The Immunosuppressant FK506 Uncovers a Positive Regulatory Cross-talk between the Hog1p and Gcn2p Pathways*

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The immunosuppressant Tacrolimus (FK506) has increased the survival rates of organ transplantation. FK506 exerts its immunosuppressive effect by inhibition of the protein phosphatase calcineurin in activated T cells. Unfortunately, FK506 therapy is associated with undesired non-therapeutic effects involving targets other than calcineurin. To identify these targets we have addressed FK506 cellular toxicity in budding yeast. We show that FK506 increased cell sensitivity upon osmotic challenge independently of calcineurin and the FK506-binding proteins Fpr1p, -2p, -3p, and -4p. FK506 also induced strong amino acid starvation and activation of the general control (GCN) pathway. Tryptophan prototrophy or excess tryptophan overcame FK506 toxicity, suggesting that tryptophan deprivation mediated this effect. Mutation of the GCN3 and -4 genes partially alleviated FK506 toxicity, suggesting that activation of the GCN pathway by FK506 was also involved in osmotic tolerance. FK506 enhanced osmotic stress-dependent Hog1p kinase phosphorylation that was not accompanied by induction of a Hog1p-dependent reporter. Interestingly, deletion of the GCN2 gene suppressed FK506-dependent Hog1p hyperphosphorylation and restored Hog1p-dependent reporter activity. Conversely, deletion of the HOGL gene impaired FK506-dependent activation of Gcn2p kinase and translation of a GCN4-LacZ reporter, highlighting functional cross-talk between the Gcn2p and Hog1p protein kinases. Taken together, these data demonstrate that both FK506-induced amino acid starvation and activation of the GCN pathway contribute to cell sensitivity to osmotic stress and reveal a positive regulatory loop between the Hog1p and Gcn2p pathways. Given the conserved nature of Gcn2p and Hog1p pathways, this mechanism of FK506 toxicity could be relevant to the non-therapeutic effects of FK506 therapy.

FK506 and cyclosporin A (CsA) are immunosuppressants used to prevent allograft rejection after organ transplantation.

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† The abbreviations used are: CsA, cyclosporin A; HOG, High-Osmolarity-Glycerol; GCN, general amino acid control; eIF-2, eukaryotic initiation factor 2; MAP, mitogen-activated protein; CRE, CAMP-response element; WT, wild type.

FK506, by complexes with the immunophilin FK506-binding protein, blocks the activity of the calcium-calmodulin-activated protein phosphatase calcineurin (1). Inhibition of calcineurin impedes nuclear translocation of the transcription factor NF-AT, which controls interleukin-2 gene expression. CsA also inhibits the NF-AT/interleukin-2 pathway through complex formation with another immunophilin, cyclophilin (1). FK506 and CsA therapies are associated with non-therapeutic toxicities (2). Among them, posttransplant diabetes mellitus is frequently observed (3). Recent studies have confirmed the greater diabetogenic potential of FK506 versus CsA in transplant patients (3, 4), suggesting that calcineurin inhibition does not mediate this effect. Unfortunately, the molecular mechanisms of FK506-induced posttransplant diabetes mellitus still remain obscure. Because of its tractable genetics and ease of manipulation, Saccharomyces cerevisiae is an excellent eukaryotic model to identify target(s) for drugs that have unknown mechanisms of action (5).

High osmolarity in yeast triggers the HOG mitogen-activated protein (MAP) kinase pathway, leading to increased transcription of stress-responsive genes and to intracellular accumulation of glycerol (6, 7). Changes in external osmolarity are sensed by two transmembrane proteins acting independently and converging at the level of the MAP kinase Pbs2p, which controls dual phosphorylation and activation of Hog1p (8). Active Hog1p translocates to the nucleus and regulates part of the osmotic gene expression by direct phosphorylation of transcriptional activators like Hot1p (9) and repressors like Sko1p (10, 11). Sko1p binds to CRE-like sequences found in the promoters of several genes involved in adaptation to high osmolarity such as the ENA1 gene, encoding an Na⁺-ATPase (10). Additionally, nuclear Hog1p associates with the transcriptional machinery and modulates promoter binding of these transcription factors (12, 13). Hog1p also regulates its own intracellular distribution by induction of its nuclear export mediated by its kinase activity (14). Other responses to osmotic stress include inhibition of nutrient uptake, protein synthesis, and the expression of genes encoding ribosomal proteins in yeast (6, 15). Although it remains unclear how osmotic stress transiently down-regulates protein synthesis, recent evidence suggests that it might be mediated by the Hog1p MAP kinase pathway itself (16, 17).

