Calcineurin-dependent growth of an FK506- and CsA-hypersensitive mutant of *Saccharomyces cerevisiae*

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The immunosuppressants FK506 and cyclosporin A (CsA) bound to their receptors, FKBP12 or cyclophilin, inhibit the Ca^{2+} /calmodulin-dependent protein phosphatase, calcineurin, preventing T cell activation or, in yeast, recovery from α -mating factor arrest. Vegetative growth of yeast does not require calcineurin, and in strains sensitive to FK506 or CsA, growth is inhibited by concentrations of drug much higher than those required to inhibit T cell activation or recovery from mating factor arrest. We now describe the isolation of a mutant of *Saccharomyces cerevisiae* which is 100–1000-fold more sensitive to the growth inhibitory properties of these drugs. The mutation (*fks1*) also confers a slow growth phenotype which is partially suppressed by exogenously added Ca^{2+} and exacerbated by EGTA. Simultaneous disruption of the two genes (*CNA1* and *CNA2*) encoding the alternative forms of the catalytic A subunit of calcineurin, or of the gene (*CNBI*) encoding the regulatory B subunit, is lethal in an *fks1* mutant. Disruption of the gene encoding FKBP12 (*FKBI*) or the major, cytosolic cyclophilin (*CPHI*) in *fks1* cells results in the loss of hypersensitivity to the relevant drug. Overexpression of *CNA1* or *CNA2*, in conjunction with *CNBI*, results in a significant decrease in hypersensitivity to FK506 and CsA. The results show that the hypersensitivity of the *fks1* mutant is due to the inhibition of calcineurin phosphatase activity by the receptor–drug complexes. The growth dependence of the mutant on the Ca^{2+} /calcineurin signal pathway provides an important tool for studying in yeast certain aspects of immune suppression by these drugs.

Introduction

FK506 and cyclosporin A (CsA) are potent immunosuppressants, which inhibit an intermediate Ca^{2+} -dependent step in T cell activation, blocking interleukin-2 (IL-2) production (reviewed in Sigal & Dumont, 1992; Schreiber, 1992). The macrolide FK506 binds with high affinity to the members of a family of highly conserved and ubiquitous proteins referred to as FK506 binding proteins (FKBPs). CsA is a cyclic undecapeptide which binds to members of another family of highly conserved and ubiquitous proteins called cyclophilins. The complex of binding protein with drug, FKBP12–FK506 or

cyclophilin–CsA, binds and inhibits the Ca^{2+} - and calmodulin-dependent protein phosphatase, calcineurin, suggesting that inhibition of calcineurin might be the mechanism of immunosuppression (Liu *et al.*, 1991a). Transient overexpression of calcineurin in activated Jurkat human T cell lines containing the IL-2 promoter linked to a reporter gene resulted in decreased sensitivity of IL-2 promoter activity to FK506 or CsA (O'Keefe *et al.*, 1992; Clipstone & Crabtree, 1992). These results indicate that calcineurin is a component of the T cell receptor signal transduction pathway, and that the drugs act by causing a loss of calcineurin function.

FK506 and CsA are also antibiotics which inhibit the growth of certain strains of yeast and fungi. The antifungal properties of these drugs and the existence of FKBPs, cyclophilins, and calcineurins in yeast and fungi have prompted genetic examinations of the mode of action of the drugs in these organisms. CsA-resistant mutants of *Saccharomyces cerevisiae* and *Neurospora crassa* either lack cyclophilin, or contain an altered receptor which fails to bind CsA, indicating that cyclophilin mediates CsA cytotoxicity in these organisms

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Abbreviations: CsA, cyclosporin A; FKBP, FK506 binding protein; IL-2, interleukin-2; NPD, nonparental ditype; PD, parental ditype; SC medium, synthetic complete medium; T, tetatype; YPAD, yeast extract/peptone/adenine/dextrose; YPAG, yeast extract/peptone/adenine/glycerol; YPD, yeast extract/peptone/dextrose.

Table 1. *S. cerevisiae* strains

Strain	Characteristics	Derivation
YFK005	<i>MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52</i>	Brizuela <i>et al.</i> (1991)
YFK007	<i>MATα ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52</i>	Brizuela <i>et al.</i> (1991)
YFK132	Same as YFK007 except <i>fks1-1 rho</i> ⁻	EMS mutagenesis
YFK188	Same as YFK007 except <i>fkbl-2::URA3</i>	Transformation (Brizuela <i>et al.</i> , 1991)
YFK426-1A	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cnb1::LYS2</i>	Foor <i>et al.</i> (1992)
YFK426-5A	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cnb1::LYS2</i>	
YFK429-1D	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52 trp1-Δ1 cnal::LEU2 cna2::URA3</i>	
YFK429-3A	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52 trp1-Δ1 cna2::URA3</i>	
YFK429-5A	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52 trp1-Δ1 cnal::LEU2</i>	
YFK440	YFK005 × YFK132 (<i>FKS1/fks1-1</i>)	Mating
YFK440-7D	<i>MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK462	YFK007 × YFK440-7D (<i>FKS1/fks1-1</i>)	Backcross #1
YFK462-8C	<i>MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK462-11A	<i>MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK531	YFK007 × YFK462-8C (<i>FKS1/fks1-1</i>)	Backcross #2a
YFK531-5A	<i>MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK532	YFK007 × YFK462-11A (<i>FKS1/fks1-1</i>)	Backcross #2b
YFK532-2B	<i>MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK532-2D	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK532-5D	<i>MATα ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK532-7C	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK532-10B	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK547	Same as YFK007 except <i>cph1::ADE2</i>	Transformation (Foor <i>et al.</i> , 1992)
YFK620	YFK532-2B × YFK426-1A (<i>CNB1/cnb1::LYS2 FKS1/fks1-1</i>)	Mating
YFK622	YFK007 × YFK532-2B (<i>FKS1/fks1-1</i>)	Backcross #3
YFK622-4D	<i>MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK622-5D	<i>MATα ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 fks1-1</i>	Meiotic segregant
YFK628	Same as YFK531-5A except <i>cph1::ADE2</i>	Transformation
YFK629	Same as YFK531-5A except <i>fkbl-2::URA3</i>	Transformation
YFK688	YFK007 × YFK622-4D (<i>FKS1/fks1-1</i>)	Backcross #4
YFK768	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cnal::LEU2 cna2::URA3 cnb1::LYS2</i>	(Foor <i>et al.</i> , 1992)
YFK801	Same as YFK532-7C except <i>cph1::ADE2</i>	Transformation
YFK803	Same as YFK532-7C except <i>fkbl-2::URA3</i>	Transformation
YFK804	Same as YFK532-10B except <i>cnal::LEU2</i>	Transformation
YFK805	Same as YFK532-10B except <i>cna2::URA3</i>	Transformation
YFK835	<i>MATα his4 leu2 lys2 pep4-3 ura3-52</i>	CD24 from C. Douglas, Merck
YFK836	Same as YFK007 except <i>bar1::ADE2</i>	Transformation
YFK837	Same as YFK132 except <i>bar1::ADE2</i>	Transformation

