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FKBP12 Physically and Functionally Interacts with Aspartokinase in *Saccharomyces cerevisiae*

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The peptidyl-prolyl isomerase FKBP12 was originally identified as the intracellular receptor for the immunosuppressive drugs FK506 (tacrolimus) and rapamycin (sirolimus). Although peptidyl-prolyl isomerases have been implicated in catalyzing protein folding, the cellular functions of FKBP12 in *Saccharomyces cerevisiae* and other organisms are largely unknown. Using the yeast two-hybrid system, we identified aspartokinase, an enzyme that catalyzes an intermediate step in threonine and methionine biosynthesis, as an in vivo binding target of FKBP12. Aspartokinase also binds FKBP12 in vitro, and drugs that bind the FKBP12 active site, or mutations in FKBP12 surface and active site residues, disrupt the FKBP12-aspartokinase complex in vivo and in vitro. *fpr1* mutants lacking FKBP12 are viable, are not threonine or methionine auxotrophs, and express wild-type levels of aspartokinase protein and activity; thus, FKBP12 is not essential for aspartokinase activity. The activity of aspartokinase is regulated by feedback inhibition by product, and genetic analyses reveal that FKBP12 is important for this feedback inhibition, possibly by catalyzing aspartokinase conformational changes in response to product binding.

Immunophilins are a family of ubiquitously expressed intracellular receptors for the structurally unrelated immunosuppressive drugs cyclosporine, FK506 (also known as tacrolimus) and rapamycin (also known as sirolimus). The immunophilins are divided into two evolutionarily unrelated families: the cyclophilins, which bind to and mediate the cellular response to cyclosporine, and the FKBPs (for FK506-binding proteins), which mediate the effects of FK506 and rapamycin. Cyclosporine and FK506 bind cyclophilin A and FKBP12 to form structurally distinct complexes that inhibit a common target, the Ca^{2+}-calmodulin-regulated serine-threonine phosphatase calcineurin, whose activity is required for the initial Ca^{2+}-dependent step in the activation of T lymphocytes via the T-cell receptor (4, 12, 17, 19, 20, 31, 32, 39). The target of the FKBP12-rapamycin complex was first identified in *Saccharomyces cerevisiae* as the TOR1 and TOR2 kinase homologs (26), and the mammalian TOR homolog has recently been identified (6, 11, 42, 43).

The two immunophilin protein families share no primary or tertiary structural homology, yet both are enzymes that catalyze peptidyl-prolyl *cis-trans* isomerization and are thus thought to participate in protein-folding pathways (23, 45). Although a multitude of prolyl isomerases have been identified and their role in immunosuppressant action has been elucidated, much remains to be learned concerning the normal cellular functions of these highly conserved proteins. A cellular function has been elucidated for one member of the cyclophilin class of immunophilins, ninaA. ninaA is a *Drosophila* cyclophilin homolog that is required for proper folding and localization of rhodopsin (13, 46, 48, 50). ninaA forms a stable complex with rhodopsin, and decreased levels of ninaA result in rhodopsin misfolding and accumulation in the endoplasmic reticulum, indicating the importance of a stoichiometric interaction that might not have a catalytic role (2).

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FKBP family members ranging in size from 12 to 80 kDa have been identified in bacteria, yeast, and vertebrates, indicating an early evolutionary origin and functional roles beyond an immune system function (25, 38). The best-characterized FKBP is the 12-kDa isoform, but little is known about the cellular role of the FKBP12 protein in the absence of drugs. A number of proteins have been identified that physically associate with FKBP12, including the type I receptor for transforming growth factor β (56, 57) and the ryanodine and IP_3 receptor Ca^{2+} channels (5, 7, 29, 51). FKBP12 may regulate the gating of these large Ca^{2+} channels by targeting calcineurin to the channel to facilitate regulatory cycles of phosphorylation and dephosphorylation (5, 29). FKBP12 also physically interacts with the zinc finger transcription factor YY1 and a 48-kDa protein, FAP48, but the physiological significance of these interactions remains to be established (9, 58). FKBP12 is required for activity of the P-glycoprotein multidrug resistance pump when expressed in yeast (28). Finally, a bacterial FKBP homolog, FkpA, has been implicated as a chaperone or protein-folding catalyst in the maturation of outer membrane proteins in *Escherichia coli* (37).

Genetic studies have demonstrated that the cytoplasmic immunophilins cyclophilin A and FKBP12 mediate cyclosporine and FK506 actions, respectively, in yeast (4, 26, 27, 30, 53). Yeast *fpr1* mutants lacking FKBP12 are viable and resistant to both FK506 and rapamycin. However, these studies revealed little about the normal cellular roles of FKBP12 in the absence of FK506 or rapamycin; thus, the biological functions of FKBP12 remain largely unknown.

As an approach to define the cellular functions of FKBP12, we sought to identify proteins that interact with FKBP12. We have employed the yeast two-hybrid screening system originally described by Fields and Song (16), which has the ability to detect protein-protein interactions in vivo. We identified the biosynthetic enzyme aspartokinase as a binding partner of FKBP12 in vivo and in vitro. Aspartokinase is the first enzyme in the methionine-threonine biosynthetic pathway in *S. cerevisiae* and is feedback inhibited by products of this pathway, homoserine and threonine (49).