FK506 inhibits amino acid uptake in yeast and induces translation of the transcriptional activator Gen4p (18). GCN4 translation is controlled by a regulatory pathway activated by amino acid or purine starvation and called the general control pathway (GCN) (19). In response to nutrient deprivation, Saccharomyces cerevisiae induces phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2) at serine 51 by the Gen2p protein kinase (19). Translational regulation of GCN4 is mediated by four short open reading frames located in...
the 5'-untranslated region of GCN4 (uORFs). In cells without nutrient limitation, the uORFs block GCN4 translation efficiently (19, 20). Upon amino acid or purine deprivation, Gen2p-dependent phosphorylation of eIF-2α leads to reduced active eIF-2 levels, thus allowing increased GCN4 translation because of bypassing the uORFs. High levels of Gen4p stimulate expression of genes involved in amino acid biosynthesis (21).

We report here that FK506 increased the sensitivity of yeast cells to osmotic stress by inducing amino acid starvation and strong activation of the GCN pathway. We found that activation of the GCN pathway regulated the activity of the Hog1 MAP kinase pathway by distinct mechanisms. At least one of these mechanisms involved a positive regulatory loop between the Gen2p and Hog1p protein kinases that has not been described previously. These results also suggest that transient inhibition of protein synthesis by osmotic stress might be regulated by Hog1p and Gen2p at the initiation step. Given the conserved nature of the Gen2p and Hog1p pathways, these FK506-dependent events could be of significance to the second effects of FK506 treatment.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—The fpr1, -2, -3, and -4 strains, lacking the corresponding immunophilins and the quadruple fpr1–4 mutant strain, have the JK393 da genetic background (MATa his hMLo leu2-3, 112 met1 trpl ura3-52). These mutants together with their isogenic parental strain have been described previously (22). The rest of the strains used in this study have the W3031a genetic background (MATa ade2-1, can1-100, his3-11, leu2-3, trpl1, ura3-1). The cnbl1 strain, harboring a disruption of the calcineurin regulatory subunit CNB1, has been described (23). The LEU2, URA3, HIS3, and TRP1 strains were generated by transformation of the W3031a strain with the YcpIP1 (LEU2), YcpIP4 (URA3), YcpIP7 (HIS3), and YcpIP15 (TRP1) plasmids, respectively (24). The cnb1, -2, -3, and -4 strains have also been described (25). Likewise the hog1 strain, lacking the HOGl gene, has been described (26). Yeast cells were transformed by standard procedures (27).

The URS CRE**<sub>Sac</sub>**-CYC1-LacZ pmP224 reporter plasmid has been described previously (10). The plasmid p180, expressing a GCN4-LacZ fusion including the entire GCN4 5'-non-coding region with four upstream open reading frames inserted into YCP50, a low copy-number plasmid marked with URA3, has also been described (28). The HIS4-LacZ p377 reporter plasmid has been described previously (29).

**β-Galactosidase Assays**—Yeast cells transformed with the appropriate reporter plasmids were grown selectively in S.D. medium and then diluted in YPD. Exponential cultures were treated with FK506 and/or sorbitol as indicated. β-Galactosidase activity was determined at the indicated times as described elsewhere (30) and represented as β-galactosidase activity units. Data are the mean ± S.E. from three independent transformants, each one measured in duplicate.

