FKBP12 Controls Aspartate Pathway Flux in Saccharomyces cerevisiae
To Prevent Toxic Intermediate Accumulation

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Prolyl isomerases are widely conserved, ubiquitous enzymes that catalyze cis-trans isomerization of peptidyl-prolyl bonds, a reaction that can be rate limiting for protein folding. Founding members of this group of enzymes are cyclophilin A, previously identified as the cyclosporine A receptor (25, 31, 75), and the structurally unrelated enzyme FK506 binding protein FKBP12 (35). A third family of prolyl isomerases, known as the parvulins, was discovered for bacteria (60, 61) and later was found to be conserved in many other organisms.

Cyclophilin A and FKBP12 mediate the immunosuppressive effects of cyclosporine A and FK506 in mammals by forming complexes with these drugs that bind to and inhibit the functions of calcineurin in T-cell activation (for a review, see reference 68). FKBP12 is also the receptor for the drug rapamycin, and the FKBP12-rapamycin complex inhibits the functions of the Tor proteins (36, 44). Both cyclophilin A and FKBP12 are conserved in budding yeast (where they are encoded by the CPR1 and FPR1 genes, respectively) and mediate calcineurin inhibition by cyclosporine A and FK506 (11, 26, 30, 37, 38, 52, 55, 78, 83) and Tor inhibition by rapamycin (36, 44). Saccharomyces cerevisiae expresses seven other cyclophilins (Cpr2 to Cpr8), three other FKBP's (Fpr2 to Fpr4), and a single parvulin (Ess1) (for a recent review, see reference 6).

With the exception of Ess1, all yeast prolyl isomerases are dispensable for growth (18, 32, 34). However, cyclophilin A does become essential in cells compromised for Ess1 function, suggesting a functional overlap between these two structurally unrelated prolyl isomerases (4). Thus far, the endogenous functions that have been defined for these proteins are relatively specific and devoted to restricted interaction partners. For example, cyclophilin A is required for glucose-stimulated transport of fructose-1,6-bisphosphatase into the vacuole import and degradation vesicles (12). In addition, cyclophilin A promotes proper subcellular localization of the essential zinc-finger protein Zpr1 (2). Cyclophilin A interacts with two different histone deacetylase complexes that regulate meiosis, the Sin3-Rpd3 and Set3 complexes, and recent studies have revealed a nuclear role for Cpr1 in controlling the expression of key meiosis-specific genes (4, 5, 58). Cpr3 is a mitochondrial cyclophilin that accelerates protein refolding after mitochondrial import (17, 19, 49, 66, 67). Cpr6 and Cpr7, like their human homolog cyclophilin 40, interact with and regulate the activity of the molecular chaperone Hsp90 (15, 20, 22, 47, 71, 76). Ess1, the first eukaryotic parvulin, was originally associated with pre-mRNA processing and termination (33, 34) and more recently with transcription and chromatin modification (4, 51, 84–86).

Yeast FKBP12 interacts with calcineurin in the absence of FK506, and genetic evidence implicates this interaction in negatively regulating calcineurin function, suggesting that this could be one of the cellular functions of this prolyl isomerase (14). A search for yeast proteins interacting with FKBP12 in the yeast two-hybrid system identified the enzyme aspartokinase (AK) as an FKBP12 binding partner (1). AK catalyzes the first reaction in the conversion of aspartic acid into the amino acid homoserine, a branch point in synthesis of threonine and methionine. Studies by Alarcon and Heitman (1) suggest that FKBP12 influences AK feedback inhibition by threonine, the main point of regulatory control in the aspartate pathway (3, 48, 57, 62).
More recently, a yeast synthetic lethal genetic screen with fpr1 mutations identified the HMO1 gene, which encodes a high-mobility-group (HMG) protein also conserved in humans (21, 45). Hmo1 is a nuclear protein that functions in stabilizing chromatin structure and plasmid maintenance (46) and as an RNA polymerase I factor (27). FKB12 and Hmo1 interact physically, possibly to regulate Hmo1 self-association (21).