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
JK9-3da	*MAT***a** *trp1 his4 leu2-3,112 ura3-52 rme1 GAL*$^+$ *HML***a**	27
JHY2-1c	JK9-3da *fpr1::ADE2-2*	8
KDY81-18c	JK9-3da *fpr1::ADE2 fpr2::URA3 fpr3::URA3* Δ*fpr4::G418*	14a
CAY3	JK9-3da Δ*hom3::G418*	This study
MLY40	Σ1278b *ura3-52 MAT*α	32a
MLY88	Σ1278b *ura3-52 fpr1 MAT*α	32a
MLY55	Σ1278b *ura3-52 leu2::hisG* Δ*gap1:: LEU2 MAT*α	32a
CTY10-5d[a]	*MAT***a** *trp1-901 his3-200 leu2-3,112 ade2 gal4 gal80 URA3::lexAop-lacZ*	S. Fields
CAY10[a]	CTY10-5d *TOR2-3 fpr1::ADE2*	This study
Y190[a]	*MAT***a** *trp1-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80 URA3:: GAL-lacZ LYS2::Gal-HIS3*	24
SMY4[a]	Y190 *TOR1-2 fpr1::ADE2*	8

[a] Two-hybrid strain.

We find that aspartokinase binds to the FKBP12 active site, and either FKBP12 binding drugs (FK506 or rapamycin) or mutations perturb the FKBP12-aspartokinase complex. FKBP12 is not required for aspartokinase expression, stability, or activity but is important for feedback inhibition of the enzyme in vivo. One model consistent with our findings is that FKBP12 catalyzes conformational changes that result in inhibition of aspartokinase activity in response to product binding. Finally, aspartokinase is conserved in bacteria, fungi, and plants (but not in animals) and is thus the most evolutionarily diverse FKBP12 target yet identified.

MATERIALS AND METHODS

Yeast strains and preparation of media. Isogenic derivatives of strains JK9-3da, Σ1278b, Y190, and CTY10-5d were constructed by one-step gene disruptions and are listed in Table 1. The Δ*hom3::G418* allele was constructed by PCR-mediated disruption (34, 54) with the G418 resistance cassette from plasmid pFA6-KanMX2 by using oligonucleotides 5′-CGGCCCAAACAATAATGTCG CTGTCGTTTGTTCCGCCCCAGCTGAAGCTTCGTACGC-3′ and 5′-ACC AACTAATGAAACAATAGACAACTTCTTTGTGATATCGCATAGGCCA CTAGTGGATCTG-3′. The *TOR2-3* mutation in strain CAY10 was isolated as a spontaneous rapamycin-resistant allele and was shown to be a TOR2 S1975R mutation by genetic linkage and DNA sequence analysis. Complete (YPD) medium, and minimal (SD) medium were prepared as previously described (47). Minimal proline (SDP) medium is identical to SD medium, except that it contains 0.17% yeast nitrogen base without amino acids or ammonium sulfate and 0.1% L-proline is used as the sole nitrogen source. Where indicated, this medium was supplemented with different concentrations of α-amino-β-hydroxyvaleric acid (hydroxynorvaline) or 100 mg of L-lysine/liter and 100 mg of D-histidine/liter. Where necessary, minimal medium was supplemented with the appropriate amino acids.

Expression vectors. The construction of plasmids pLexA-FPR1, pGBT9-FPR1, and pGBT9-FPR2; FKBP12 mutants; and plasmids pFP101 (FKBP12) and pFP102 (F43Y FKBP12 mutant) has been described previously (8, 28, 33). Aspartokinase and the aspartokinase truncations were generated by PCR amplification with primers 5′-CG**GAATTC**ATGCCAATGGATTTCCAACCTACA TCA-3′ and 5′-CGC**GGATCC**TTAAATTCCAAGTCTTTTCAA-3′ for full-length aspartokinase, primers 5′-CG**GAATTC**ATGCCAATGGATTTCCAAC CTACATCA-3′ and 5′-CGC**GGATCC**ATCTACGACTAACTTGTACTT-3′ for the truncation-expressing residues 1 to 394, primers 5′-CCG**GAATTC**GAT CCTCGTAAGGTTCCTGAA-3′ and 5′-CGC**GGATCC**TTAAATTCCAAGTC TTTTCAA-3′ for the region containing residues 261 to 527, primers 5′-CCG **GAATTC**GATCCTCGTAAGGTTCCTGAA-3′ and 5′-CGC**GGATCC**ATCTA CGACTAACTTGTACTT-3′ for the region containing residues 261 to 394, and primers 5′-CCG**GAATTC**AAGTACAAGTTAGTCGTAGAT-3′ and 5′-CG**GG ATCC**TTAAATTCCAAGTCTTTTCAA-3′ for the region containing residues 388 to 527. The primers have either *Eco*RI or *Bam*HI sites shown in boldface. The PCR products were gel purified, digested with the corresponding restriction enzymes, cloned in the two-hybrid vector pGAD424, and confirmed by sequencing.

Yeast two-hybrid system. The yeast reporter strains CAY10 and SMY4 were cotransformed with pLexA-FPR1 plasmid and yeast genomic libraries (10) or with pGBT9-FPR1 plasmid and rescued library plasmids, respectively, by the lithium acetate method (44). Double transformants were plated on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator medium (10). β-Galactosidase assays were performed as described previously (8).

Western blot analysis and affinity chromatography. Procedures for affinity purification of the His6-FKBP12 protein, preparation of yeast protein extracts, FKBP12 affinity chromatography, and Western blot detection were performed as previously described (8). The rabbit polyclonal antiserum against aspartokinase was kindly provided by S. Carl Falco (40).