**Yeast Growth Assays**—Standard methods for yeast culture and manipulations were used (31). Synthetic medium (S.D.) contained 2% glucose, 0.67% yeast nitrogen base without amino acids (Difco), and the amino acids, purine, and pyrimidine bases required by the strains of interest. YPD medium contained 2% glucose, 2% peptone, and 1% yeast extract. FK506 was kindly provided by Fujisawa Inc., dissolved in ethanol, and diluted in YPD at the corresponding doses.

For analysis of cell growth by drop test, logarithmically growing cells in liquid YPD medium were 10-fold serially diluted, and volumes of around 3 μl were dropped with a stainless steel replicator (Sigma) on solid plates containing 2% Bacto-Agar (Difco) and YPD medium with the corresponding doses of FK506 and/or sorbitol (Sigma) as indicated. Growth was recorded after 2–5 days in all cases.

For viability assays, exponentially growing cultures in liquid YPD were collected by centrifugation, and an equal number of cells were plated onto YPD plates containing the corresponding doses of FK506 and/or sorbitol. Colonies were quantitated after 2–5 days in all cases. Data represented are the mean ± S.E. of at least two independent experiments, each one done in duplicate.

The ability of yeast cells to grow in various liquid conditions was tested by adding equal amounts of cells from overnight cultures in YPD to 3 ml of YPD supplemented with the corresponding doses of FK506 and/or sorbitol. OD<sub>600</sub> was measured at the indicated time points. Growth inhibition (%) for a given strain in FK506-containing medium was expressed as the ratio of the OD<sub>600</sub> obtained for this strain to the OD<sub>600</sub> of the control culture.

**Immunoblotting**—Strains were grown in liquid YPD medium to mid-log phase and then treated with the corresponding doses of FK506 and/or sorbitol at the indicated times. After treatment, equal numbers of cells were collected by centrifugation and resuspended in 20% tri-chloroacetic acid. Cells were lysed by glass-beads vortexing. Insoluble protein extracts were pelleted by centrifugation and resuspended in alkaline Laemmli buffer. 20 μg of total cellular protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Protran<sup>®</sup>, Schleicher & Schull) filters. Uniform gel loading was confirmed by Ponceau S staining of membranes after transfer. Phosphorylated Hog1p was detected with an antiphospho-p38 antibody (Upstate) from New England Biolabs (NEB). Phosphorylated eIF-2α was detected with an antiphospho-eIF-2α antibody (Ser<sup>51</sup>) from New England Biolabs. Immunocomplexes were visualized by enhanced chemiluminescence detection (Pierce) using a HRP-conjugated goat anti-rabbit IgG (Bio-Rad). The figures show an experiment representative of at least two independent ones with essentially identical results.

**RESULTS**

**FK506 Confers Sensitivity to Osmotic Stress in Yeast Independently of Calcineurin and FK506-binding Proteins**—Previous evidence has revealed the existence of new targets of FK506 other than calcineurin in budding yeast (32). To identify these targets, we screened for FK506 effects on yeast cell growth under different conditions. We used the WT W303.1a strain and the cnb1 mutant, which lacks the calcineurin regulatory subunit CNB1 and has no detectable calcineurin activity. Interestingly, we found that FK506, in addition to inhibiting growth under normal conditions, dramatically increased cell sensitivity to sorbitol in both strains (Fig. 1A). CA didn’t induce any detectable osmotic sensitivity at doses 20-fold higher than those used for FK506 (Fig. 1A). Other candidate targets mediating this sensitivity could be one or some of the immunophilins known to be intracellular targets of FK506. S. cerevisiae has four of these proteins encoded by the FPR1, -2, -3, and -4 genes, respectively (22, 33). It was shown previously that neither fpr1, -2, -3, and -4 nor the quadruple fpr1–4 mutants were resistant to FK506 toxicity at high doses (22, 33).

