 and rapamycin than those measured in the Bio-Gel resin assay, where true equilibrium conditions do not prevail, and resin hydrophobicity can interfere. Alternatively, these drugs may interact differently with binding and PPIase sites, which may not fully overlap.

Cloning and Sequencing of *FKB2*, the Yeast Gene Encoding FKBP-13. PCR primers designed by reverse translation of the N-terminal amino acid sequence of yFKBP-13 (Fig. 2*B*) were used to amplify a DNA fragment encoding the predicted region of the N terminus. This DNA sequence was used as a probe to identify four phage clones from a yeast genomic library. The phages contained overlapping inserts from the same region of the genome (Fig. 2*A*). A 1.8-kb *Sal* I fragment from phage clone H2 was subcloned into pBluescript II KS(+) (Stratagene) (Fig. 2*A*), and the region encoding yFKBP-13 was sequenced (Fig. 2*B*). The open reading frame encodes a 135-amino acid protein with a predicted molecular weight of 14,486. There are 16 (or 17, depending on which AUG triplet is the initiating codon) amino acids at the N terminus not found in the purified protein, which are presumably removed by processing. The mature yFKBP-13 is highly similar to other FKBPs (Fig. 3) and consists of 118 amino acid residues with a molecular weight of 12,446. We have named the gene encoding this protein (the second yeast FKBP) *FKB2*.

Membrane Association of yFKBP-13. Since *FKB2* encodes a protein with a putative membrane-localizing signal sequence, soluble and membrane fractions were prepared from protoplasts. The majority (93%) of the yFKBP-13 activity was found in the membrane fraction, while 92% of the yFKBP-12 activity was found in the soluble fraction.

***FKB2* Is a Nonessential Gene That Does Not Mediate FK506 or Rapamycin Sensitivity.** *FKB2* null alleles were constructed by blunt-end ligation of the 1.7-kb *Bam*HI *HIS3* fragment from plasmid YEp6 (20) in both orientations into a *Pac* I site (Fig. 2) in codon 20 in the open reading frame of the mature protein (Fig. 4*A*). The alleles were introduced into the diploid strain YFK319 (*FKB1/fkb1-Δ2::URA3*) by one-step gene replacement, yielding the *fkb2* heterozygotes YFK323 (sense) and YFK325 (antisense). Their presence was confirmed by Southern hybridization analysis (Fig. 4*B*). Nine of 11 tetrads from YFK323 and 12 of 16 from YFK325 yielded four viable spores. His^+ and His^- segregated 2:2 in every case. Ura^+ and Ura^- also segregated 2:2, independently of His^+. In 7 of 9 tetrads from YFK323 and 8 of 12 from YFK325 one spore was His^+ Ura^+. In 2 of 12 tetrads from YFK325 two spores were His^+ Ura^+. These results show that *FKB1*

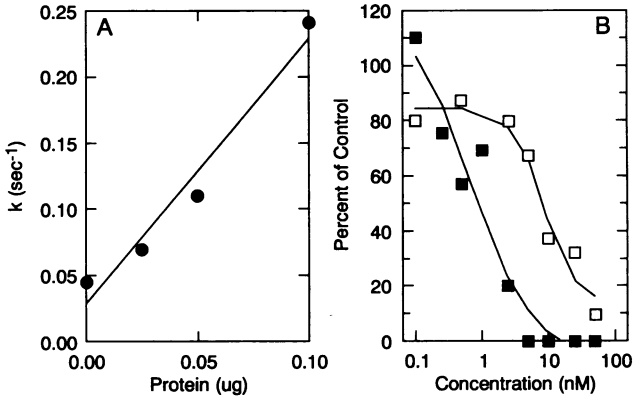
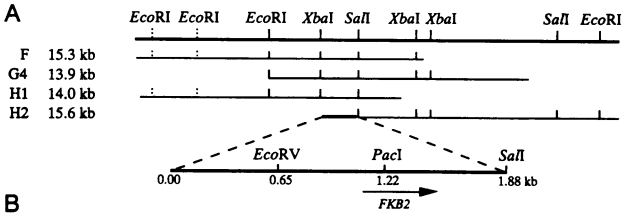


FIG. 1. (*A*) PPIase activity of pure yFKBP-13 was determined as described for Table 2 with the substrate *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide. (*B*) PPIase activity of yFKBP-13 (1 nM) was measured in the presence of various amounts of FK506 (□) or rapamycin (■) with *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (70 μM).