In this study, we extended the analysis of FKB12 cellular functions by conducting a systematic search for yeast mutations that exhibit synthetic lethality with an fpr1 mutation. In this screen, we found that in addition to hmo1Δ, a mutation in the HOM6 gene encoding homoserine dehydrogenase also conferred lethality in an fpr1Δ mutant. Homoserine dehydrogenase catalyzes the last step in the synthesis of homoserine from aspartate. We present evidence that loss of FKB12 function in a hom6 mutant leads to toxic accumulation of aspartate β-semialdehyde, the substrate of homoserine dehydrogenase, through deregulation of AK activity. Our results indicate that FKB12 is a key component governing metabolic flux through the homoserine biosynthetic cascade.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides used in this work are listed in Table 1.

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Two-hybrid interaction assays. Yeast two-hybrid host strain PJ69-4A was transformed with plasmids expressing the Gal4 DNA binding domain (BD) and Gal4 activation domain (AD) fusion proteins. Transformants were grown in liquid synthetic dextrose (SD) medium supplemented with adenine, uracil, histidine, and leucine or in the same medium with 100 μg of 1-phenylalanine/liter or with 10 μg of 5-fluoroorotic acid/liter, and induction of the lacZ reporter gene was measured as β-galactosidase activity as described previously (14).

Western blot analysis. For Western blot analysis of expression of AK and Fpr1, yeast strains expressing these proteins were cultured in liquid YPD medium. Whole-cell protein extracts were prepared by glass bead disruption in lysis buffer A (20 mM HEPES [pH 7.4], 20 mM KCl, 0.5 mM EDTA, and a cocktail of protease inhibitors consisting of 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin A/ml, and 0.001% aprotinin), using a FastPrep instrument (FP 120; Bio 101, Savant). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Immun-Blot; Bio-Rad), probed with rabbit polyclonal antiserum against Fpr1 (13) or with rabbit polyclonal antiserum against aspartokinase (59), kindly provided by S. Carl Falko. Reactions were detected with ECL (Amersham Biosciences).

AK purification and assays. AK partial purification was as described previously (24). AK activity was measured with an enzymatic assay that couples ADP formation with NADH depletion, using the pyruvate kinase/lactate dehydrogenase system (63).

RESULTS

fpr1Δ hmo6Δ double mutants are inviable. To elucidate cellular functions of FKB12 in yeast, we conducted a search for mutations in yeast genes that result in a lethal phenotype when combined with an fpr1Δ mutation. By using a high-

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TABLE 2. Yeast strains used in this study

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throughput assay recently developed by Pan et al. (54), we identified a deletion of the HOM6 gene, which encodes homoserine dehydrogenase, as a candidate synthetic lethal mutation.

To validate these results, the synthetic lethal interaction between the fpr1Δ and hom6Δ mutations was tested by classic tetrad analysis. To this end, fpr1Δ and hom6Δ single mutants were first constructed by replacing the entire FPR1 and HOM6 open reading frames with nourseothricin and G418 resistance modules, respectively. The resulting fpr1Δ::nat and hom6Δ::kan strains were crossed to obtain an FPR1/fpr1Δ::nat HOM6/ hom6Δ::kan doubly heterozygous mutant diploid strain. As shown in Fig. 1A, this diploid strain sporulated to produce haploid meiotic progeny that were resistant to nourseothricin (Nat+) or to G418 (G418+) but not to both drugs. This finding indicates that the fpr1Δ hom6Δ double mutant is inviable and supports the results obtained in the high-throughput screen. Microscopic observation of meiotic products with an inferred fpr1Δ hom6Δ genotype (deduced from the genotype of their tetrad siblings) revealed that these spores germinate and undergo a limited number of cell divisions prior to growth cessation (data not shown). Neither the fpr1Δ mutation nor the hom6Δ mutation exhibited synthetic lethality, with the met15Δ0 or lys2Δ0 mutation also segregating in this cross (data not shown).