Thus, we tested the effect of FK506 on the growth under osmotic stress in both strains (Fig. 1A). Asp didn’t induce any detectable osmotic sensitivity at doses 20-fold higher than those used for FK506 (Fig. 1A). Other candidate targets mediating this sensitivity could be one or some of the immunophilins known to be intracellular targets of FK506. S. cerevisiae has four of these proteins encoded by the FPR1, -2, -3, and -4 genes, respectively (22, 33). It was shown previously that neither fpr1, -2, -3, and -4 nor the quadruple fpr1–4 mutants were resistant to FK506 toxicity at high doses (22, 33).

The effect of FK506 on the growth under osmotic stress in both strains (Fig. 1B) had similar results. As shown in Fig. 1B, disruption of the immunophilin genes did not improve cell growth in the presence of sorbitol and FK506, in agreement with previous findings (22, 33). Therefore, FK506-induced sensitivity to high osmolarity in yeast was independent of the known targets calcineurin and immunophilins.

**Tryptophan Prototrophy or Excess Tryptophan Overcomes FK506-induced Osmotic Stress Sensitivity in Yeast**—FK506 impairs S. cerevisiae cell growth by inhibition of tryptophan, histidine, and leucine import in yeast strains auxotrophic for these amino acids (18, 34). Accordingly, prototrophy for one of these amino acids or an excess of tryptophan or histidine added exogenously to the growth medium rescued the growth defect (34). We assessed whether amino acid prototrophy was also able to modulate the FK506 osmotic phenotype in a set of WT strains prototrophic for histidine, leucine, tryptophan, or uracil by testing the growth of these strains in sorbitol-containing medium with FK506. As shown in Fig. 2A, only the WT-TRP1 strain was able to grow in the presence of FK506. Consistent with this, excess tryptophan in the growth medium also relieved FK506 osmotic sensitivity in the WT and the cnb1 mutant (Fig. 2B). Therefore, these results confirm that tryptophan availability alleviates the FK506 sensitivity to osmotic stress in a calcineurin-independent manner.

FK506-induced Tryptophan Deprivation Activates the General Control Pathway—FK506 blocks amino acid import, generating starvation of amino acids, and stimulates GCN4 translation (18, 34). To determine whether GCN4 translation was
dependent on general control pathway activation, we tested the effect of FK506 on the p180 GCN4-lacZ reporter in the WT and gcn1, -2, -3, and -4 yeast strains. The p180 reporter contains the entire GCN4 5′-untranslated region driving expression of the LacZ gene. GCN1 together with GCN20 encode adaptor proteins, which bind to ribosomes and interact with GCN2 kinase mediating its activation by uncharged tRNAs (35, 36). GCN3 encodes the α subunit of eIF-2B, a multisubunit guanine nucleotide exchange factor that regulates levels of GTP-bound eIF-2α. Phosphorylated eIF-2α binds to Gcn3p and blocks eIF-2B exchange function favoring selective translational induction of GCN4 (37–39). When expression from the GCN4-LacZ reporter was measured 3 h after FK506 addition, 3-fold induction was seen in the WT strain but was absent in all of the gcn mutants tested (Fig. 3A). We also tested a HIS4-LacZ reporter containing the HIS4 promoter driving the LacZ gene in the WT and gcn1, -2, and -3 strains. Expression of HIS4-LacZ increases after amino acid starvation in a Gcn4p-dependent manner (40). As expected, following FK506 addition there was a 4-fold increase in HIS4-LacZ expression after 3 h in the WT strain, which was abolished in the gcn mutants (Fig. 3B). Interestingly, tryptophan prototrophy or addition of excess tryptophan also abolished GCN4-LacZ induction by FK506 (Fig. 3C). Therefore, these data show that FK506 induces GCN4 control by activating the GCN pathway. Furthermore, these findings confirm that starvation for tryptophan is the most relevant event triggering GCN4 expression in response to FK506.