The yeast gene encoding aspartokinase, *HOM3*, was PCR amplified with two flanking primers (CGCGGATCCATGCCAATGGATTTCCAACCTACATCA [789] and CGCGGATCCTTAAATTCCAAGTCTTTTCAA [637]), cleaved with *Bam*HI, cloned in the Invitrogen His_6 expression plasmid pTrcHisA, and confirmed by DNA sequence analysis. The resulting plasmid was introduced into the Invitrogen host strain TOP10. Cells expressing His_6-tagged aspartokinase (H6-AK) were grown at 37°C overnight in a 20-ml culture of FB medium (25 g of tryptone, 7.5 g of yeast extract, 1 g of glucose, 6 g of NaCl, 50 ml of 1 M Tris [pH 7.6] per liter) with ampicillin (50 μg/ml). The culture was centrifuged, and the pellet was resuspended in 1 liter of FB with ampicillin (50 μg/ml) and grown at 37°C to an optical density at 600 nm of 0.7. IPTG (isopropyl-β-D-thiogalactopyranoside) (2 mM) was added, and the culture was incubated for 4 h at 37°C. Cells were collected by centrifugation at 10,000 rpm (Sorvall RCSB; GS3 rotor) for 10 min, resuspended in 10 ml of ice-cold lysis buffer (40 mM HEPES [pH 7.4], 200 mM KCl, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride), and lysed by sonicating 10 times for 1 min with 1-min periods of cooling. Cell lysate was clarified by centrifugation at 40,000 rpm for 30 min in a Beckman Ti-70 rotor. Four milliliters (packed volume) of Ni^{2+} nitriloacetic acid-agarose resin (Qiagen) (preequilibrated in lysis buffer) was added to the clarified cell lysate, and the suspension was gently stirred for 30 min at 4°C. The resin-cell extract mix was loaded onto an econocolumn (Bio-Rad) and washed sequentially with 250 ml of lysis buffer, 100 ml of 10 mM imidazole, 50 ml of 20 mM imidazole, and 50 ml of 50 mM imidazole, all in lysis buffer. H6-AK was eluted in 4 ml of 200 mM imidazole in lysis buffer, dialyzed with lysis buffer, and coupled to Affi-Gel as described previously for FKBP12 (8).

Aspartokinase activity assays. Aspartokinase activity was measured by monitoring ADP production by using the pyruvate kinase-lactate dehydrogenase coupled assay (55). The standard assay mixture contained 15 μg of partially purified protein, 10 mM aspartic acid, 10 mM ATP, 30 mM $MgSO_4$, 0.3 mM NADH, 180 mM KCl, 100 mM HEPES (pH 7.6), 4.5 mM phosphoenolpyruvate, 10 U of pyruvate kinase, and 15 U of lactate dehydrogenase in a final volume of 0.5 ml. Depletion of NADH was monitored by measuring the optical density at 340 nm, and the oxidation of NADH was coupled to ADP production.

RESULTS

FKBP12 interacts with aspartokinase in vivo. The normal cellular roles of the FKBP12 protein in the absence of FK506 and rapamycin are largely unknown. The identification and characterization of proteins in a cell with which a given protein interacts is often helpful in understanding the function of that protein. We used the yeast two-hybrid system to identify proteins that interact with FKBP12.

The entire yeast FKBP12 protein was fused to the DNA binding domain of LexA (pFLEX1) (8). Strain CAY10 is derived from the two-hybrid reporter strain CTY10-5d, and it contains a LexA promoter-driven *E. coli lacZ* reporter gene, is deleted for FKBP12 (Δ*fpr1*), and contains a *TOR2-3* mutation that confers rapamycin resistance. Strain CAY10 was cotransformed with pFLEX1 and three libraries of random yeast genomic DNA fragments fused to the GAL4(AD) coding sequence. A total of 60,000 double transformants were screened, and positive candidates were identified as blue colonies on X-Gal medium (10). Thirty-four colonies that displayed β-galactosidase activity were identified, and library plasmids were rescued and retransformed in the reporter strain SMY4 in combination with a GAL4(BD)-FKBP12 fusion plasmid or an extraneous GAL4(BD) fusion plasmid. Note that the DNA binding domain fused to FKBP12 was switched from LexA to GAL4 as a measure of specificity. Five clones remained positive, and partial sequence analysis revealed that two genes had been identified. The first encodes SPP41 and was previously

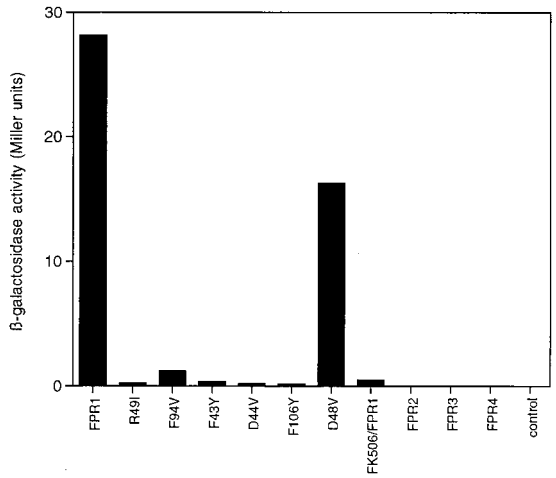

FIG. 1. Specificity of the FKBP12-aspartokinase interaction in vivo. GAL4-aspartokinase fusion protein was expressed with GAL4-FKBP12 (FPR1) fusion protein in the presence or absence of FK506 (10 μg/ml) or with mutant forms of FKBP12 (R49I, F94V, F43Y, D44V, F106Y, and D48V) or other members of the FKBP family (FPR2, FPR3, and FPR4) as GAL4 fusion proteins in the SMY4 two-hybrid host strain (Table 1). β-Galactosidase activities were measured by chlorophenol red-β-D-galactopyranoside assay.

identified as a potential negative regulator of *PRP3* and *PRP4* gene expression (36). The second encodes aspartokinase and is the subject of this report. FKBP12 and aspartokinase also interacted in the two-hybrid assay when aspartokinase was fused to the GAL4 DNA binding domain and FKBP12 was fused to the GAL4 activation domain.