(Tropschug *et al.*, 1989). FK506-resistant mutants of *N. crassa* lack FKBP12 (Barthelmess & Tropschug, 1993). However, similar experiments have not clearly delineated the role of an FKBP12–FK506 complex in mediating FK506 sensitivity in yeast. Disruption of the *FKB1* gene encoding the yeast homologue of FKBP12 reduces sensitivity to FK506 only slightly (Brizuela *et al.*, 1991; Heitman *et al.*, 1991). It does, however, eliminate sensitivity to the FK506-related macrolide, rapamycin (Koltin *et al.*, 1991; Brizuela *et al.*, 1991). Rapamycin, which has been found to bind to most FKBP12s, does not block IL-2 production, but inhibits later events required for full T cell activation. Consistent with this, the mammalian FKBP12–rapamycin complex does not bind or inhibit mammalian calcineurin (Liu *et al.*, 1991a). Mutations in three yeast *FKR* genes conferring FK506 resistance also have no effect on FKBP12 activity and map to loci distinct from *FKB1* (Brizuela *et al.*, 1991). Furthermore, the inhibition of yeast growth occurs at a

concentration of FK506 more than 1000-fold higher than that which inhibits T cell activation. It is therefore likely that the mechanism of toxicity in wild-type yeast cells is unrelated to the mechanism of inhibition of T cell activation.

Calcineurin is a heterodimer consisting of one catalytic and one regulatory subunit. In yeast two genes (*CNA1* and *CNA2*) encoding alternative catalytic subunits (A1 and A2), and one gene (*CNB1*) encoding the regulatory B subunit, have been described. Disruption of these genes has shown that calcineurin is not required for vegetative growth (Cyert *et al.*, 1991; Cyert & Thorner, 1992; Kuno *et al.*, 1991; Liu *et al.*, 1991b; Nakamura *et al.*, 1992). Thus, inhibition of calcineurin by FK506 or CsA would not explain the growth inhibitory properties of these drugs. However, yeast calcineurin has been implicated in recovery from α -mating factor arrest: mutants lacking calcineurin fail to resume growth in the continued presence of α -factor (Cyert *et al.*, 1991; Cyert

& Thorner, 1992). In line with these observations, we found that recovery from mating factor arrest is highly sensitive to FK506 and CsA, at levels 100–1000-fold lower than those required to inhibit vegetative growth (Foor *et al.*, 1992). Moreover, this sensitivity requires FKBP12 or cyclophilin, respectively. In addition, we were able to demonstrate that, just as for mammalian cells, FK506 bound to yeast FKBP12 appears to form a complex with yeast calcineurin (Foor *et al.*, 1992). Thus, the interactions of these receptor–drug complexes with calcineurin are highly conserved.

Here we describe the isolation and characterization of a mutation, *fks1*, which renders the vegetative growth of yeast cells sensitive to levels of FK506 or CsA as low as those previously found to inhibit recovery from mating factor arrest. Hypersensitivity requires the presence of FKBP12 or cyclophilin. We surmised that, unlike wild-type strains, the mutant would require calcineurin for growth. Evidence is presented that this is indeed the case.

Methods

Yeast strains, media, and genetic methods. Yeast strains (Table 1) were grown at 28 °C (unless otherwise indicated) on synthetic complete medium (SC), yeast extract/peptone/dextrose (YPD), YPD supplemented with adenine (YPAD), or yeast extract/peptone/glycerol supplemented with adenine (YPAG) (Brizuela *et al.*, 1991; Sherman *et al.*, 1986). Supplements were added, when needed, after sterilization. Concentrated stock solutions of the antibiotics were prepared in methanol or ethanol. The amounts of solvent added to the media had no effect on growth. Standard methods were used for genetic analysis (Sherman *et al.*, 1986) and DNA transformation (Becker & Guarente, 1991). Tetrad dissections were performed on YPAD containing 10 mM-CaCl₂. The construction and use of *fkb1::URA3* (Brizuela *et al.*, 1991), *cph1::ADE2*, *cnal::LEU2*, *cna2::URA3* and *cnb1::LYS2* (Foor *et al.*, 1992) disruption fragments have been described. The *bar1::ADE2* allele was constructed by synthesizing a *BAR1* DNA fragment by polymerase chain reaction [residues 1 to 2729 of the sequence given by MacKay *et al.* (1988)] and replacing the 1.4 kb *XbaI*–*StuI* fragment within the *BAR1* gene with the 3.8 kb *BamHI* *ADE2* fragment from p669 (provided by P. Linder; Foor *et al.*, 1992).

Growth inhibition assays. Sensitivity to α -factor was determined by halo assay as described by Cyert *et al.* (1991). A replica plate assay was used to score the antibiotic sensitivities of various strains (Brizuela *et al.*, 1991). Briefly, strains were patched onto solid YPAD medium lacking antibiotic, incubated for 2 d and then replicated onto solid medium with or without antibiotic, followed by growth for 1–2 d. The lowest concentration of antibiotic preventing growth (minimum inhibitory concentration; MIC) was determined by inoculating a suspension of actively growing cells into medium containing serial dilutions of antibiotic. Tubes (16 mm) containing 1.0 ml of medium were inoculated with ca. 2×10^4 cells for wild-type (YFK007) or 2×10^5 cells for *fks1* strains and incubated with shaking until growth of a control culture without antibiotic reached an OD₆₀₀ of 1.0–1.6.