Table 2. PPIase substrate specificities of yFKBP-13

Xaa	k_{cat}/K_m, $\mu M^{-1} \cdot sec^{-1}$	
	yFKBP-13	yFKBP-12
Gly	0	2
Ala	0	17
Val	7	95
Leu	54	82
Phe	28	21
His	2	0
Lys	0	12

PPIase activities with peptide substrates (70 μM) of the structure *N*-succinyl-Ala-Xaa-Pro-Phe-*p*-nitroanilide were assayed (15). Reactions (1.0 ml) were conducted at 25°C in 100 mM Tris·HCl (pH 7.5). The concentration of yFKBP-13 and yFKBP-12 was 1 nM, determined as described previously (18). Recombinant yFKBP-12 was expressed in *Escherichia coli* using an expression plasmid provided by A. Marcy and K. Chan (Merck Research Laboratories) and purified by gel filtration. The recombinant and native proteins behave identically in ligand-binding assays (data not shown).

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AATTATAATCAATAAATAAAGCATATATAATGCACATTTTTAACATCTGATTACTCGCAT 60
CGTTTCTGGAAGAAAATAGCTAATATTCGTTATTTATGGCATCACGATTATCTCACCAGT 120
TACCCGTTTATGCTCTTGAAGAGATTTAGCATTACTGCCAGCGCATCTTCAAATACAGGT 180
TTATATGAGACCCATTACTATAACCCTAAGAAGAGAAAAAGGAGTGCTTTCGTTTTCAAT 240
CACATTCTAGTTTACAGTAATTGAGTCTCGATGATGTTTAATATTTACCTTTTCGTCACT 300
                                M  M  F  N  I  Y  L  F  V  T
TTTTTTTCCACCATTCTTGCAGGTTCCCTGTCAGATTTGGAAATCGGTATTATCAAGAGA 360
F  F  S  T  I  L  A  G  S  L  S  D  L  E  I  G  I  I  K  R
                   G  S  L  S  D  L  E  I  G  I  I  K  R
                PacI
ATACCGGTAGAAGATTGCTTAATTAAGGCAATGCCAGGTGATAAAGTTAAGGTTCATTAT 420
I  P  V  E  D  C  L  I  K  A  M  P  G  D  K  V  K  V  H  Y
I  P  V  E  D  X  L  I  K  A  M  P  G  D  K  V  K  V  X  Y
ACAGGATCTTTATTAGAATCGGGAACTGTATTTGACTCAAGTTATTCAAGAGGCTCTCCT 480
T  G  S  L  L  E  S  G  T  V  F  D  S  S  Y  S  R  G  S  P
T  X  S  L
ATCGCTTTTGAACTTGGCGTTGGCAGAGTAATTAAAGGTTGGGATCAAGGTGTTGCCGGC 540
I  A  F  E  L  G  V  G  R  V  I  K  G  W  D  Q  G  V  A  G
ATGTGCGTTGGCGAAAAAAGAAAGCTGCAAATTCCAAGTTCTTTGGCCTACGGAGAAAGA 600
M  C  V  G  E  K  R  K  L  Q  I  P  S  S  L  A  Y  G  E  R
GGTGTCCCAGGCGTCATTCCTCCAAGTGCTGATTTGGTGTTTGATGTCGAATTGGTAGAC 660
G  V  P  G  V  I  P  P  S  A  D  L  V  F  D  V  E  L  V  D
GTGAAATCAGCCGCCTAGGTAAATTGGCACTCAGGCACATATCTGCAACAAGATGCATGG 720
V  K  S  A  A
AGTACGTATGTGGCAAACTTTTTTTTATAGTTGGAAAATATATCCATATGAGGATGCCAG 780
CCATCATGTCAAGTTTCAATGTAATTATGGTTGTGTATATTATGTTTGCAGCAAAATAAA 840
ATAAAAGGGCTCTAACTACAATATATTAATATAAAGAAATGTTATTCAATCTAGTTTGGG 900
TATGAATTTTTTCCCTCTTAATAAAAATAGTTTAAGTAGTTTGTTCAGTCTTCTTATCGC 960
TGGTAAACATTTTTTGGGAATTTGGTTTTAAACTATCAAAAAAATCCTGAAAAAAGTCTT 1020
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FIG. 2. (*A*) Restriction map of the region including *FKB2*. The order of the three *Eco*RI fragments at the left end of the map was not established. The positions of the ambiguous sites are indicated by the dotted vertical lines. The lines below the map indicate the regions carried in each of the four phage clones F, G4, H1, and H2. A more detailed restriction map of the 1.8-kilobase (kb) *Sal* I fragment from phage H2 is shown at the bottom. The position and orientation of *FKB2* are indicated by the arrow. (*B*) Nucleotide sequence of *FKB2* (GenBank accession no. M90767). The predicted amino acid sequence of the open reading frame is presented below the nucleotide sequence and that obtained by Edman degradation analysis of purified yFKBP-13 is presented below the predicted one. Upstream TATA boxes and the *Pac* I site are underlined.