To address the question of sequence requirements for FKBP12 binding to aspartokinase, we tested a series of FKBP12 mutants in which single amino acid substitutions were introduced. Some of these mutations perturb the active site and reduce prolyl isomerase activity with peptide substrates in vitro (F43Y, D44V, and F106Y), while others are required, or are near residues that are required, for FKBP12-FK506 binding to calcineurin (R49I, F94V, and D48V) (1, 14, 52). The interaction of these FKBP12 mutants with aspartokinase was quantified by measuring β-galactosidase activities in the two-hybrid assay. As shown in Fig. 1, only wild-type FKBP12 and the D48V FKBP12 mutant protein interacted strongly with aspartokinase. In contrast, mutations at FKBP12 active site or surface residues abrogated FKBP12-aspartokinase binding in the two-hybrid assay. We have previously demonstrated that these mutant fusion proteins are stably expressed as detected by Western blot analysis (33). Two immunosuppressive drugs, FK506 and rapamycin, bind to the FKBP12 active site, and both drugs effectively competed with aspartokinase for FKBP12 binding (Fig. 1). These findings demonstrate that drug binding or mutations within the active site or surface residues decrease FKBP12 binding to aspartokinase, suggesting that the interaction is specific, occurs at both the FKBP12 active site and surrounding surface residues, and may be functionally important.

Although aspartokinase was identified as a binding partner for FKBP12, yeast cells express three other closely related FKBP proteins (FPR2, FPR3, and FPR4), one or more of which might be the physiologically relevant aspartokinase binding partner. To test this possibility, we assessed whether aspartokinase would interact with FPR2, FPR3, or FPR4 in the two-hybrid assay. As shown in Fig. 1, only FKBP12, and not the FPR2 (FKBP13), FPR3 (FKBP70), or FPR4 (FKBP60) proteins, interacted with aspartokinase, further demonstrating the specificity of the FKBP12-aspartokinase interaction. In the case of FPR3 and FPR4, which are acidic proteins, fusions to the GAL4(AD) were required. Whereas GAL4(BD)-aspartokinase readily interacted with GAL4(AD)-FKBP12 (32 U), no interaction was detected with either the GAL4(AD)-FPR3 or the GAL4(AD)-FPR4 fusion protein. In other studies, the GAL4(AD)-FPR3 and GAL4(AD)-FPR4 fusion proteins were found to interact with the ribosomal protein S24 in the two-hybrid system, indicating that these fusion proteins are expressed and functional (14a).

To assess aspartokinase structural features required for FKBP12 binding, we made a number of constructs encoding full-length aspartokinase or various regions of aspartokinase (from residues 1 to 394, 261 to 527, 261 to 394, and 388 to 527) fused to the GAL4 activation domain. None of the truncated forms of aspartokinase interacted with FKBP12 in the two-hybrid assay (data not shown), indicating that intact aspartokinase, or two noncontiguous regions of the protein, may be necessary for FKBP12 binding. Finally, the addition or omission of threonine, a product of the aspartokinase pathway responsible for feedback inhibition, had little or no effect on the strength of the FKBP12-aspartokinase interaction detected in the two-hybrid assay (data not shown).

FKBP12 interacts with aspartokinase in vitro. To confirm the specific interaction between FKBP12 and aspartokinase in vitro, we tested aspartokinase binding to an FKBP12 affinity matrix. Yeast FKBP12 was His_6 tagged (H6-FKBP12) at the amino terminus, subcloned into a bacterial expression vector, overexpressed in bacteria, and purified on a Ni^{2+} affinity column. By this approach high levels of pure H6-FKBP12 were readily recovered, and the recombinant protein was fully active and FK506 sensitive in an in vitro prolyl isomerase assay (data not shown).

To produce the affinity chromatography matrix, H6-FKBP12 protein was coupled to Affi-Gel 10 beads. To test if aspartokinase binds to the FKBP12 affinity matrix, beads loaded with FKBP12 were incubated with or without 20 μM FK506 prior to the addition of cell extracts. Cell extracts were prepared from the wild-type strain JK9-3da and isogenic mutants lacking FKBP12 (*fpr1*) or aspartokinase (*hom3*). Proteins retained by the FKBP12 affinity matrix were eluted, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting with a polyclonal antiserum to aspartokinase (Fig. 2A).

With extracts from wild-type or *fpr1* mutant cells we detected binding of aspartokinase (visualized as an ~65-kDa protein) to the FKBP12–Affi-Gel beads in the absence of FK506 (Fig. 2A, lanes 1 and 3). This binding is specific because no aspartokinase binding was observed with Affi-Gel beads alone (Fig. 2A, lane 7) or with extracts lacking aspartokinase (Fig. 2A, lanes 5 and 6). As with the two-hybrid assay, the in vitro FKBP12-aspartokinase interaction was disrupted by FK506 (Fig. 2A, lanes 2 and 4). These findings provide additional support for the specificity of the interaction and underscore the fact that the FKBP12 active site is required to form the complex.