Isolation of mutants hypersensitive to FK506 and CsA. YFK005 and YFK007 were mutagenized with EMS by standard procedures (Sherman *et al.*, 1986), plated on YPAD, and grown at 25 °C for 5 d. Colonies were replicated onto SC containing 0, 1, or 10 μ g FK506 ml⁻¹ and the replicates were incubated 1–2 d at 30 °C. Putative hyper-

sensitive mutants were reisolated from the plates lacking FK506 and retested before further analysis. All mutants grew poorly on SC compared to the parent. Their growth and sensitivity to FK506 were greater on YPAD, making this the preferred medium for scoring the hypersensitive phenotype.

Construction of plasmids carrying the *CNA1*, *CNA2* and *CNB1* genes. Individual phage clones containing the calcineurin genes were identified from a yeast genomic DNA library of strain S288C in λ -DASH (Stratagene, cat. no. 943901) by hybridization to probes synthesized from yeast genomic DNA by PCR (Foor *et al.*, 1992). The *CNA1*, *CNA2* and *CNB1* genes were mapped to 4.3 kb *HindIII*, 4.3 kb *BglII* and 1.3 kb *EcoRV* DNA fragments within isolated phage clones, respectively. The *CNB1* fragment was inserted into the *SmaI* site of pBluescript II KS(+) in the *lacZ* orientation and transferred as a *BamHI*–*EcoRI* fragment to the *TRP1*-selectable, single-copy and multicopy yeast shuttle vectors YCplac22 and YEplac112 (Gietz & Sugino, 1988), giving plasmids YCp-B and YEp-B, respectively. The *CNA1* fragment was inserted into the *HindIII* site and the *CNA2* fragment into the *BamHI* site of YEp-B, giving YEp-A1-B and YEp-A2-B, respectively.

Purification of proteins. Recombinant yeast FKBP12 was purified from *Escherichia coli* (Nielsen *et al.*, 1992). Yeast calmodulin was purified using an unpublished procedure (M. H. Pausch & J. Thorner, University of California, Berkeley; personal communication) from a protease deficient strain (YFK835) containing plasmid pEL1 in which the yeast *CMD1* gene is expressed from the *GAL1* promoter (Davis & Thorner, 1989). Yeast calcineurins were purified from strain YFK768 (*cnal cnal2 cnb1*) containing either YEp-A1-B or YEp-A2-B by DEAE-cellulose chromatography followed by affinity chromatography on yeast calmodulin coupled to an agarose support essentially as described by Cyert & Thorner (1992) except that the concentration of EGTA in the DEAE column buffer was increased from 1 to 3 mM. The level of calcineurin during the purification was monitored by formation of the 100 kDa complex of calcineurin with FKBP12–FK506 as detected by Superose 12 chromatography with [³H]dihydro-FK506 as described by Foor *et al.* (1992). Whole cell extracts were prepared as previously described (Foor *et al.*, 1992; Nielsen *et al.*, 1992). Samples of crude extracts contained 0.4 mg of protein in 0.20 ml. Excess [³H]dihydro-FK506 (104 nM), recombinant yeast FKBP12 (1.2 μ g ml⁻¹) and native yeast calmodulin (0.7 μ g ml⁻¹) were added to the samples before loading. Under these conditions, all of the calcineurin is present in the complex (J. Nielsen & M.-J. Hsu, unpublished observations). The formation of the complex is thus an accurate measure of the amount of calcineurin present in a sample. Calcineurin phosphatase activity was assayed as described by Liu *et al.* (1991a). The substrate was a synthetic peptide corresponding to the phosphorylation site of the RII subunit of cAMP-dependent protein kinase, which was phosphorylated with [γ -³²P]ATP at the serine residue. Reactions were started by the addition of 1 μ M substrate and incubated at 30 °C for 10 min. Less than 10% of the substrate was utilized in all cases. Values from reactions lacking enzyme were subtracted from those obtained with enzyme. Protein was measured with the Bio-Rad Protein Assay with bovine serum albumin as the standard.

Results

Isolation of FK506-hypersensitive mutants

Although inhibition of recovery from mating factor arrest in yeast is a good model for the inhibition of T cell activation by FK506 (Foor *et al.*, 1992), it is difficult to quantify the effects of the drugs or to select mutants experimentally. For this reason, we decided to investigate

Table 2. Effect of Ca^{2+} on drug sensitivities of the *fksl* mutant

The wild-type (WT) strain (YFK007) and the *fksl* strain (YFK532-7C) were grown in YPAD with or without 10 mM- CaCl_2 . Antibiotic modes of action: amphotericin B (membrane integrity); anisomycin (protein synthesis); cycloheximide (protein synthesis); 5-fluorocytosine (DNA synthesis); ketoconazole (sterol synthesis); rapamycin (unknown); trifluoperazine. 2HCl (calmodulin antagonist). The results for FK506 and CsA were determined with twofold dilutions of drug; trifluoperazine with 5 $\mu\text{g ml}^{-1}$ increments; and the other drugs with threefold dilutions.

Strain	Ca^{2+}	MIC ($\mu\text{g ml}^{-1}$)								
		FK506	CsA	Ampho- tericin B	Aniso- mycin	Cyclo- heximide	5-Fluoro- cytosine	Ketocon- azole	Rapa- mycin	Trifluo- perazine
WT	—	50	> 100	0.5	20	0.03	10	1	0.003	15
WT	+	50	> 100	0.5	20	0.03	10	1	0.003	20
<i>fksl</i>	—	0.03	2	0.2	20	0.03	10	1	0.003	15
<i>fksl</i>	+	0.03	4	0.5	20	0.03	10	1	0.003	15

mutants of yeast hypersensitive to FK506 in the hope that hypersensitivity would prove to be more useful for investigating these interactions than recovery from mating factor arrest. The parental strains (YFK005 and YFK007), which are sensitive to about 50 $\mu\text{g FK506 ml}^{-1}$, were mutagenized with EMS. We tested about 2100 isolates on drug plates containing 1 and 10 $\mu\text{g FK506 ml}^{-1}$ and identified six hypersensitive mutants. One mutant (YFK132) was sensitive to 0.1 $\mu\text{g FK506 ml}^{-1}$. YFK132 was also sensitive to 10 $\mu\text{g CsA ml}^{-1}$, while its parent (YFK007) was insensitive to 100 $\mu\text{g ml}^{-1}$. The mutant grew significantly more slowly than its parent, and also failed to grow on glycerol as a sole source of carbon.