A

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                                              -1+1
yFKBP-13   MMFNIYLFVTFFSTIL-------A|GSLSDLEIGIIKR  13
hFKBP-13   MRLSWFRVLTVLSICLSAVAS|TGAEGKRKLQIGVKKR  16
yFKBP-12                          MSEVIEGNVKIDRISP  15
hFKBP-12                                MGVQVETISP   9
NcFKBP-14                         MTIPQLDGLQIEVQQE  15

yFKBP-13   IPVEDCLIKAMPGDKVKVHYTGSLLESGTVFDSSYSR  50
hFKBP-13   --VDHCPIKSRKGDVLHMHYTGK-LEDGTEFDSSLPQ  50
yFKBP-12   ---GDGATFPKTGDLVTIHYTGT-LENGQKFDSSVDR  48
hFKBP-12   ---GDGRTFPKRGQTCVVHYTG-MLEDGKKFDSSRDR  42
NcFKBP-14  ---GQGTRETRRGDNVDVHYKG-VLTSGKKFDASYDR  48

yFKBP-13   GSPIAFELGVGRVIKGWDQGVAGMCVGEKRKLQIPSS  87
hFKBP-13   NQPFVFSLGTGQVIKGWDQGLLGMCEGEKRKLVIPSE  87
yFKBP-12   GSPFQCNIGVGQVIKGWDVGIPKLSVGEKARLTIPGP  85
hFKBP-12   NKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPD  79
NcFKBP-14  GEPLNFTVGQGQVIKGWDEGLLGMKIGEKRKLTIAPH  85

yFKBP-13   LAYGERGVPGVIPPSADLVFDVELVDVKSAA       118
hFKBP-13   LGYGERGAPPKIPGGATLVFEVELLKIERRTEL     120
yFKBP-12   YAYGPRGFPGLIPPNSTLVFDVELLKVN          113
hFKBP-12   YAYGATGHPGIIPPHATLVFDVELLKLE          107
NcFKBP-14  LAYGNRAVGGIIPANSTLIFETELVGIKGVQKGE    119
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B

Percent Identities

	yFKBP-13	hFKBP-13	yFKBP-12	hFKBP-12	NcFKBP-14
yFKBP-13	(100)	55	52	47	47
hFKBP-13	55	(100)	44	44	44
yFKBP-12	52	44	(100)	57	43
hFKBP-12	47	44	57	(100)	44
NcFKBP-14	47	44	43	44	(100)

FIG. 3. (*A*) Sequence alignment of yFKBP-13 (this work), hFKBP-13 (7), hFKBP-12 (5, 6), yFKBP-12 (11–13), and *Neurospora crassa* FKBP (NcFKBP-14) (19). Residues identical in four or more of the sequences shown are enclosed by lines. Dashes indicate gaps introduced to maximize sequence homology. The processing sites for yFKBP-13 and hFKBP-13 are indicated by vertical lines, and the conserved cysteine residues by dots above the sequence alignment. Residues with side chains in contact with FK506 bound to hFKBP-12 are indicated with arrows above the sequence alignment. The numbering of each sequence begins with the first amino acid of the mature protein. (*B*) Percent identity of amino acids between each pair of sequences. The values are based on comparisons of mature proteins.

and *FKB2* are not linked and that cells disrupted in either or both are viable. Southern hybridization analysis of yeast chromosomes separated by pulsed-field gel electrophoresis showed that *FKB2* is located on chromosome IV (data not shown). Superose chromatography of cell extracts mixed with labeled FK506 confirmed that the *fkb2* mutations abolished detectable yFKBP-13 activity (Fig. 4*C*). The wild-type, *fkb1*, *fkb2*, and *fkb1 fkb2* strains exhibited no significant difference in growth rate on YPA medium containing glucose, glycerol, or acetate as the carbon source and were not heat-sensitive for growth on YPAD. We also compared the effects of the *fkb1* and *fkb2* null mutations on sensitivity to FK506 and rapamycin (Fig. 4 *D* and *E*). As shown previously (14), the *fkb1* mutation confers rapamycin resistance but results in little or no change in sensitivity to FK506. The *fkb2* mutation caused no significant change in FK506 or rapamycin sensitivity in *FKB1* or *fkb1* genetic backgrounds.