These in vitro experiments do not rule out the possibility that FKBP12 and aspartokinase might interact via a bridging cellular protein. To address this question, in vitro binding experiments were performed with purified proteins. H6-AK was affinity purified on Ni^{2+} affinity columns, and H6-AK was coupled to Affi-Gel 10 beads. Binding experiments were performed with H6-FKBP12 (purified as described above). Proteins were eluted and subjected to SDS-PAGE, and Western blotting was performed with a polyclonal antibody against

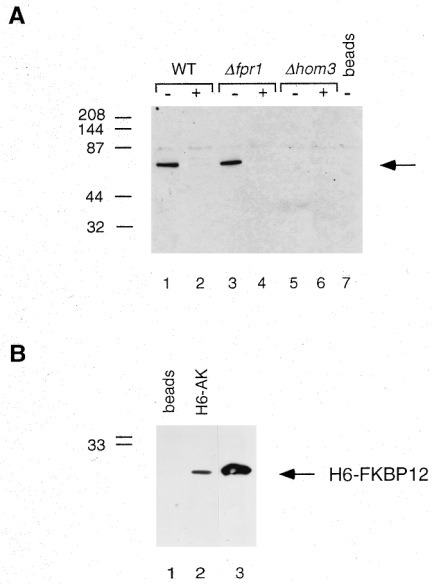

FIG. 2. FKBP12 and aspartokinase interact in vitro. (A) Binding assays with purified H6-FKBP12 coupled to Affi-Gel 10 beads were performed with cell extracts from wild-type strain JK9-3da (WT) (lanes 1 and 2) and isogenic mutant strains lacking FKBP12 (Δ*fpr1*; strain JHY3-3d) (lanes 3 and 4) and aspartokinase (Δ*hom3*; strain CAY3) (lanes 5 and 6) in the absence (−) or presence (+) of FK506 (20 μM). Lane 7 is a control binding assay in which Affi-Gel beads without H6-FKBP12 were incubated with wild-type extract. Bound proteins were eluted, fractionated by SDS-PAGE, and analyzed by Western blotting probed with an antibody against aspartokinase. The arrow indicates the position of migration of aspartokinase. (B) Binding assays were performed with H6-AK affinity matrix and 3 μg of purified H6-FKBP12 (lane 2). Lane 1 is a control experiment in which Affi-Gel beads were incubated with 3 μg of purified H6-FKBP12. Six nanograms of purified H6-FKBP12 was electrophoresed as a control (lane 3).

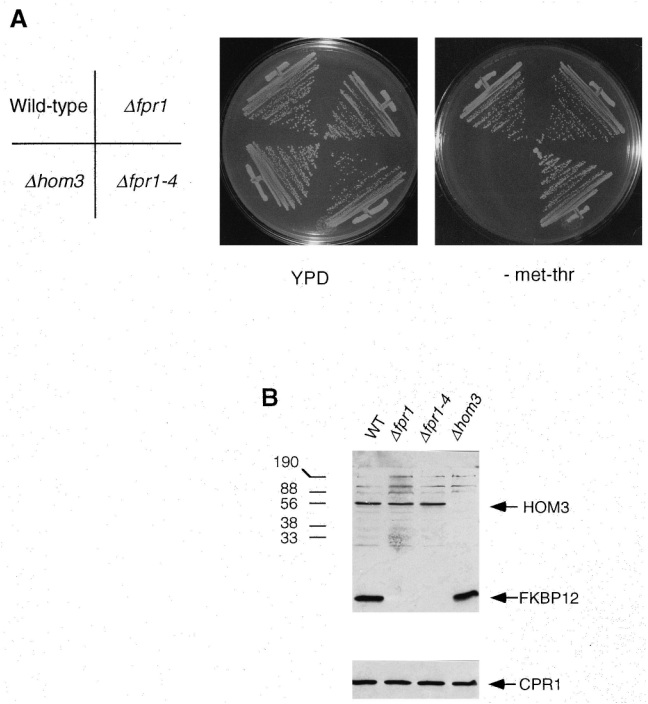

FIG. 3. FKBP12 is not required for aspartokinase expression or stability. (A) Wild-type strain JK9-3d and isogenic mutants lacking FKBP12 (Δ*fpr1*), all yeast FKBPs (Δ*fpr1-4*), or aspartokinase (Δ*hom3*) were grown for 72 h at 30°C on YPD or synthetic medium lacking methionine, threonine, or both methionine and threonine (−met-thr). (B) Fifty micrograms of protein extracts from wild-type strain JK9-3d (WT) and isogenic mutants lacking FKBP12 (Δ*fpr1*), all yeast FKBPs (Δ*fpr1-4*), or aspartokinase (Δ*hom3*) were analyzed by Western blot with antisera against aspartokinase and FKBP12. Twenty-five micrograms of protein from the same extracts was analyzed by Western blot for cyclophilin A to verify that equal amounts of protein were loaded.

FKBP12. We readily detected FKBP12 binding to aspartokinase–Affi-Gel beads (Fig. 2B, lane 2), and this binding was specific because no FKBP12 binding was observed with Affi-Gel beads alone (Fig. 2B, lane 1). These findings confirm that aspartokinase and FKBP12 form a direct complex in vitro.

FKBP12 is not required for aspartokinase expression or activity. In *S. cerevisiae*, threonine and methionine are synthesized from aspartate through a common metabolic pathway that leads to homoserine, the point at which the pathways diverge. Aspartokinase is the first enzyme of the methionine-threonine biosynthetic pathway. To determine if FKBP12 binding is required for aspartokinase function, we tested if *fpr1* mutants lacking FKBP12 were auxotrophic for methionine or threonine. Yeast mutants lacking FKBP12 are viable, but grow more slowly than isogenic wild-type strains, with the doubling time increased by 15 to 30% (27). Mutants lacking FKBP12 have no additional growth defect on media lacking methionine and/or threonine (Fig. 3A and data not shown); thus, FKBP12 is not essential for aspartokinase activity. In addition, we found no difference in the amount of aspartokinase expressed in wild-type versus *fpr1* mutant strains lacking FKBP12 (Fig. 3B).