Disruption of the GCN Pathway Alleviates FK506-induced Osmotic Sensitivity—Recently, a functional connection between the GCN pathway and tolerance to salt stress has been described (25). Disruption of the GCN pathway increased tolerance to NaCl suggesting that (over)activation of this pathway had toxic effects on cell growth under salt stress. If the same was true for FK506, then its toxicity should be modulated by inactivation of the GCN pathway. To test this possibility, we analyzed the growth of the WT and WT-HIS3, LEU2, TRP1, and URA3 strains on YPD plates containing FK506 (50 μg/ml) and/or sorbitol (1 M) supplemented or not with 4-fold excess tryptophan (4xTrp). Drop tests were done as described under “Experimental Procedures.”
sensitivity by measuring the growth of the WT and the gcn1, -2, -3, and -4 mutants under osmotic stress in the presence or absence of FK506. As a control we used the WT-TRP1 strain, which is resistant to FK506 toxicity. Unexpectedly, FK506 inhibited cell growth to the same extent in all the strains tested (Fig. 4B), suggesting that resistance to FK506 toxicity in the gcn3 and -4 mutants was due to increased cell viability rather than improved cell growth under stress conditions. To test this hypothesis we measured the ability of each mutant to form viable colonies in sorbitol-containing plates with or without FK506. As shown in Fig. 4C, the colony-forming ability of the WT-TRP1 strain remained unaffected in the presence or absence of FK506. The number of viable colonies in FK506-containing medium was substantially higher in the gcn3 and -4 mutants than in the WT-trp1, gcn1, and gcn2 mutants as expected. Interestingly, deletion of GCN3 and -4 did not fully
FK506 strongly augmented Hog1p phosphorylation by the Hog1p phosphorylation status revealed an unexpected rescue FK506 toxicity as tryptophan prototrophy did in the WT strain. Therefore, inactivation of the GCN pathway only partially suppressed FK506 toxicity, and tryptophan starvation also contributed to FK506 osmotic sensitivity. Overall, these findings indicate that FK506 activation of the GCN pathway somehow modulates cell sensitivity to osmotic stress.

FK506 Enhances Hog1p Phosphorylation by Osmotic Stress via Gcn2p—Osmotic stress leads to activation of the HOG MAP kinase pathway through dual phosphorylation of the Thr-174 and Tyr-176 residues of the Hog1p MAP kinase by its dual specificity kinase Pbs2p. These phosphorylation events are necessary and sufficient to allow nuclear translocation of Hog1p, leading to induction of expression of target genes (14) and tolerance to osmotic stress. Phosphorylation of Hog1p can be monitored by Western blot analysis using a commercially available antibody specifically recognizing the phosphorylated residues. We explored whether the HOG pathway was involved in the resistance to FK506 toxicity observed in some GCN pathway mutants. WT and gcn1, -2, -3, and -4 cells were preincubated or not with FK506 for 1 h, osmostressed for 45 min, and harvested for total protein extraction. Western blot analysis of the Hog1p phosphorylation status revealed an unexpected result. FK506 strongly augmented Hog1p phosphorylation by osmotic stress in the WT and the gcn1, -3, and -4 mutants (Fig. 5). Surprisingly, hyperphosphorylation of Hog1p was fully abolished in the gcn2 mutant, suggesting that Gen2p mediated this effect. To further confirm this result we tested the effect of FK506 on the pMP224 reporter, containing a CRE-like sequence driving expression of the LacZ gene (see “Experimental Procedures”). This sequence is the binding site for the transcriptional repressor Sko1p, known to be a direct substrate of the Hog1p MAP kinase (11). Hog1p phosphorylation inactivates Sko1p repressor function, leading to transcriptional derepression of some HOG pathway-dependent genes (10, 11).

