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Genetics

Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains

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ABSTRACT **The immunophilin–immunosuppressant complexes cyclophilin–cyclosporin A (CsA) and FKBP12–FK506 inhibit the phosphatase calcineurin to block T-cell activation. Although cyclophilin A, FKBP12, and calcineurin are highly conserved from yeast to man, none had previously been shown to be essential for viability. We find that CsA-sensitive yeast strains are FK506 hypersensitive and demonstrate that calcineurin is required for viability in these strains. Mutants lacking cyclophilin A or FKBP12 are resistant to CsA or FK506, respectively. Thus, both the immunosuppressive and the antifungal actions of CsA and FK506 result from calcineurin inhibition by immunophilin–drug complexes. In yeast strains in which calcineurin is not essential, calcineurin inhibition or mutation of calcineurin confers hypersensitivity to LiCl or NaCl, suggesting that calcineurin regulates cation transport.**

Cyclosporin A (CsA), FK506, and rapamycin inhibit T cells by blocking signal transduction (1–3). CsA is the primary immunosuppressant for graft rejection and graft-versus-host disease; FK506 and rapamycin are in clinical trials. CsA and FK506 both prevent T-cell responses to antigen presentation, and rapamycin inhibits T-cell responses to interleukin 2. These natural products are also antifungal and antiparasitic.

Two types of intracellular immunosuppressant-binding proteins, termed immunophilins, have been identified by drug affinity chromatography. CsA binds to cyclophilins (4–6); FK506 and rapamycin bind to FK506-binding proteins (FKBPs) (7–9). Cyclophilins and FKBPs are ubiquitous, highly conserved, and expressed in multiple intracellular compartments (1–3). While cyclophilins and FKBPs share no primary or tertiary homology, both catalyze peptidylprolyl isomerization and may fold proteins *in vivo* (5–9).

Drug binding inhibits proline isomerization but does not cause immunosuppression (10, 11). Instead biochemical (10, 12) and genetic findings (13–15) reveal that CsA, FK506, and rapamycin form toxic protein–drug complexes. One target of the cyclophilin–CsA and FKBP12–FK506 complexes is the calcium-regulated serine (threonine)-specific protein phosphatase calcineurin (10, 12). CsA and FK506 inhibit calcineurin and interfere with T-cell responses to antigen (16, 17), neutrophil chemotaxis (18), stoma aperture in plants (19), and recovery of yeast from pheromone cell cycle arrest (20).

Our studies capitalize on the conserved nature of immunophilins and calcineurin and examine their functions in *Saccharomyces cerevisiae* (14, 21). Growth of most yeast strains is not inhibited by CsA. In a previous study, CsA-resistant mutants of an unusual CsA-sensitive strain, IL993/5c, were reported to lack cyclophilin A or express mutants that do not bind CsA (13). Because the previously identified target of the cyclophilin A–CsA complex, calcineurin, is not essential for viability in several yeast strains (22–24), CsA could inhibit a different protein that is essential in yeast (2, 20). Alternatively, calcineurin could be essential in CsA-sensitive strains (2). We have now tested these models.

MATERIALS AND METHODS

Yeast Strains. Strains are listed in Table 1. Isogenic strains derived from the CsA-sensitive strain IL993/5c were constructed. CsA-resistant mutants arise at high frequency, and CsA sensitivity was confirmed at each step. IL993/5c was transformed with the *ura3-52* allele (pMRFW2, *Bam*HI/*Sma* I cleaved) by selection on 5-fluoroorotic acid (5-FOA) (25). The resulting 5-FOA-resistant strain (TB2) is Ura$^-$ and rarely reverts to Ura$^+$. Mating type was HO switched to yield *MAT***a** *ura3* strain TB3. A *leu2::hisG-URA3-hisG* allele was introduced by transformation with *Bgl* II-cleaved pNKY85 and selection for Ura$^+$ (TB6), followed by 5-FOA selection (43) to yield *ura3 leu2::hisG* strain TB23 (26). The *ilv5* mutation was reverted on SD-ilv medium (TB24).

Plasmids and Gene Disruptions. The *CPR1* gene was subcloned on a 2-kb *Eco*RI fragment into the *Eco*RI site of *CEN URA3* plasmid pRS316 (27), to yield plasmid pTB4a. The *CPR1* gene (28) in pUC18 was deleted/disrupted by inserting between two internal *Msc* I sites the *ADE2* gene as a blunted 2.25-kb *Bgl* II fragment from pFL39 (29); integration was targeted with *Bam*HI/*Xho* I. The *CMP1* gene was PCR amplified from genomic DNA (primers CCGGGGATCCAGATCTACTACTGGGAAACAAAAGGG and GGCCGGGGATCCTTATCACAGTTGTGGCTTTTTCTCCGC), purified, *Bam*HI cleaved, and cloned in *CEN LEU2* vector pRS315 (27) to yield pRCAN. The calcineurin B gene, *CNB1* (24, 30), was PCR amplified from genomic DNA (primers GCAGAATTCATCCATAGAAGCATTTTTATTTC and CCAGAATTCCTTATTGTTTGTTACATATCA), and the 809-bp PCR product was cloned in the pUC18 *Eco*RI site to yield pCNB4. The *CNB1* gene was disrupted at an internal blunted *Sty* I site with *ADE2* (as above), yielding pCSAD12. A PCR product spanning *cnb1::ADE2* was used for disruptions. *cpr1::LEU2-1*, *fpr1::URA3-3*, *fpr1::ADE2-2*, *cmp1::LEU2*, *cmp2::URA3*, and *cmp1::hisG* were disrupted as described (21, 22, 30) and confirmed by PCR and known phenotypes. Transformations were as described (31).