To test if FKBP12 was required for normal levels of aspartokinase activity, aspartokinase was partially purified by the method of Ramos et al. (41) from cell extracts of the wild-type strain JK9-3da and isogenic mutants lacking FKBP12 (*fpr1*) or aspartokinase (*hom3*). Aspartokinase activity was assayed as previously reported (55) by using pyruvate kinase and lactate dehydrogenase in a coupled system. ADP formed in the aspartokinase reaction is phosphorylated to ATP at the expense of phosphoenolpyruvate, and the resulting pyruvate is reduced by NADH. The oxidation of NADH was followed spectrophotometrically at 340 nm. No differences were observed between wild-type and mutant extracts lacking FKBP12 (Fig. 4), indicating that FKBP12 is not essential for aspartokinase activity.

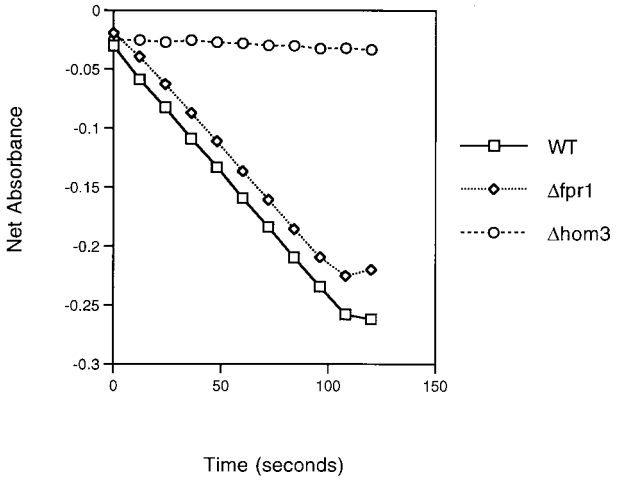

FIG. 4. FKBP12 is not required for aspartokinase activity. Aspartokinase was partially purified from isogenic wild-type (WT), FKBP12 mutant (Δ*fpr1*), and aspartokinase mutant (Δ*hom3*) strains, and aspartokinase activity was assayed by the pyruvate kinase-lactate dehydrogenase coupled assay, in which NADH oxidation was measured by plotting the net absorbance at 340 nm versus time.

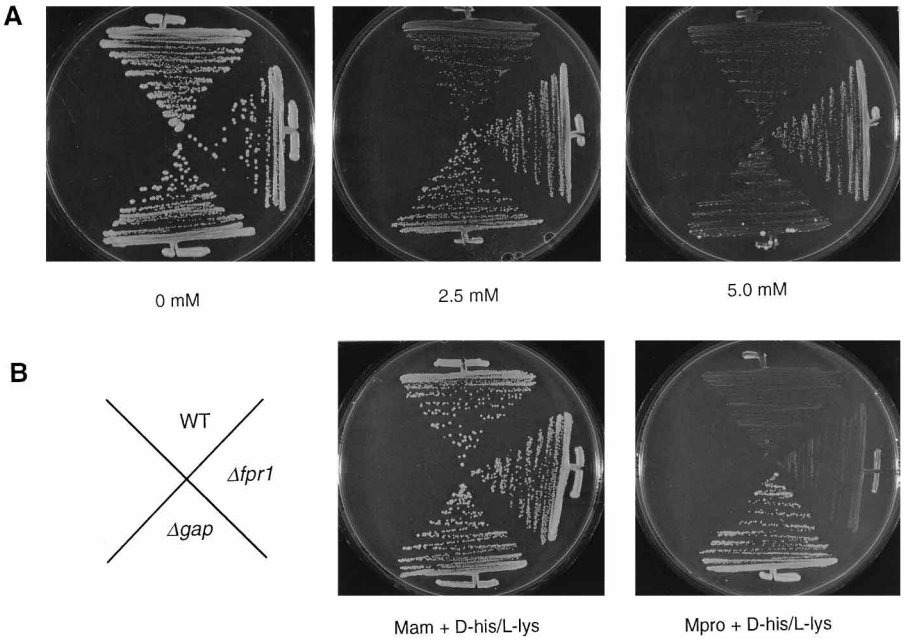

FIG. 5. FKBP12 is required for feedback inhibition of aspartokinase by hydroxynorvaline. (A) Wild-type strain MLY40 (WT) and the isogenic mutants lacking FKBP12 (MLY88; *fpr1*) and aspartokinase (MLY55; Δ*gap1*) were grown for 96 h at 30°C on minimal proline medium lacking or containing 2.5 or 5 mM hydroxynorvaline. (B) The strains described for panel A were grown for 96 h at 30°C on either minimal ammonium (Mam) or minimal proline (Mpro) medium containing 100 μg of D-histidine/ml and 100 μg of L-lysine/ml (D-his/L-lys).

FKBP12 regulates feedback inhibition of aspartokinase. The regulation of the threonine-methionine biosynthetic pathway occurs at two levels: transcriptional regulation and a posttranslational regulation of enzyme activity. Aspartokinase activity is regulated posttranslationally by feedback inhibition in response to threonine. Hydroxynorvaline is a toxic structural analog of threonine. Cells grown in the presence of hydroxynorvaline produce smaller quantities of the amino acids synthesized through this pathway, which become limiting for growth.