DISCUSSION

Eukaryotic FKBPs consist of an extensive, highly conserved core region (Gly[26] to Leu[111] of yFKBP-13) flanked by divergent N and C termini (Fig. 3*A*). The core region includes all residues known from structural studies of hFKBP-12 with bound FK506 (21) to be directly involved in forming the ligand binding pocket. Of the residues whose side chains interact with FK506, all are completely conserved (Fig. 3*A*), except Phe[46] of hFKBP-12, which is replaced with Ile[54] in yFKBP-13 and Leu[52] in the binding protein from *N. crassa*, and His[87], which is replaced with Val[95] in yFKBP-13 and

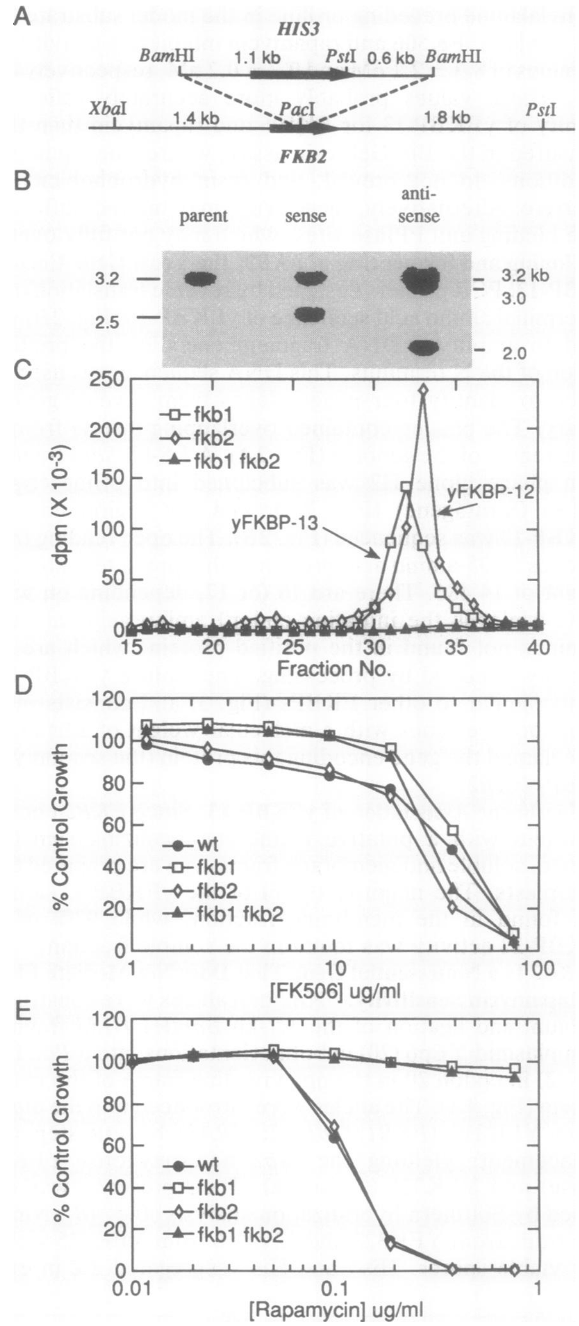

FIG. 4. Disruption of *FKB2*. (*A*) Restriction enzyme map of the *fkb2-1*::*HIS3* allele. Solid arrows indicate the location and direction of transcription of *FKB2* and *HIS3*. The *fkb2-2*::*HIS3* allele contains the *HIS3* fragment inserted in the opposite (antisense) orientation. (*B*) Southern hybridization analysis of DNAs from strains YFK319 [*FKB2*/*FKB2* (parent)], YFK323 [*FKB2*/*fkb2-1*::*HIS3* (sense)] and YFK325 [*FKB2*/*fkb2-2*::*HIS3* (antisense)]. The DNA was digested with *Xba* I plus *Pst* I and probed with labeled DNA prepared from the 1.8-kb *Sal* I fragment carrying *FKB2*. (*C*) FK506-binding activities of *fkb1*, *fkb2*, and *fkb1 fkb2* strains. Whole cell extracts of meiotic progeny derived from YFK325 (*FKB1*/*fkb1*-Δ2::*URA3* *FKB2*/*fkb2-2*::*HIS3*) were subjected to chromatography on Superose 12 with [^{3}H]dihydro-FK506. (*D* and *E*) FK506 and rapamycin drug sensitivities of sister tetratype meiotic progeny derived from YFK325. Results are presented as the average of two tetrads that were each assayed three times.

similar hydrophobic residues in the other binding proteins. These alterations may account for some of the differences in affinity for FK506 and rapamycin as well as PPIase activity among these proteins.