Viability of fpr1Δ hom6Δ double mutants was rescued by ectopic expression of plasmid-borne copies of FPR1 or HOM6, indicating that lethality of the double mutant is attributable to deficiencies in FPR1- and HOM6-encoded functions. The FPR1/fpr1Δ::nat HOM6/hom6Δ::kan diploid strain was transformed with URA3-selectable plasmids expressing either the wild-type FPR1 gene or the HOM6 gene, and the resulting strains produced Ura+ Nat+ G418+ segregants (Fig. 1B). All Ura+ Nat+ G418+ meiotic segregants were sensitive to counterselection of the URA3 plasmid-borne marker with 5-fluoroorotic acid (9), indicating that the FPR1- or HOM6-expressing plasmids are required for viability. In control experiments, sporulation of the FPR1/fpr1Δ::nat HOM6/hom6Δ::kan diploid strain transformed with a URA3 control vector failed to produce any viable fpr1 hom6 (Ura+ Nat+ G418+) meiotic products (Fig. 1B).

Expression of an FKBP12 mutant protein with reduced prolyl-isomerase activity restores viability of fpr1Δ hom6Δ double mutants. We next addressed whether FKBP12 enzymatic activity is required for function. The FPR1/fpr1Δ::nat HOM6/hom6Δ::kan diploid strain was transformed with a centromere-based LEU2 plasmid expressing the Fpr1<sup>F43V</sup> mutant, altered in an amino acid residue conserved in mammalian FKBP12 and important for prolyl-isomerase activity (77), and analyzed by tetrad dissection. As shown in Fig. 2, expression of the Fpr1<sup>F43V</sup> rescued viability of fpr1Δ hom6Δ double mutants, indicating that full FKBP12 prolyl-isomerase activity is not required for viability of these mutants. We note that the growth rate of fpr1Δ hom6Δ colonies expressing Fpr1<sup>F43V</sup> was lower than that of those expressing wild-type Fpr1 from the same LEU2 vector in a control experiment (Fig. 2), and this result could be attributable to reduced expression of the Fpr1<sup>F43V</sup> mutant, as previously observed (39).

fpr1Δ is not synthetically lethal with other hom mutations. The yeast HOM6 gene encodes homoserine dehydrogenase, which catalyzes the last step in conversion of aspartic acid to homoserine, the common precursor in synthesis of threonine and methionine. The first two steps in this pathway are catalyzed by AK and aspartate β-semialdehyde dehydrogenase, which are encoded by the HOM3 and HOM2 genes, respectively (Fig. 3A). hom3, hom2, and hom6 mutants are all auxotrophic for threonine and methionine and must therefore import these amino acids from the culture medium to survive. Because the fpr1Δ mutation was not synthetically lethal with a
met15 mutation causing methionine auxotrophy, one possible model to explain the synthetic lethal phenotype of fpr1Δ hom6Δ double mutants is that FKBP12 might be required for efficient threonine uptake in yeast. One prediction of this model is that fpr1Δ hom3Δ and fpr1Δ hom2Δ double mutants would exhibit a lethal phenotype, similar to that observed for fpr1Δ hom6Δ double mutants. To test this, we constructed G418-resistant hom3Δ::kan and hom2Δ::kan single mutants, mated these with the fpr1Δ::nat strain described above, and isolated FPR1/fpr1Δ::nat HOM3/hom3Δ::kan and FPR1/fpr1Δ::nat HOM2/hom2Δ::kan diploid strains. As shown in Fig. 3B, sporulation of these strains produced viableNat' G418' spores that exhibited no growth defect, indicating that the fpr1Δ hom3Δ and fpr1Δ hom2Δ double mutants are viable and therefore capable of efficient threonine uptake. Thus, the synthetic lethal interaction observed between hom6 and fpr1 is gene specific and is not observed with other hom mutations.