One possibility is that FKBP12 catalyzes aspartokinase conformational changes that occur during product inhibition to reduce enzyme activity. Wild-type yeast cells grown in the presence of 2.5 or 5 mM hydroxynorvaline exhibited a growth defect (Fig. 5A). In contrast, isogenic FKBP12 mutant cells grew better than wild-type cells at the same concentrations of hydroxynorvaline, suggesting that FKBP12 is required for proper feedback inhibition by hydroxynorvaline. The colony size of the FKBP12 mutant strain was somewhat reduced on medium containing hydroxynorvaline compared to that on control medium, indicating that this is a relative rather than absolute resistance. Importantly, FKBP12 mutant cells were as resistant as (at 2.5 mM) or more resistant than (at 5 mM) Δ*gap1* mutant cells, which lack the general amino acid permease that, at least in part, transports hydroxynorvaline (Fig. 5A). These growth effects were quantified by liquid culture assays in synthetic minimal proline medium containing several different concentrations of hydroxynorvaline (Table 2). The doubling time for all three strains was ~4 h in control medium, whereas in medium with 2.5 mM hydroxynorvaline the doubling time for the wild-type strain was increased to >24 h, but it was increased to only ~9 h for the Δ*gap1* mutant strain, and it was increased to ~12 h for the *fpr1* mutant strain (Table 2). These observations further support the conclusion that strains lacking FKBP12 are relatively, but not absolutely, hydroxynorvaline resistant.

To further investigate a role for FKBP12 in feedback inhibition of aspartokinase, an *fpr1* mutant strain lacking FKBP12 was transformed with either a centromeric plasmid lacking or encoding wild-type FKBP12 or the FKBP12 active site mutant F43Y. Expression of wild-type FKBP12, but not of the active site mutant, fully restored feedback inhibition by hydroxynorvaline (Fig. 6). These findings reveal that FKBP12 is necessary for proper feedback inhibition of aspartokinase by hydroxynorvaline and that the integrity of the FKBP12 active site is required for this function.

Because the macrolide binding site of FKBP12 participates in binding to aspartokinase, and FKBP12 mutants (*fpr1*) are resistant to the toxic effects of hydroxynorvaline, we tested whether the growth defect observed in wild-type strains grown in the presence of hydroxynorvaline could be blocked by an excess of FK506. As shown in Fig. 7, 100 μg of FK506/ml overcame the toxic growth-inhibitory effects of 2.5 mM hydroxynorvaline, further confirming that the drug binding, active site of FKBP12 is involved in binding to aspartokinase and that FKBP12 binding to aspartokinase is important for proper regulation by feedback inhibition.

An alternative explanation for these observations, namely, that FKBP12 is required for the general amino acid permease GAP1 function of importing hydroxynorvaline into the cell

TABLE 2. Growth rates in liquid cultures with hydroxynorvaline

Strain	Doubling time (h)			
	0[a]	2.5	5	10
Wild type (MLY40)	4	>24	>24	>24
fpr1 (MLY88)	4	12	14	18
Δ*gap1* (MLY55)	4	9	>24	>24

[a] Concentration of hydroxynorvaline (millimolar).

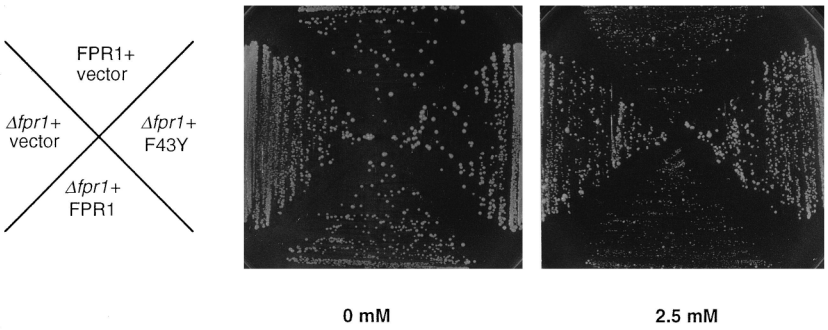

FIG. 6. Wild-type FKBP12, but not an active site mutant, complements the aspartokinase feedback inhibition defect in FKBP12 mutant strains. Yeast strains expressing (*FPR1*; strain JK9-3d) or lacking (Δ*fpr1*; strain JHY2-1c) FKBP12 were transformed with plasmids expressing no FKBP12 (vector), wild-type FKBP12 (FPR1; plasmid pFP101), or the FKBP12 active site mutant F43Y (plasmid pFP102). Cells were grown for 96 h at 30°C on minimal proline medium lacking (0 mM) or containing (2.5 mM) hydroxynorvaline.

(Fig. 5A), was excluded by the finding that GAP1 was functional in FKBP12 mutant cells (*fpr1*) to import the toxic amino acids D-histidine and L-lysine (Fig. 5B). Moreover, the resistance of aspartokinase to feedback inhibition by hydroxynorvaline that was observed in the FKBP12 mutant strains (*fpr1*) was not a nonspecific consequence of the modest growth defect of these strains, because the F43Y FKBP12 active site mutant complements the growth defect of *fpr1* mutant strains (14a) but does not complement and restore feedback inhibition of aspartokinase. Thus, taken together, our findings support the conclusion that FKBP12 regulates feedback regulation of aspartokinase.

DISCUSSION

The evolutionarily conserved cytoplasmic protein FKBP12 is known to bind FK506 and rapamycin to mediate immunosuppression, but much less is known about its normal physiological roles. We used the yeast two-hybrid system to identify proteins that interact with FKBP12 in vivo. In this study we demonstrated that FKBP12 interacts with aspartokinase in the two-hybrid system. Mutations within the FKBP12 active site disrupt binding to aspartokinase, and structural and biochemical studies have demonstrated that these residues are involved in the binding of proline-containing peptides and the catalysis of peptidyl-prolyl isomerization (1, 52). FKBP12 surface residues required for FKBP12-FK506 binding to calcineurin (1) are necessary for aspartokinase binding. Finally, FK506 and rapamycin bind to and inhibit FKBP12 and abolish the FKBP12-aspartokinase interaction, again indicating that aspartokinase binds to the FKBP12 active site. The FKBP12-aspartokinase interaction is specific, as aspartokinase did not interact with other members of the FKBP family (Fig. 1). Finally, by affinity chromatography, we showed that aspartokinase binds FKBP12 in vitro (Fig. 2A), that FK506 inhibits this interaction, and that the interaction is direct (Fig. 2B). Taken together, these findings establish that FKBP12 and aspartokinase specifically interact in vivo and in vitro and suggest that aspartokinase might be a relevant FKBP12 substrate or target in vivo.