Genetic analysis of YFK132

To determine whether the phenotypes of YFK132 were the result of a single mutation, tetrad analyses were performed on crosses between the mutant and wild-type strains. The FK506-hypersensitive and slow-growth phenotypes were recessive in all of the diploid isolates. All 15 tetrads with four viable spores from the cross of YFK132 with YFK005 yielded two FK506-hypersensitive and two nonhypersensitive spore colonies. FK506-hypersensitive colonies were slow growing, while nonhypersensitive colonies grew normally. All segregants were capable of growth on glycerol, although we found that those which were hypersensitive to drug and slow-growing produced daughter cells which failed to grow on glycerol at a higher frequency than the nonhypersensitive and fast growing segregants. Spore viability was low (ca. 72%), probably due to additional EMS-induced mutations in YFK132. To improve spore viability, *MAT α* *fksl* meiotic segregants were repeatedly backcrossed to YFK007 to generate diploid YFK688 (Table 1). The viability of spores from this diploid was 88%. All 24 tetrads with four viable spores from this diploid yielded

two FK506-hypersensitive and two nonhypersensitive spore colonies. FK506-hypersensitive colonies were also sensitive to CsA and grew slowly, while the nonhypersensitive colonies were CsA resistant and grew normally. These results indicated that the drug-hypersensitive and slow-growth phenotypes of YFK132 resulted from mutation of a single nuclear gene, which we have called *FKS1* (FK506 sensitive), while the failure to grow on glycerol was due to a cytoplasmic petite mutation.

Effect of Ca^{2+} and drugs on the growth of the *fksl* mutant

Further physiological examination of the *fksl* mutant revealed that its growth rate increases upon the addition of CaCl_2 to the growth medium. For example, the doubling time of the *fksl* strain YFK532-7C decreased from 3.3 h in YPAD to 2.1 h in YPAD supplemented with 10 mM- CaCl_2 , the optimum level, while that of the wild-type strain (YFK007) increased slightly from 1.5 to 1.6 h. Growth rates were not increased by higher concentrations of NaCl or KCl, and only a slight effect was seen with MgCl_2 . Growth of the mutant was strongly inhibited by 4 mM-EGTA, a Ca^{2+} chelator, while that of the wild-type strain was unaffected. The addition of CaCl_2 had no effect on sensitivity of the mutant to FK506 and resistance to CsA increased only slightly (Table 2). The mutant exhibited little or no difference from the wild-type strain in its sensitivity to a number of other antibiotics, in the presence or absence of CaCl_2 , including amphotericin B, anisomycin, cycloheximide, 5-fluorocytosine, ketoconazole, lovastatin, and trifluoperazine (Table 2). These antibiotics are structurally and functionally distinct and have diverse modes of action. Thus, the hypersensitivity of the *fksl* mutant to the two immunosuppressants FK506 and CsA appears to be highly specific.

Fig. 1. Inhibition of growth of the *fks1* mutant by FK506. YFK132 (*fks1*) was grown in liquid YPAD medium. Growth was monitored in a Klett–Summerson colorimeter with a red (no. 66) filter. At the indicated time FK506 ($1 \mu\text{g ml}^{-1}$) was added to one of two cultures. ○, Culture without FK506; ●, culture with FK506.

Inhibition of growth by FK506 did not occur immediately upon addition of drug to an exponentially-growing culture. Rather, the growth rate decreased gradually over more than one generation, with the culture density increasing 3.3-fold before cessation of growth (Fig. 1). Drug-inhibited cells remained viable throughout the course of the experiment, even after growth had ceased (data not shown). Microscopic examination of the fully inhibited culture showed that the cells had stopped growing at all stages of the cell cycle as judged by the proportion of budded cells and the range of sizes of the buds (data not shown). No unusual morphological characteristics were observed.

Requirement for FKBP12 or cyclophilin

Disruption of *FKB1* in an *fks1* haploid strain resulted in loss of hypersensitivity to FK506 (Fig. 2a), with no effect on hypersensitivity to CsA (Fig. 2b) or the slow growth phenotype. Conversely, disruption of *CPH1* resulted in loss of hypersensitivity to CsA (Fig. 2b), with no effect on hypersensitivity to FK506 (Fig. 2a) or the slow growth phenotype. This indicated that each drug acts by binding to its specific cytosolic binding protein. In addition, since disruption of the gene encoding the binding protein for one drug did not alter hypersensitivity to the other drug or the slow growth phenotype, *fks1* cannot map to either *FKB1* or *CPH1*. It was previously shown that disruption of *FKB1* results in resistance to rapamycin (Brizuela *et al.*, 1991; Koltin *et al.*, 1991). The same was true in the *fks1* background (Fig. 2c). A genetic cross of an *fks1* haploid strain with

Fig. 2. Sensitivity of *fks1* cells to FK506 and CsA is mediated by their respective binding proteins, FKBP12 (*fkb1*) and cyclophilin (*cph1*). YFK007 (wild-type) (●), YFK532-7C (*fks1*) (○), YFK801 (*fks1 cph1*) (■), and YFK803 (*fks1 fkb1*) (□) were grown in liquid YPAD medium containing 10 mM- CaCl_2 in the presence or absence of (a) FK506, (b) CsA, or (c) rapamycin. Representative results from a single experiment are presented as the fraction of control growth in the absence of antibiotic.

Table 3. Genetic analysis of the *fks1-1* mutation

Diploids were created by crossing the indicated parents and sporulated, and tetrads were dissected. Adenine, leucine and lysine prototrophies were used as markers to follow the *cph1*, *cnal*, and *cnb1* loci, respectively. Uracil prototrophy was used as marker to follow the *fkb1* and *cna2* loci. FK506 hypersensitivity was determined on YPAD supplemented with 1 µg FK506 ml⁻¹. CsA sensitivity was determined on YPD supplemented with 10 µg CsA ml⁻¹ for crosses YFK694 and YFK700. Ascus types: PD, parental ditype; NPD, nonparental ditype; T, tetratype. Ascus types were determined from 4-spore tetrads, and 3- and 2-spore tetrads when ascus types could be deduced.