Expression of this reporter was measured in the different mutants used previously for the Hog1p phosphorylation study. As shown in Fig. 6, osmotic stress induction of the pMP224 reporter was blocked by FK506 in the WT and the gcn1, -3, and -4 mutants, despite the fact that Hog1p was hyperphosphorylated under these conditions. Interestingly, sorbitol induction of the reporter was unaffected by FK506 in the gcn2 mutant, further reinforcing the hypothesis that Gen2p was mediating FK506 effects. The absence of reporter expression in the WT and gcn1, -3, and -4 mutants was not due to a transient delay in transcriptional activation as we obtained the same results up to 3 h later (data not shown). Therefore, these data demonstrate that FK506 augmented Hog1p phosphorylation and perturbed Hog1p-dependent transcriptional induction via Gen2p, revealing a functional link between Hog1p and Gen2p kinases. In addition, these results suggest that suppression of FK506 toxicity by deletion of GCN3 and -4 genes was mediated by other unrelated mechanisms.

**gcn2 Mutant Sensitivity to FK506 Is due to Amino Acid Starvation and Not to Gen4p-dependent Gene Expression**—Deletion of GCN2 reduced Hog1p phosphorylation and restored Hog1p-dependent gene expression in the presence of FK506, but it did not relieve FK506 toxicity (Fig. 4). Therefore, other mechanisms were also contributing to FK506 sensitivity of the gcn2 strain. It has been described that GCN4 activation is harmful to cells under salt stress (25), probably because of enhancement of the expression of GCN4-regulated genes, which might create some metabolic problems under stress conditions. In addition, there are several instances in which GCN4 translation can be stimulated in a Gen2p-independent manner (41, 42). We reasoned then that the FK506 toxicity observed in the gcn2 mutant could be explained by increased GCN4 translation and subsequent Gen4p-dependent expression by Gen2p-independent mechanisms. Thus, we measured the expression

![Cross-talk between Hog1p and Gcn2p Pathways](Image 318x462 to 562x737)

**Fig. 6.** FK506 abolishes Hog1p-dependent transcriptional induction via Gcn2p. Induction of the pMP224 CRE\textsubscript{ENA1}–CYC1-LacZ reporter in WT and GCN pathway mutant strains treated with (filled bars) or without (open bars) FK506 (50 μg/ml) for 1 h and exposed or not to sorbitol (1 M) for 1 h. β-Galactosidase activity was measured and represented as described under “Experimental Procedures.”
of the GCN4-LacZ and HIS4-LacZ reporters in the WT and gcn mutant strains under osmotic shock with or without FK506. As shown in Fig. 7A, activation of GCN4 induced by FK506 was abolished in all the mutants tested under osmotic shock. Moreover, expression of the HIS4-LacZ reporter was also inhibited (Fig. 7B), ruling out the possibility that a Gen4p-regulated gene product would mediate the FK506 sensitivity of the strain lacking GCN2.

FK506 toxicity in the gcn2 mutant could also be explained by the inability of this mutant to induce tryptophan biosynthesis under tryptophan starvation produced by FK506. If that were the case, then addition of excess tryptophan should alleviate the osmotic-sensitive phenotype of this mutant. Therefore, we tested the colony-forming ability of the gcn2 mutant in sorbitol with FK506 in a medium containing 4-fold excess tryptophan. As shown in Fig. 7C, excess tryptophan fully rescued the FK506 sensitivity of the gcn2 mutant, thus showing that tryptophan starvation was responsible for this effect. Excess tryptophan also relieved FK506 toxicity in the gcn3 and gcn4 mutant strains (data not shown). As these mutants were partially resistant to FK506 osmotic sensitivity, this result suggests that tryptophan deprivation was also involved in the FK506 sensitivity of these strains but to a lesser extent than in the gcn2 mutant.