The sequence of yFKBP-13 is most similar to that of hFKBP-13, while that of yFKBP-12 is similar to that of hFKBP-12 (Fig. 3*B*). The core region of hFKBP-13 is 64% identical to that of yFKBP-13 and only 51% identical to that of hFKBP-12. The region of similarity of yFKBP-13 to hFKBP-13 extends an additional 20 residues upstream of the core region, with 9 identical residues and 3 highly conserved. The upstream region of yFKBP-12 is highly similar to that of hFKBP-12, with 11 of 19 residues identical and 2 highly conserved. This region diverges significantly from the same regions of yFKBP-13 and hFKBP-13. In addition, Cys^{19} and Cys^{75} of yFKBP-13 are also found in hFKBP-13 but not in other FKBPs. It has been suggested (7) that hFKBP-13 may be localized to a compartment with an oxidizing environment that promotes the formation of a disulfide bond between these two residues. The x-ray structure of hFKBP-12 indicates they would be within disulfide bond-forming distance of each other (7, 21).

Both yFKBP-13 and hFKBP-13 are membrane-bound and possess putative N-terminal signal sequences. The signal sequence of yFKBP-13 is atypical, in that it lacks any positively charged residue(s) near its N terminus. In this respect yFKBP-13 resembles the product of the yeast gene *MEL1*, although the latter does have a positive charge near its cleavage site (22). It has been suggested that the four C-terminal residues (RTEL) of hFKBP-13 may be an endoplasmic reticulum retention sequence (7). No similar sequence is seen in yFKBP-13, although there is a short C-terminal extension beyond the conserved core.

The similarity of yFKBP-13 to hFKBP-13 and of yFKBP-12 to hFKBP-12 (and other mammalian FKBP-12s) suggests that these proteins form two evolutionarily related groups with distinct functions. Our preliminary results show that yFKBP-12 interacts with bovine calcineurin, inhibiting its phosphatase activity toward phosphopeptide substrate, as reported for hFKBP-12 (3), whereas yFKBP-13 does not interact (unpublished results). The difference in compartmentalization is also consistent with the idea that the two types act on different substrates, with the membrane-bound forms possibly involved in protein translocation. Certain cyclophilins found in both yeast and mammals also have signal sequences (23, 24), as does the cyclophilin-like *ninaA* gene product of *Drosophila melanogaster* (25). Thus, these PPIases, which are otherwise unrelated to FKBPs, also occur as two types, cytosolic and membrane-bound.

We did not detect any alteration in viability or growth rate on various carbon sources upon disruption of *FKB2*. In addition, we found no effect of the *fkb2* null allele on sensitivity to FK506 or rapamycin, in either the presence or the absence of intact *FKB1*. Alterations in drug sensitivity might have been seen if yFKBP-13 could act as a significant binding reservoir, either sparing or providing ligand to the sensitive target. It is clear, however, that such is not the case for either drug. The finding that rapamycin sensitivity is affected by *fkb1*, but not by *fkb2* mutations, also suggests that these binding proteins have distinct functions *in vivo*. Koser *et al.* (23) recently described similar findings for the yeast cyclophilin Cyp2, which also possesses a signal sequence. *cyp2* null mutations confer no apparent change in either growth rate or cyclosporin sensitivity. Indeed, even triple disruptions of the PPIase-encoding genes *CYP1*, *CYP2*, and *RBP1* (equivalent to *FKB1*) were viable. It would obviously be of interest to test a quadruple mutant also lacking *FKB2*. Mutations in *ninaA* result in defective photoreceptors, due to improper translocation of rhodopsin (25). The nature of the defect in *Drosophila* suggests that disruption of PPIase genes in any organism may cause rather subtle defects.

Since mutants lacking yFKBP-13, or yFKBP-13 and yFKBP-12, are unaltered in their sensitivity to FK506, it is likely that yeast possesses an additional target for this drug and perhaps another member of the FKBP family. The product of the FK506-responsive yeast gene *FKR3* is an attractive candidate for this target (14). The *fkr3* mutant is resistant to FK506 and possesses a temperature-sensitive growth defect that is rescued by the drug. Although a third binding protein is not detected in extracts from *fkb1 fkb2* mutants by the methods published here, such a protein may be of relatively low abundance or not detectable under these assay conditions.

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