Cross	Parents	Relevant genotype	Ascus type			Spore viability (%)
			PD	NPD	T	
YFK694	YFK622-4D × YFK188	<i>fks1-1</i> × <i>fkb1-2::URA3</i>	3	3	9	85
YFK700	YFK622-4D × YFK547	<i>fks1-1</i> × <i>cph1::ADE2</i>	3	3	9	88
YFK618	YFK532-5D × YFK429-5A	<i>fks1-1</i> × <i>cnal::LEU2</i>	27	4	42	81
YFK624	YFK532-5D × YFK429-3A	<i>fks1-1</i> × <i>cna2::URA3</i>	10	16	44	80
YFK620	YFK532-2B × YFK426-5A	<i>fks1-1</i> × <i>cnb1::LYS2</i>	11	9	45	66

an *fkb1::URA3* strain confirmed that *fks1* does not map to *FKB1* (Table 3). The PD:NPD:T ratio was very close to the theoretical 1:1:4 expected for the random assortment of independent and unlinked genes. Parallel results were obtained in a cross of an *fks1* haploid strain with a *cph1::ADE2* strain (Table 3).

Growth of the *fks1* mutant is calcineurin-dependent

Since FK506 and CsA, when bound to their receptors in extracts of mammalian cells, are inhibitors of calcineurin (Liu *et al.*, 1991a), we suspected that growth of the *fks1* mutant would be calcineurin-dependent. Haploid strains were transformed in separate experiments with purified DNA fragments carrying the *cnal::LEU2*, *cna2::URA3*, and *cnb1::LYS2* alleles, and genomic DNA isolated from the transformants was analysed by Southern transfer hybridization. Those in which *CNA1* or *CNA2* was disrupted remained hypersensitive to FK506 with no change in the MIC (30 ng ml⁻¹). Disruptants of *CNA2*, however, showed a pronounced decrease in growth rate on YPAD relative to the *fks1* parent (data not shown). The greater effect of *cna2::URA3* compared to *cnal::LEU2* on the growth of the *fks1* mutant is similar to their effects on recovery from α -factor arrest in the wild type (Foor *et al.*, 1992). We were unable to disrupt *CNB1*, or simultaneously the two *CNA* genes, in the *fks1* mutant. These results were consistent with the idea that growth of the *fks1* mutant is calcineurin-dependent.

To prove this, we crossed *fks1* strains with strains containing disruptions of the calcineurin genes. In the cross between *fks1* and *cnb1::LYS2* only a small fraction (8/73) of the dissected tetrads had 4 viable spores, while a large proportion had three (33/73) or two (27/73) viable spores. All of the tetrads with four viable spores were parental ditype with two *fks1* and two *cnb1::LYS2*

spores. Of the 33 tetrads with three viable spores, 30 were tetratype with wild-type, *fks1*, and *cnb1::LYS2* viable spores and one inviable spore predicted to be *fks1 cnb1::LYS2*; the remaining 3 were parental ditype with one *fks1* and two *cnb1::LYS2* viable spores and one inviable spore predicted to be *fks1*. Of the 27 tetrads with two viable spores, 15 were tetratype with one wild-type and one *cnb1::LYS2* viable spore. Of the remaining 12, nine were nonparental ditype with two wild-type viable spores and two inviable spores predicted to be *fks1 cnb1::LYS2*, and 3 were of unknown type with one *fks1* and one *cnb1::LYS2* viable spore. Thus, none of 63 *fks1 cnb1::LYS2* spores was viable, establishing that disruption of *CNB1* in the presence of the *fks1* mutation is lethal to the spores. The number of tetrads of each type in this cross are listed in Table 3. The PD:NPD:T ratio is very close to 1:1:4 consistent with *FKS1* and *CNB1* being independent and unlinked genes.

We also crossed *fks1* strains with *cnal::LEU2* or *cna2::URA3* strains (Table 3). Spores with *fks1* and *cnal::LEU2* alleles or *fks1* and *cna2::URA3* alleles were viable. The PD:NPD:T ratios indicated that the *fks1* mutation does not map to *CNA1* and *CNA2*. The data suggest that *cnal::LEU2* and *fks1* are linked at a distance of about 48 cM. When we crossed *fks1 cnal::LEU2* and *cnal::LEU2 cna2::URA3* strains, the results were qualitatively similar to those obtained in the cross with *cnb1::LYS2*. There was a significant drop in the level of spore viability, and none of 49 spores predicted from tetrad analysis to be *fks1 cnal::LEU2 cna2::URA3* was viable (detailed data not presented). Thus, in an *fks1* mutant simultaneous disruption of the two genes encoding the alternative catalytic subunits of calcineurin or disruption of the single gene encoding the common regulatory subunit yielded inviable spores. These findings parallel the effects of calcineurin gene

Fig. 3. Calcineurin overexpression in *fks1* cells confers FK506 and CsA resistance. YFK531-5A (*fks1*) transformants containing plasmid YEp-A1-B (□), YEp-A2-B (○), or YEp-lac112 (vector) (△) were grown in liquid YPAD medium containing 10 mM-CaCl₂ (to ameliorate the slow-growth defect of the *fks1* strain) in the presence or absence of (a) FK506 or (b) CsA. Representative results from a single experiment are presented as the fraction of control growth in the absence of antibiotic. The experiment was repeated with essentially the same results.

disruptions on recovery from mating factor arrest (Cyert *et al.*, 1991; Cyert & Thorner, 1992; Foor *et al.*, 1992) and are consistent with the idea that the two yeast calcineurins have redundant, or at least overlapping, functions.

To determine whether *fks1 cnb1::LYS2* double mutants merely fail to germinate or are also unable to grow vegetatively, we transformed a plasmid carrying *CNB1* and *TRP1* (YCp-B) into diploid YFK620 (*FKS1/fks1 CNB1/cnb1::LYS2*). In 57 tetrads from this strain, spore viability was ca. 72% and 18 *fks1 cnb1::LYS2* spores were recovered. All of the *fks1 cnb1::LYS2* spores which grew also carried the *CNB1* plasmid as judged by their Trp⁺ phenotype. Thus, the *CNB1* gene on the plasmid complemented the defect of the *fks1 cnb1::LYS2* mutant. We also grew independent

fks1 and *fks1 cnb1::LYS2* meiotic segregants containing the *CNB1* plasmid for several generations nonselectively to allow plasmid loss, and then measured the fraction of cells which had retained or lost the plasmid. All of the colonies from the *fks1 cnb1::LYS2* haploids remained Trp⁺ (ca. 900 were tested), whereas only a small fraction (6%) of the *fks1* colonies remained Trp⁺ (ca. 1700 were tested). The failure to lose the *CNB1* plasmid in *fks1 cnb1::LYS2* double mutants demonstrated that vegetative growth of the *fks1* mutant is calcineurin-dependent.