**Hog1p Regulates Gen2p Activation Induced by FK506**—We have shown above that Gen2p activation induced by FK506 strongly influenced Hog1p function and Hog1p-dependent gene expression. We then tested whether Hog1p was also able to regulate the FK506-dependent activation of Gen2p. This possibility was explored by studying FK506-induced phosphorylation of eIF-2α, a known physiological substrate of the Gen2p kinase, in the WT and hog1 mutant strains. We analyzed the phosphorylation status of eIF-2α by immunoblot analysis using a commercially available polyclonal antibody that specifically recognizes eIF-2α phosphorylated at serine 51 (see “Experimental Procedures”). As expected, FK506 treatment induced sustained phosphorylation of eIF-2α in the WT strain in a time-dependent manner (Fig. 8A). Remarkably, loss of Hog1p function negatively affected eIF-2α phosphorylation by FK506. We confirmed this result by measuring FK506 induction of the GCN4-LacZ reporter in the hog1 strain. Again, no GCN4-LacZ expression was observed in the hog1 mutant, thus showing that Hog1p function was required for efficient Gen2p activation and GCN4 translation by FK506. Accordingly, the hog1 mutant was more sensitive to growth inhibition by FK506 than the WT strain (Fig. 8C). Taken together, these data demonstrate that Hog1p also modulates Gen2p, further supporting a functional connection between both protein kinases.

**DISCUSSION**

In the present study we report that the immunosuppressant FK506 increased yeast sensitivity to osmotic stress independent of its well established targets calcineurin and immunophillin. FK506 strongly activated the GCN pathway by inducing amino acid deprivation as described by Heitman et al. (18). Accordingly, tryptophan prototrophy or excess tryptophan overcame FK506 toxicity, suggesting that amino acid starvation mediates FK506 toxicity. In addition, genetic disruption of the GCN pathway partially alleviated FK506-induced osmotic sensitivity, showing that activation of the GCN pathway was also toxic to cells under osmotic shock. We found that the GCN pathway regulated the function of the Hog1 MAP kinase pathway through distinct mechanisms. One of them involved positive functional cross-talk between the Gen2p and Hog1p protein kinases, which has not been described before. Overall, these results extend our knowledge of coordination between signal transduction pathways under stress conditions. As both Hog1p and Gen2p pathways are conserved in higher eukaryotes, this mechanism of action could be relevant for the non-therapeutic effects of FK506 treatment.

The most relevant event triggering FK506-induced osmotic sensitivity seems to be amino acid starvation, and more specifically tryptophan starvation. It was shown previously that FK506 inhibited amino acid import and activated the GCN pathway (18). Accordingly, FK506 toxicity was relieved by adding excess tryptophan and by expression of either the TRP1 gene or those genes encoding tryptophan transporters TAT1-TAT2 (18, 34). However, it is still not known how FK506...
impairs amino acid transport by TAT1-TAT2. This effect is not attributable to inhibition of calcineurin or any of the known immunophilins in yeast as strains deficient for these genes are still sensitive to FK506 toxicity (22, 33). Similar phenotypes with respect to tryptophan auxotrophy and rescue by excess tryptophan or expression of TAT2/TRP1 genes have been reported in several studies on perturbations of sphingolipid metabolism (43–46), high pressure (47), and volatile anesthetics (48). Moreover an erg6 mutant defective in ergosterol biosynthesis is also defective in tryptophan uptake (49). Tryptophan import is inherently cold-sensitive, and many cold-sensitive mutants in yeast are tryptophan auxotrophs or have mutations in tryptophan permeases or tryptophan biosynthesis (50–52). Thus, tryptophan transport appears to be a weak link in yeast physiology, becoming limiting for cell growth under a variety of stress conditions. FK506 may inhibit Tat1p-Tat2p function, turnover, and/or trafficking by altering plasma membrane lipid composition. Interestingly, FK506 inhibits directly the function of PDR5 (53), an ABC transporter known to control the phospholipid content of plasma membrane (54). Additionally, deletion of the TOR-regulated NPR1 kinase, which regulates TAT2 tryptophan permease turnover, alleviates FK506 toxicity (55). Further experiments are required to strengthen this hypothesis.