Deletion of HOM3 or HOM2 suppresses lethality of fpr1Δ hom6Δ double mutants. An alternative model to explain the lethal phenotype of fpr1Δ hom6Δ double mutants is that these strains accumulate toxic levels of the substrate of homoserine dehydrogenase (Hom6), aspartate β-semialdehyde (ASA). In this model, introduction of a mutation earlier in the pathway will block ASA formation and restore viability of fpr1Δ hom6Δ mutant strains. We therefore tested whether hom3Δ and hom2Δ mutations suppress lethality of the fpr1Δ hom6Δ double mutant. fpr1 hom3Δ and fpr1 hom2Δ double-mutant strains were crossed with a hom3Δ::hph mutant, and FPR1/fpr1 HOM3/hom3Δ::hph and FPR1/fpr1 HOM2/hom2Δ::hph diploid strains heterozygous at three loci were isolated. Sporulation of these diploids produced no viable fpr1 hom6Δ double mutants (Nat' G418' Hyg'), confirming synthetic lethality of fpr1 and hom6Δ mutations in these crosses (Fig. 4). In contrast, viable fpr1Δ hom3Δ hom6Δ and fpr1Δ hom2Δ hom6Δ triple-mutant strains (Nat' G418' Hyg') were readily isolated, and the growth of these triple mutants was indistinguishable from that of the wild type. These results support a model in which ASA accumulation is toxic and results in the lethal phenotype observed in fpr1Δ hom6Δ mutants.

Deletion of HOM6 is deleterious to strains expressing an AK mutant resistant to feedback inhibition. In yeast, flux through the homoserine biosynthetic pathway is governed through feedback inhibition of AK by threonine (Fig. 3A). Mutations that render AK resistant to feedback inhibition lead to threonine overproduction (3, 48, 57, 62, 63, 72).

Interestingly, AK was identified as a binding partner of FKBP12 during a search for yeast proteins interacting with FKBP12 in the yeast two-hybrid system (1). In these studies,
yarn cells with a deletion of the FPR1 gene or exposed to the FKBP12-specific inhibitor FK506 exhibited resistance to the toxic threonine analog hydroxynorvaline. Mutant cells expressing a feedback-resistant AK are also resistant to hydroxynorvaline (62), suggesting that FKBP12 regulates AK inhibition by threonine (1). If loss of FKBP12 function results in deregulation of AK activity, deletion of FPR1 in a hom6Δ mutant could lead to ASA accumulation, in accordance with our results. In addition, this model predicts that a HOM3 mutant allele encoding a feedback-resistant AK would also exhibit synthetic lethality with a hom6Δ mutation. To test this model, we constructed a yeast strain expressing a feedback-resistant AK by integrating the HOM3-R7 mutant allele, which was originally isolated from a threonine-overproducing yeast strain (57; Velasco et al., unpublished). The HOM3-R7 mutant strain was then crossed with a hom3 hom6Δ double mutant to obtain a HOM3-R7/hom3::kanMX4 HOM6/hom6::hphMX4 diploid strain. Tetrad analysis of this diploid revealed that most HOM3-R7 hom6Δ recombinants were inviable, and those few that did survive grew poorly (Fig. 5). Taken together, these results indicate that dysregulation of AK activity becomes toxic to cells defective in homoserine dehydrogenase activity and support the hypothesis that accumulation of ASA impairs growth.

FKBP12-AK interaction is induced by threonine, and reduced by a mutation in HOM3 that renders AK resistant to
feedback inhibition. FKBP12 and AK have been shown to physically interact both in vivo and in vitro (1). To study whether this interaction influences AK feedback inhibition by threonine, we studied the effect of threonine on FKBP12 binding to wild-type and feedback-resistant mutant AK. We first studied FKBP12-AK interaction in vitro by affinity chromatography by assaying binding of wild-type and mutant AK to recombinant His6-Fpr1 protein that had been produced in bacteria and coupled to an agarose matrix. As shown in Fig. 6A, both wild-type and feedback-resistant AK interacted with FKBP12 in the absence of exogenous threonine. Addition of threonine to the binding reactions increased FKBP12 interaction with wild-type AK but not with feedback-resistant AK, suggesting that threonine enhances FKBP12 binding to wild-type AK but less so with a feedback-resistant AK mutant.