Expression of *fks1-1* and mating type control

Recovery from mating factor arrest is calcineurin-dependent and, as such, resembles the growth of the *fks1* mutant. It seemed possible that the mutant might be growing in a state similar to that seen during recovery from mating factor arrest, and that the *fks1* mutation might therefore be signalling the presence of α -factor even in its absence. In this case one might expect the *fks1* mutant to be relatively resistant to the growth arrest induced by exposure of cells to α -factor, since the cells would already be in a state of recovery. However, the mutant and the wild-type strains did not differ in their response to α -factor (data not shown). To test this further, we disrupted *BAR1*, the gene encoding α -factor protease (MacKay *et al.*, 1988). Mutations in this gene confer hypersensitivity to α -factor. Strains YFK836 (*bar1*) and YFK837 (*fks1 bar1*) were both hypersensitive to α -factor and did not differ from each other in their response to it.

It also seemed possible that the *fks1* phenotypes might be under mating type control and only be expressed in *MATa* haploid cells. However, in the crosses described above, we found that the phenotypes were expressed equally well in *MATa* segregants. Expression of the *fks1* phenotypes could also be under cell type control and not be expressed in homozygous *fks1* diploids. Four *MATa fks1* and four *MATa fks1* segregants were crossed generating 16 *fks1* homozygous diploids. All of these diploids were hypersensitive to FK506 and grew even more slowly than *fks1* haploids (data not shown). Thus, expression of the *fks1* phenotypes is not under mating type control.

Overexpression of calcineurin

Since growth of the *fks1* mutant is calcineurin-dependent, we postulated that hypersensitivity to FK506 and CsA was due to inhibition of this enzyme by the drugs. To test this *in vivo*, we overexpressed calcineurin. The presence of a multicopy plasmid carrying *CNA1* and *CNB1* (YEp-A1-B), or *CNA2* and *CNB1* (YEp-A2-B) in the *fks1*

Fig. 4. Overexpression of calcineurin as measured by the formation of the 100 kDa FKBP12-FK506 binding complex in extracts of *fks1* cells. See Methods for details of sample preparation and Superose 12 chromatography. YFK531-5A (*fks1*) transformants containing plasmid YEp-A1-B (□), YEp-A2-B (○), or YEplac112 (vector) (Δ) were grown in liquid YPAD medium containing 10 mM-CaCl₂. Plasmid loss was 47%, 37%, and 20%, respectively. Each column profile presents data from a single experiment. The experiment was repeated with independent transformants with essentially the same results.

mutant increased resistance to FK506 and CsA compared to a control strain with the vector alone (Fig. 3). These results parallel those seen in Jurkat T cell lines overexpressing calcineurin (O'Keefe *et al.*, 1992; Clipstone & Crabtree, 1992). Yeast cells with YEp-A2-B were significantly more resistant to both drugs than those with YEp-A1-B (Fig. 3). Moreover, the presence of YEp-A2-B decreased the doubling time of the mutant in the absence of Ca²⁺ from 3.2 to 2.8 h, and in the presence of Ca²⁺, from 2.2 to 2.0 h.

The level of overexpression of calcineurin in the *fks1* cells with the plasmids was quantified as follows. We previously demonstrated in crude yeast extracts that FK506 binds to yeast FKBP12 and forms a 100 kDa complex with yeast calcineurin and calmodulin which can be detected by chromatography on Superose 12 (Foor *et al.*, 1992). The formation of this complex was used to determine the levels of calcineurin in strains containing the multicopy calcineurin plasmids (Fig. 4). Maximum levels of the complex were obtained only when the extracts were supplemented with purified yeast FKBP12 and calmodulin, indicating that these components were limiting in extracts from strains in which calcineurin was overexpressed. Other experiments (data not shown) demonstrated that the presence of excess FK506, FKBP12 and calmodulin, causes the complete conversion of calcineurin to the complexed form and, under these conditions, the amount of complex is an accurate measure of the level of the enzyme in the

sample. The *fks1* strain with YEp-A1-B contained 3.7-fold more calcineurin than the *fks1* strain with vector alone, while the *fks1* strain with YEp-A2-B contained 14-fold more. The ratio of the type 1 calcineurin (the form containing the A1 catalytic subunit) to type 2 (containing the A2 subunit) is similar to that obtained in extracts of strains in which calcineurin is not overexpressed (Foor *et al.*, 1992), and may simply reflect a difference in the promoter activities of *CNA1* and *CNA2*.

The overexpression of each calcineurin in strains with the *FKS1* wild-type allele was also determined. YFK768 (*cnal cna2 cnb1*) was transformed with YEp-A1-B and YEp-A2-B, and the level of calcineurin was quantified as before. The strain with YEp-A1-B contained 18-fold more calcineurin than the wild-type strain YFK007, while that with YEp-A2-B contained 42-fold more. The high levels of calcineurin in the strain with YEp-A2-B allowed us to detect the presence of FKBP12 in the 100 kDa complex by antibody staining (Fig. 5). Previously, the presence of FKBP12 in the complex was inferred from the failure of the complex to form in extracts of mutants lacking FKBP12 (Foor *et al.*, 1992). In the absence of FK506 (Fig. 5, upper panel), FKBP12 eluted in fractions 34 and 35. In the presence of FK506 (lower panel), most of the FKBP12 was found in fractions 22 to 25, which contained the calcineurin-dependent 100 kDa complex. A small amount of FKBP12 eluted in fraction 31. Control experiments with purified FKBP12 alone showed a similar change in the position of elution of FKBP12 upon the addition of drug, suggesting that drug binding induces a conformational change in the protein.