As a consequence of tryptophan deprivation, FK506 strongly induced the GCN pathway (Fig. 3). Our results demonstrate that activation of this pathway also contributes to osmotic stress sensitivity by interfering with Hog1p pathway function. Several observations support this hypothesis. Tryptophan availability blocked GCN pathway activation and alleviated osmotic sensitivity. Genetic disruption of the GCN pathway partially relieved FK506 toxicity. Most importantly, FK506 augmented osmotic stress-induced Hog1p phosphorylation and inhibited Hog1p-dependent CRE-LacZ reporter expression in a Gcn2p-dependent manner. The most relevant conclusion drawn from these findings is the existence of a functional link between Hog1p and Gcn2p kinases. Accordingly, deletion of HOG1 blocked increases in the phosphorylation of eIF-2α and GCN4 translation normally elicited by addition of FK506. Functional connections between these two pathways have been described previously mainly at the transcriptional level (25, 56). Our results further extend this link, suggesting that Gcn2p and Hog1p could directly or indirectly regulate each other. To our knowledge, this is the first evidence establishing a functional connection between these protein kinases. The mechanism underlying this bidirectional regulation remains unknown. The simplest model would involve direct phosphorylation between both kinases. However no genetic or biochemical data supporting this possibility are available so far. Alternatively, this regulatory loop could be mediated by other components controlled by Gcn2p or Hog1p. Activation of Gcn2p by new phosphorylation events, some of them yet to be identified, has been shown to occur (57, 58). One or some of these events could be dependent on Hog1p. Conversely, Gcn2p could regulate the function of activators or inhibitors of Hog1p. FK506 incremented Hog1p activation by high osmolarity that did not lead to increased expression of the Hog1p-dependent CRE-LacZ reporter. This evidence is consistent with a signaling defect downstream of Hog1p that could involve perturbations of Hog1p nucleocytoplasmic trafficking (14) as it has been described before (59). Hog1p activity is also controlled by the Ser-Thr protein phosphatases Ptc1p, -2p, and -3p and the Tyr protein phosphatase Ptp2p and -3p (60), for which function would somehow be influenced by Gcn2p. Experiments addressing these issues are currently underway.

FK506-dependent Hog1p hyperphosphorylation required Gcn2p but not Gcn1p. Monitoring of uncharged tRNA levels by the His6Res-related sequences of Gcn2p is facilitated by Gcn1p and -20p (35) under amino acid or purine starvation (28). Therefore, FK506 could induce Gcn2p activation through alternative mechanisms. Interestingly, rapamycin, an inhibitor of TOR kinases, triggers Gcn2p activation by dephosphorylation of Ser-577 mediated by the type 2A-related protein phosphatase regulator TAP42 (58). As FK506 induces sustained tryptophan deprivation and as amino acid starvation is known to modulate TOR pathway function (61), this novel mechanism of Gcn2p activation may contribute to FK506 induction of GCN4 translation.
Deletion of GCN3 and -4 partially alleviated FK506-induced osmotic stress in yeast without relieving FK506 toxic effects on the Hog1p pathway. Thus, additional mechanisms dependent on the GCN pathway are involved in FK506 toxicity. Expression of one or some Gcn4p-regulated genes may be toxic for yeast growth under osmotic stress in the presence of FK506. Indeed, GCN4 overexpression without affecting translation initiation increases sensitivity to salt stress (25). Accordingly, no GCN4 translation was detected in the gcn3 and -4 mutants. Thus, it is reasonable to think that under stress conditions, overactivation of some Gcn4p-regulated genes would create metabolic problems or elicit counteracting cellular responses, thus compromising cell integrity. Good candidates would be Gcn4p target genes encoding regulatory proteins recently identified by a whole-genome expression profiling in cells starved from histidine (62).

Inactivation of GCN3 but not GCN2 led to FK506 resistance. The sole reported function of GCN3 is to modulate eIF-2B activity in response to eIF-2α phosphorylation by GCN2 (37–39). This result would be explained if FK506 could induce the GCN pathway partly through Hog1p-dependent activation of Gcn2p induced by FK506. This suggests that, upon osmotic shock, a part of the cell defense responses to osmotic shock, thus decreasing viability under high osmolarity. Whether these responses elicited by FK506 in yeast are relevant to the secondary effects described in patients under FK506 therapy remains to be established and deserves further investigation.

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