We further analyzed FKBP12-AK interactions in vivo with the yeast two-hybrid system. The two-hybrid host strain PJ69-4A, coexpressing Gal4 DNA-binding domain-Fpr1 (Gal4BD-Fpr1) and Gal4 activation domain-AK (Gal4AD-AK) fusion proteins, was grown in a synthetic medium with or without threonine. Interactions between the fusion proteins were detected and quantified by measuring expression of the lacZ reporter gene. Similar experiments were conducted with cells coexpressing Gal4BD-Fpr1 and a Gal4AD-AK

\[ E282D \] feedback-resistant fusion protein. Results of these experiments are shown in Fig. 6B. In the absence of exogenous threonine, the interaction detected between FKBP12 and wild-type AK was considerably greater than that detected between FKBP12 and AK

\[ E282D \], indicating that the mutant AK binds FKBP12 with less affinity than wild-type AK in vivo in the presence of threonine at normal intracellular concentrations (Fig. 6B). Addition of threonine to the culture medium increased both FKBP12-AK and FKBP12-AK

\[ E282D \] binding, lending support to the hypothesis that threonine enhances these interactions. FKBP12-AK binding was nearly abolished by the presence of FK506 (Fig. 6B), in accord with previous observations (1).

In addition, we detected a low but significant level of self-interaction between both wild-type and mutant AKs in the two-hybrid assay (Fig. 6B). These results suggest that yeast AK might form homodimers, or homo-oligomers, as has been reported for this protein in other organisms (8, 23, 56, 80). AK-AK and AK

\[ E282D \]-AK

\[ E282D \] interactions were also detected in the presence of FK506, indicating that neither FKBP12 binding nor prolyl-isomerase activity is required for AK self-interaction.

The hom6Δ mutation confers sensitivity to FK506. Because FK506 prevented FKBP12-AK interaction in the two-hybrid assay, we tested whether FK506 mimics the lethal effect of an fpr1Δ mutation in a homoserine dehydrogenase-deficient mutant. As shown in Fig. 7A and B, FK506 indeed inhibited growth of the hom6Δ mutant. By microscopic examination, hom6Δ mutant cells exposed to FK506 exhibited abnormal morphologies, indicative of a cytokinesis defect (Fig. 7C). Drugs that target both FKBP12 and Hom6 might therefore be expected to exhibit a synergistic lethal effect (see Discussion).

Aspartokinase from an fpr1Δ mutant is inhibited by threonine in vitro. The results obtained thus far indicate a role for FKBP12 in the regulation of AK activity, as proposed originally by Alarcon and Heitman (1). To test this hypothesis, we assayed AK activity (in the presence and absence of threonine) from wild-type and fpr1, hom6, or HOM3-R7 feedback-resistant mutant cells. AK expression and AK partial purification was analyzed by Western blotting. As shown in Fig. 8A, all strains exhibited similar levels of AK activity in the absence of threonine. In the presence of 1 mM threonine, the AK activi-
FKBP12 controls aspartate pathway

From wild-type and *fpr1/H9004* strains were inhibited to comparable levels, indicating that FKBP12 is not required for AK inhibition in vitro by threonine. Threonine also inhibited AK activity of the *hom6/H9004* mutant, suggesting that homoserine dehydrogenase does not play a role in AK feedback inhibition.

AK activity in the wild-type, *fpr1/H9004*, or *hom6/H9004* strain was completely feedback inhibited by the addition of 5 mM threonine, whereas the feedback-resistant AK exhibited no inhibition (Fig. 8B). Addition of recombinant His6-Fpr1 to these assays did not have any detectable effect on feedback inhibition of AK from wild-type or the *fpr1Δ* mutant (data not shown).