Several models can be envisioned for a physiologically relevant interaction between FKBP12 and aspartokinase. Given the role of aspartokinase in the threonine-methionine biosynthetic pathway, and the fact that FKBP12 mutants have a slight growth defect, it was possible that FKBP12 was required for aspartokinase expression or function. However, in contrast to aspartokinase mutants (*hom3*), which are auxotrophic for threonine and methionine, *fpr1* mutant strains lacking FKBP12 are not. We also found no difference in the levels of aspartokinase expression in wild-type versus FKBP12 mutant yeast strains or in the activities of partially purified aspartokinase from wild-type and an FKBP12 mutant strain. Thus, FKBP12 is not required for aspartokinase expression, stability, or activity.

A second model is that FKBP12 is involved in feedback inhibition of aspartokinase by products, possibly by altering the structure of the enzyme and affecting its activity. Hydroxynorvaline is a toxic structural analog of threonine that feedback inhibits aspartokinase and deprives the cell of threonine and methionine. We found that FKBP12 mutant yeast strains are resistant to the toxic effects of hydroxynorvaline and that the

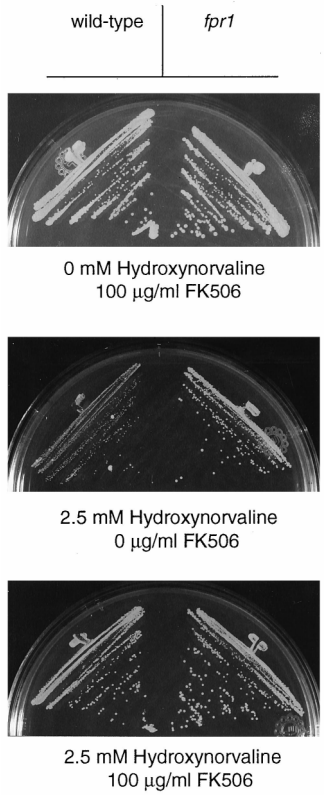

FIG. 7. FK506 reverses growth inhibition by hydroxynorvaline. Isogenic wild-type (MLY40) and FKBP12 mutant (MLY88; *fpr1*) strains were grown for 96 h at 30°C on minimal proline medium with or without hydroxynorvaline (2.5 mM) and with or without FK506 (100 μg/ml) as indicated.

wild-type FKBP12 gene complements this mutant phenotype, whereas an FKBP12 active site mutant (F43Y) fails to do so (Fig. 6). Taken together, our findings support a model in which FKBP12 plays a role in the feedback inhibition of aspartokinase. Previous studies have demonstrated that during refolding of *E. coli* aspartokinase, aspartokinase activity is recovered quickly but recovery of feedback inhibition is greatly delayed (22). An intriguing possibility is that one of the bacterial FKBP homologs might play an analogous role in facilitating conformational changes during feedback inhibition of the bacterial enzyme.

We can envision two possible models for the role of FKBP12 in modulating feedback inhibition of aspartokinase. In the first model, the active site and prolyl isomerase activity of FKBP12 are required for it to function in a traditional enzyme-substrate interaction. In this model, FKBP12 binds to and catalyzes the isomerization of one or more peptidyl-prolyl bonds in aspartokinase to result in conformational changes that inhibit aspartokinase activity upon or during product binding. In the second model, FKBP12 binds to aspartokinase to form a stable protein-protein complex, and binding rather than catalysis is the required FKBP12 function. The FKBP12-aspartokinase complex might be required for interaction with other regulatory molecules, or FKBP12 might perform a chaperone function for aspartokinase regulation. Because active site mutations that reduce FKBP12 prolyl isomerase activity with short peptide substrates in vitro and drugs that bind to the FKBP12 active site prevent both FKBP12 binding to aspartokinase and catalysis, we cannot separate binding from catalysis by these approaches. Hence, our findings are consistent with both models. A number of proline residues are conserved between evolutionarily divergent aspartokinases, and one or more of these prolines could represent targets for FKBP12 binding or catalysis. Our mutagenic studies further implicate residues both within and surrounding the FKBP12 active site in aspartokinase binding, which may be indicative of a large interaction surface between the two molecules.

Recent studies of other prolyl isomerase-protein complexes are relevant to this discussion. In the case of the *Drosophila* ninaA cyclophilin homolog, ninaA forms a very stable, stoichiometric complex with rhodopsin, and it has been suggested that it serves as a chaperone rather than as a catalyst (2). In the case of the human cyclophilin A protein and human immunodeficiency virus type 1, cyclophilin A forms a stable complex with the GAG protein, is assembled into the virion, and is required for the production of infectious virus (3, 18). The recent solution of the X-ray structure of the cyclophilin A-GAG complex reveals that cyclophilin binds to an exposed proline-containing loop on GAG and that the peptidyl-prolyl bond is in the standard *trans* configuration (21). The resulting hypothesis is that the role of cyclophilin A is binding to, and not catalysis of, the GAG protein (35). Finally, recent studies on the mammalian RanBP2 cyclophilin homolog reveal that its ability to form a stable complex with mammalian opsins is similarly dependent on the integrity of the cyclophilin active site (15). Taken together, these findings suggest that some, but perhaps not all, functions of the prolyl isomerases may be independent of catalytic activity.

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