Purification of the yeast calcineurins from overexpressing strains

It has never been directly demonstrated that the enzymic activity of yeast calcineurin is inhibited by FKBP12-FK506. The assay of the phosphatase activity of yeast calcineurin is extremely difficult in crude preparations due to the presence of other dephosphorylating enzymes. In order to test for inhibition and to further characterize the formation of the inhibitory complex, we purified the two types of calcineurin from extracts of the high-producing strains. The purification procedure consisted of DEAE-cellulose chromatography followed by affinity chromatography on immobilized yeast calmodulin. Both enzymes were nearly homogeneous as judged by silver staining of an SDS-polyacrylamide gel (data not shown). The formation of the 100 kDa complex could also be demonstrated with these highly purified yeast calcineurins, and required the addition of FK506, FKBP12 and calmodulin. The requirement for calmodulin has also been shown previously with relatively crude yeast

Fig. 5. Presence of FKBP12 in the 100 kDa complex demonstrated by Western blot. Whole cell extract of YFK768 containing plasmid YEp-A2-B was incubated in the absence (upper panel) or presence (lower panel) of FK506 and chromatographed on Superose 12 as described in Methods. Fractions from the column were concentrated by TCA precipitation and fractionated by electrophoresis in an SDS-polyacrylamide gel. FKBP12 was detected by Western blot analysis (Nielsen *et al.*, 1991) with rabbit antiserum raised against yeast FKBP12.

Table 4. *Inhibition of yeast calcineurin by FK506–FKBP12*

Phosphatase assays were performed in duplicate with 20 nM-yeast calcineurin in the absence of Mn^{2+} . The concentration of calcineurin was reduced to 1 nM in the presence of Mn^{2+} . Enzyme specific activity (SA) is reported as nmol phosphate released min^{-1} (mg calcineurin) $^{-1}$. Additions: 2 μM -yeast FKBP12, 1 μM -FK506, 1 μM -rapamycin, 1 mM- $MnCl_2$ and 0.88 mM-EGTA. Relative activities (RA) are reported separately for reactions with and without Mn^{2+} .

Assay additions	Type 1 calcineurin		Type 2 calcineurin	
	SA	RA	SA	RA
None	4.75	(1.00)	5.38	(1.00)
FKBP12	4.61	0.97	5.44	1.01
FK506	4.66	0.98	5.23	0.97
FKBP12 + FK506	0.32	0.07	0.62	0.11
FKBP12 + rapamycin	5.13	1.08	5.46	1.02
Mn^{2+}	104	(1.00)	116	(1.00)
Mn^{2+} + FKBP12	99	0.95	118	1.02
Mn^{2+} + FK506	101	0.97	110	0.95
Mn^{2+} + FKBP12 + FK506	9	0.09	15	0.13
Mn^{2+} + FKBP12 + rapamycin	99	0.95	113	0.98
EGTA – Ca^{2+} – calmodulin	0.13	—	0.15	—

preparations (Foor *et al.*, 1992) and with mammalian proteins (Wiederrecht *et al.*, 1992). The formation of the 100 kDa complex with the type 1 calcineurin, but not type 2, was stimulated by 1 mM- $MnCl_2$ added to the sample applied to the column (data not shown). This was only found with the enzyme preparation obtained after the calmodulin affinity chromatography step. $MnCl_2$ had no effect with crude extracts or fractions obtained after chromatography on DEAE-cellulose. Mammalian calcineurin contains several species of multivalent cations, including Zn^{2+} , Fe^{3+} and Ca^{2+} (Pallen *et al.*, 1988). One or more of these may have been partially removed from the yeast protein, since the calmodulin affinity chromatography step was carried out in the presence of excess EGTA. Although Mn^{2+} may substitute for one of these, it is also possible that trace contamination of the $MnCl_2$

solution with other multivalent cations is responsible for restoration of the ability of the type 1 calcineurin to form the complex with FKBP12–FK506.

Inhibition of yeast calcineurin by FKBP12–FK506

Purification of the calcineurins allowed us to test directly the inhibition of phosphatase activity by FKBP12–FK506. It has been reported that after calmodulin affinity chromatography, calcineurin is converted to a form with low specific activity in the presence of Ca^{2+} and calmodulin. Reactivation can only be achieved by preincubation with Mn^{2+} (1.0 mM) and not Ca^{2+} (Stewart *et al.*, 1982). We found this to be true with both the type 1 and type 2 preparations (Table 4). However, as shown in Table 4, both the Ca^{2+} - and Mn^{2+} -dependent activities

are potentially inhibited by the FKBP12–FK506 complex. FKBP12–rapamycin did not significantly inhibit the phosphatase activity, consistent with previous observations that rapamycin inhibits a different, Ca^{2+} -independent, signalling pathway (Sigal & Dumont, 1992).

Discussion

The yeast *fks1* mutant provides a model for the inhibition of calcineurin and therefore of T cell activation by FK506 or CsA. The strain is sensitive to levels of these drugs approaching the nanomolar concentrations which inhibit T cells. Inhibition requires the same drug receptors, FKBP12 and cyclophilin, and is mediated by the binding of the receptor–drug complexes to calcineurin and the inhibition of its enzymic activity. Inhibition of growth is a more useful phenotype for studying these interactions than is recovery from mating factor arrest.

Although other CsA-sensitive yeast mutants have been described (Tropschug *et al.*, 1989; Koser *et al.*, 1991), their sensitivity to FK506 was not reported. Tropschug *et al.* (1989) also noted an association of a petite phenotype with CsA-sensitive yeast mutants. Their results implied that a petite phenotype was necessary for CsA sensitivity. Our results show that this is not necessarily the case, but that CsA-sensitive strains segregate petites at a high frequency. However, the relationship of their mutations to the *fks1* allele is unknown.

In addition to the drug-hypersensitive phenotypes, the *fks1* mutant exhibits a slow-growth phenotype, which is partially suppressed by 10 mM- Ca^{2+} . Other Ca^{2+} -responsive mutants of yeast have also been described. A *cal1* mutant shows Ca^{2+} -dependent growth at 23 °C with a sixfold longer doubling time in the absence of Ca^{2+} than in the presence of 100 mM- CaCl_2 (Ohya *et al.*, 1984). It also fails to grow at 37 °C on YPD unless supplemented with CaCl_2 (Ohya *et al.*, 1984). This mutant arrests in the cell division cycle with a tiny bud when transferred to Ca^{2+} -poor media and incubated at the nonpermissive temperature. A *ypt1^{ts}* mutant can be rescued from growth arrest at the nonpermissive temperature by 50–100 mM extracellular Ca^{2+} but grows normally on YPD at the permissive temperature (Schmitt *et al.*, 1988). *YPT1* encodes an essential *ras*-related guanine-nucleotide-binding protein which localizes to the Golgi. The *fks1* mutant differs from the *cal1* and *ypt1* strains both in the level of Ca^{2+} required for optimum growth and its lack of a *ts* phenotype. Disruption of the *PMR1* gene, encoding a P-type ATPase localized in a Golgi-like organelle, results in secretory and growth defects, which are worsened by EGTA and ameliorated by exogenously added Ca^{2+} (Rudolph *et al.*, 1989; Antebi & Fink, 1992).