One possible role for FKBP12 in AK inhibition could be to bind and stabilize threonine-AK complexes, modulating the dynamics of AK response to changes in intracellular threonine concentrations. In this model, FKBP12 might function to

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**FIG. 7.** *hom6Δ* mutation confers sensitivity to FK506. (A) Approximately 10⁷ cells from *hom6Δ* mutant strain MAY308 were plated on solid YPD medium and exposed to 250 µg of FK506 diffusing from a paper disk. A control paper disk containing only the FK506 solvent (90% EtOH plus 10% Tween 20) was included. The plate was incubated at 30°C for 2 days and photographed. As a control, the same assay was conducted with isogenic wild-type strain BY4742. (B) Liquid YPD cultures of *hom6* (MAY308) and wild-type (BY4742) strains were exposed to 10 µg of FK506/ml or left untreated as controls, and growth was measured based on optical density at 600 nm (OD₆₀₀). (C) *hom6Δ* cells from the FK506-exposed culture described for panel B were photographed using differential interference contrast microscopy after 22 h of incubation with the drug.

**FIG. 8.** AK inhibition by threonine. (A) Western blot analysis of AK and FKBP12 in protein extracts and partially purified AK preparations obtained from the wild type (BY4741) or from an *fpr1Δ* (MAY193), *hom6Δ* (MAY308), or *HOM3-R7* (MAY315) mutant strain or from a *hom3Δ* mutant strain (MAY310) as a control. (B) AK activity in partially purified extracts obtained from the strains described above was assayed in the presence or absence of threonine (1 or 5 mM) as described in Materials and Methods and in Results.
maintain AK in an inhibited conformation, preventing it from returning to the active state. To investigate this, we assayed AK from the wild-type and fpr1Δ strains following preincubation of the enzyme preparations with threonine in the presence or absence of bacterially expressed His6-Fpr1. Threonine-treated AK preparations were then mixed with the AK assay reagents, reducing the threonine concentration, and the AK activity was then compared to that of similar AK reactions containing the same final threonine concentrations but in which AK was not preexposed to threonine. These experiments revealed no increase in threonine inhibition in AK preincubated with threonine, indicating that AK inhibition by threonine, even in the presence of FKBP12, is fully reversible in vitro. In these assays, there was no significant difference in threonine inhibition of AK from the wild type compared to results with the fpr1Δ mutant in either the presence or the absence of His6-Fpr1 (data not shown).

DISCUSSION

Here we investigated the cellular functions of yeast FKBP12 by a systematic search for mutations that are lethal when combined with an fpr1 mutation. In addition to revealing the fpr1-hom6 synthetically lethal interaction which is the subject of this report, this assay also confirmed the fpr1-hmo1 synthetically lethal interaction previously reported by Dolinski and Heitman (21).

Our results are consistent with toxic accumulation of ASA, or an ASA derivative, as being the cause of lethality in fpr1Δ hom6Δ double-mutant strains. The hypothesis that ASA accumulation inhibits growth of fpr1Δ hom6Δ double mutants is supported by the fact that hom3Δ and hom2Δ mutations, which both abolish ASA formation, rescue the viability of fpr1Δ hom6Δ double mutants. In this model, FKBP12 regulates metabolic flux to prevent ASA accumulation. Deletion of HOM6 alone does not lead to lethal levels of ASA, likely because exogenous threonine imported from the medium inhibits AK activity, reduces Asp-P, and limits ASA formation (Fig. 3A). hom6Δ mutants expressing a feedback-resistant AK enzyme are severely growth impaired or inviable, even though these strains express FKBP12, again supporting the model that increased flux through the pathway is lethal when the pathway is blocked by mutation at the Hom6 step.

In solution, ASA is an unstable compound that polymerizes (16, 50, 64, 79). We tested the hypothesis that ASA might cyclize to produce a compound analogous to 1-azetidine-2-carboxylic acid, a toxic proline analogue. Recent studies have shown that yeast strains in the \( \Sigma1278b \) background are naturally resistant to 1-azetidine-2-carboxylic acid and that this resistance is due to the expression of MPRI or MPRI2, which are homologous genes encoding acetyltransferases that detoxify this proline analogue (43, 74). MPRI and MPRI2 are not present in strains in the S288C background studied here. A plasmid expressing MPRI did not rescue viability of fpr1Δ hom6Δ double mutants, suggesting that ASA conversion into 1-azetidine-2-carboxylic acid is not the basis for ASA toxicity. The mechanism of ASA toxicity is currently unknown and might be addressed through different genetic approaches, including isolation of additional mutations suppressing lethality of fpr1Δ hom6Δ double mutants or screening for high-copy suppressors of these double-mutant strains. We have observed morphological changes in FK506-exposed hom6Δ mutant cells that appear to be the result of cytokinesis defects.

Interestingly, our studies reveal a potential new target for antifungal drug therapy. We show that the widely used immunosuppressant FK506 inhibits growth of a hom6Δ mutant, probably by blocking FKBP12-AK interaction, and thereby mimicking the effects of an fpr1Δ mutation. In theory, combination of FK506 (or preferably, a nonimmunosuppressive derivative of FK506) with a homoserine dehydrogenase-specific inhibitor could reproduce the lethal effect of an fpr1Δ hom6Δ double mutation. Such a combination therapy would target a biosynthetic pathway that is conserved in fungi but not in mammals. The natural compound 5-hydroxy-4-oxonorvaline (HON), a toxic amino acid produced by Streptomyces akiyoshii, is a very efficient inhibitor of homoserine dehydrogenase (40, 82, 87, 88). HON inhibits growth by blocking synthesis of homoserine, and it has shown activity against different fungal organisms (87). In addition, HON has established activity in treating systemic Candida infections in mice (87). Preliminary assays revealed no inhibitory effect of HON on fpr1 mutants in YPD medium, indicating that HON uptake is reduced in this medium. Further studies will be necessary to identify other homoserine dehydrogenase inhibitors with potential use in combination with FK506 in new antifungal therapies. Indeed, combination therapies with FK506 and azole antifungals have proven highly effective against Aspergillus fumigatus and Candida species (53, 73).

We have observed that threonine enhances FKBP12-AK interactions, indicating that this amino acid induces structural changes in AK that result in increased affinity for FKBP12. These results, together with the genetic data described above and the observations reported by Alarcon and Heitman (1), suggest a role for FKBP12 in AK feedback regulation. In support of this model, FKBP12 shows reduced affinity for a mutant AK carrying the E282D substitution, affecting a highly conserved amino acid residue required for feedback regulation by threonine (Velasco et al., unpublished). The E282D substitution could decrease AK affinity for threonine, rather than for FKBP12, because an increasing threonine concentration has a clear positive effect on FKBP12-AK binding in vivo.

Thus, one possibility is that AK feedback regulation involves threonine-induced interaction with FKBP12. However, our results indicate that AK inhibition by threonine in vitro does not require FKBP12, because AK activities from the wild type and an fpr1Δ mutant exhibit similar threonine sensitivities. These results indicate that FKBP12 is not required for providing AK with a conformational state competent for subsequent threonine inhibition and suggest that FKBP12-AK interaction is not necessary for AK feedback regulation under these in vitro conditions. Western blot analysis of the partially purified extracts from the wild-type strain used in the AK assays revealed the presence of FKBP12, although the fractionation procedure used for AK enrichment might result in reduced FKBP12/AK concentration ratios in the partially purified AK preparations. However, addition of bacterially expressed, His6-tagged yeast FKBP12 to the AK reactions did not increase threonine sensitivity of AK obtained from the wild-type or fpr1Δ strain (data not shown).

What then is the role of FKBP12 in AK feedback inhibition?
FKBP12 might interact with threonine-inhibited AK complexes to stabilize them in an inactive state. In our studies, AK preincubation with threonine in the presence of endogenous Fpr1 or bacterially expressed His6-Fpr1 did not result in a detectable increase in AK inhibition. In this regard, we cannot rule out the possibility that the His6-Fpr1 fusion protein used in these studies fails to promote AK inhibition, despite the fact that His6-Fpr1 interacts with AK physically in vitro. Alternatively, FKBP12-AK interaction might be reduced in the chemical environment where AK activity is routinely assayed in vitro or feedback inhibition might require additional proteins that are lost during AK partial purification. Future studies will address the molecular mechanism by which FKBP12 exerts a regulatory influence over AK activity in vivo.

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