However, unlike *fks1* strains, *pmr1* strains appear to grow normally on YPD (Antebi & Fink, 1992) and only grow poorly on synthetic minimal medium containing low concentrations of Ca^{2+} . Although the *fks1* mutation confers a phenotype which differs from those of the *cal1*, *ypt1*, and *pmr1* mutations, some of these differences could be due to allele-specific effects; its exact relationship to these other mutations, if any, awaits further work.

What is the nature of the *fks1* mutation? One possibility is that the mutation increases permeability to the drugs. This is probably not the case. Recovery from mating factor arrest in wild-type *FKS1* cells is inhibited by concentrations of FK506 or CsA as low as those inhibiting the growth of the *fks1* mutant. In addition, the mutant is unaltered in its sensitivity to several other antifungal drugs (Table 2) showing that the permeability to these drugs has not changed. It is therefore likely that the drugs enter both wild-type and mutant cells equally well and inhibit calcineurin and that the growth of the mutant is inhibited because the mutation has caused calcineurin to become essential.

The recessive character of the *fks1* mutation suggests that it may result in the loss of function of its gene product. The slow growth phenotype, Ca^{2+} -responsiveness and calcineurin-dependence of the *fks1* mutant imply that this loss of function may be partially replaced by calcineurin, which is activated by Ca^{2+} . Yeast calcineurin is thought to be located exclusively in the cytoplasm (Cyert & Thorner, 1992). The cytosolic level of Ca^{2+} in haploid cells increases from 70 nM, in cells exposed to 0.1 mM extracellular Ca^{2+} , to 350 nM in cells suspended in media containing 10 mM- Ca^{2+} (Halachmi & Eilam, 1993). There is little further increase in cytosolic levels when the extracellular level of Ca^{2+} is increased above 10 mM. We found that 10 mM extracellular CaCl_2 was optimal for stimulating the growth of the *fks1* mutant; higher levels produced no further increase in growth rate. *In vitro* the Ca^{2+} /calmodulin-dependent activity of mammalian calcineurin is stimulated most significantly in the range 100–900 nM- Ca^{2+} (Stewart *et al.*, 1982). These considerations suggest that the stimulation of the growth of the mutant by exogenously added Ca^{2+} is due to the activation of calcineurin, an idea supported by the observation that overexpression of the type 2 calcineurin stimulates growth. The magnitude of this effect is rather small compared to that caused by Ca^{2+} , suggesting that Ca^{2+} may also exert its effect through a component other than calcineurin. It is possible that the *FKS1* gene product is a protein phosphatase, with a specificity overlapped by that of calcineurin. However, the component compensating for the loss of the *FKS1* gene product may not be calcineurin but rather a component which is calcineurin-dependent.

Thus, the *FKS1* gene product itself need not be a phosphatase.

The kinetics of inhibition of growth upon the addition of drug to an exponentially growing culture is consistent with the idea that the compensating component may be downstream of calcineurin. The binding of FK506 or CsA to their binding proteins and the inhibition of calcineurin phosphatase activity occurs rapidly *in vitro* (J. Nielsen, unpublished observations). If drug is quickly taken up by cells, one would expect that hyperphosphorylation of calcineurin substrates would occur soon thereafter. The inhibition of growth by the drugs, however, requires several hours, implying that the synthesis or accumulation of the growth-rate-limiting component may be inhibited by hyperphosphorylation of a calcineurin substrate, and that growth ceases only after dilution of this component by further growth after the addition of drug.

Growth of the hypersensitive mutant and recovery of wild-type cells from mating factor arrest are both calcineurin-dependent. We considered that *FKS1* might encode a function the loss of which leads to a state of constitutive pheromone recovery. However, such a condition should result in insensitivity to α -factor, but the *fks1* mutant is as sensitive as the wild type. In addition, components of the mating pathway are generally under a/α control and not expressed in a/α diploids, yet a/α *fks1/fks1* diploids are slow-growing and fully drug-hypersensitive. The question also arises whether growth of the hypersensitive mutant and recovery from mating factor arrest also depend upon dephosphorylation of the same phosphoprotein by calcineurin. Our data do not directly address this issue. An increase in internal Ca^{2+} has been observed late in the response to mating factor (Iida *et al.*, 1990). Deprivation of Ca^{2+} during this period results in a loss of viability. It is interesting to speculate that FK506 and CsA inhibit recovery from mating factor arrest by preventing calcineurin from transmitting this Ca^{2+} signal. If this is the case, growth of the hypersensitive mutant is different from the pheromone response, since these cells maintain viability during growth arrest caused by the drugs.

Mating factor arrest and recovery are transient phenomena. Under normal conditions, in the absence of cells of the opposite mating type, haploid cells eventually return to a non-calcineurin-dependent, drug-insensitive state. The hypersensitive mutant, however, is continuously calcineurin-dependent. The slow growth of the mutant may be due to a low basal activity of calcineurin in the absence of elevated levels of cytosolic Ca^{2+} . Alternatively, transient increases in Ca^{2+} occurring at some point during the cell cycle may be sufficient to sustain growth. If this is the case, one might expect the *fks1* mutant to display a nonlinear progression through

the cell cycle. Microscopic examination of this strain did not reveal any blatant alteration in the number of cells in various stages of the cell cycle, either when the cells were growing or when growth had ceased due to the presence of drug.

Like wild-type yeast cells, many mammalian cell types are refractory to FK506 and CsA action. An understanding of why wild-type yeast cells are relatively resistant to these drugs, while *fks1* cells are highly sensitive may provide insights into why some mammalian cells respond to these immunosuppressants while others do not. The *fks1* mutant also provides a powerful genetic background for further studies of the mechanism of action of FK506 and CsA, as well as the role of calcineurin *in vivo*. Genetic selections for FK506- and/or CsA-resistant mutants in *fks1* strains should isolate interesting mutations in FKBP12, cyclophilin, calcineurin, and other gene products which lie in the Ca^{2+} /calcineurin-dependent signalling pathway.

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