Medium and Assay of Pheromone Response. Media were as described (21, 32). In some cases, medium was prepared with CsA in vehicle [30% (vol/vol) ethanol/70% (vol/vol) cremophor EL] and control medium with 1% vehicle alone. Pheromone response was assayed with α-factor (0–10 μM) and β-galactosidase expression measured from *CEN* or 2-μm *URA3 FUS1-lacZ* reporter plasmids pSB231 and pSB234 (33) with chlorophenol red β-D-galactopyranoside.

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Abbreviations: CsA, cyclosporin A; FKBP, FK506-binding protein; TOC, target of cyclosporin A; YPD, yeast extract/peptone/dextrose.

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Table 1. Yeast strains

Strain	Relevant genotype
	Isogenic JK9-3d series
JK9-3dα	*MATα ura3-52 leu2-3,112 trp1 his4 rme1 HMLa*
JK9-3da	*MATa ura3-52 leu2-3,112 trp1 his4 rme1 HMLa*
JHY58	JK9-3da *cnb1::ADE2 Δade2*
TB68	JK9-3da *cmp1::LEU2 cmp2::URA3*
	K42 series
K42	*MATa ade2 trp1* CsA^s
JHY31-11d	*MATa ura3-52 leu2-3,112 trp1* CsA^s
TB25	JHY31-11d *cpr1::LEU2-1*
	Isogenic IL993/5c series
IL993/5c	*MATα* ρ^o *ilv5* CsA^s
TB2	*MATα* ρ^o *ilv5 ura3* CsA^s
TB3	*MATa* ρ^o *ilv5 ura3* CsA^s
TB23	*MATα* ρ^o *ilv5 ura3 leu2::hisG* CsA^s
TB24	*MATa* ρ^o *ura3* CsA^s
TB26	*MATα* ρ^o *ilv5 ura3 leu2::hisG cpr1::LEU2-1*
TB27	*MATα* ρ^o *ilv5 leu2::hisG cpr1::LEU2-1*
TB40	*MATa* ρ^o *ura3 leu2::hisG cpr1::LEU2-1*
TB62	*MATa* ρ^o *ura3* CsA^s *fpr1::URA3-3*
TB63	*MATa* ρ^o *ura3 leu2::hisG cpr1::LEU2-1 fpr1::URA3-3*
TB70	*MATα* ρ^o *ilv5 ura3 leu2::hisG* CsA^s *cmp1::LEU2*
TB71	*MATα* ρ^o *ilv5 ura3 leu2::hisG* CsA^s *cmp2::URA3*
TB88	*MATα* ρ^o *ilv5 ura3 leu2::hisG* CsA^s *cmp1::hisG*
TB121	*MATα* ρ^o *ilv5 ura3 leu2::hisG* CsA^s *cmp1::hisG cmp2::URA3* (pRCAN)
	Isogenic Y190 series
Y190	*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL-lacZ LYS2::GAL-lacZ*
SMY1	Y190 *fpr1::ADE2-2*
SMY2	Y190 *cnb1::ADE2*
CHY1	Y190 *cpr1::LEU2-1*

CsA^s, CsA sensitive.

Sequencing. The *CPR1* locus was PCR amplified from genomic DNA (34) (primers GAATTCGGATCCCCGCTAATACTACCATGTCC and AGATCTGGATCCTATTGTTCCAGGCAGAGCGG). PCR products of 546 bp were extracted with phenol/$CHCl_3$/isoamyl alcohol (25:24:1, vol/vol), isopropanol precipitated, and ExoCircumVent (New England Biolabs) thermal cycle sequenced with PCR primers and primers CGGCGGTAAGTCTATCTACGG and CTGTCGTGGTGCTTCTTGAAG.

CsA Import, CsA-Binding Assays, and Western Analysis. To assay CsA import, cells were incubated with [Membt-β-^{3}H]-CsA (Amersham; specific activity = 11.1 Ci/mmol; 1 Ci = 37 GBq) and pelleted through a 10% (wt/vol) sucrose cushion, and cell-associated CsA was quantitated by scintillation. LH-20 CsA binding was assayed as described (4, 32). Cyclophilin A was detected by Western analysis (ECL; Amersham) with rabbit polyclonal antisera.

RESULTS

Cyclophilin A–CsA Complex Is Toxic in Yeast. To determine the mechanism of CsA toxicity, we isolated CsA-resistant mutants of the CsA-sensitive yeast strain IL993/5c (13). We first engineered isogenic derivatives with auxotrophic mutations to serve as selectable markers for transformation. We confirmed that the yeast homolog of mammalian cyclophilin A (encoded by *CPR1*) is required for CsA toxicity in yeast. Disruption of the nonessential *CPR1* gene (21, 28) conferred resistance to growth inhibition at up to 200 μg of CsA per ml (cyclophilin A mutant TB26; Fig. 1). CsA-binding activity is dramatically diminished in this cyclophilin A mutant strain (Fig. 2*A*). Introduction of the cyclophilin A gene on a low-copy-number centromeric plasmid restored CsA sensitivity (Fig. 1), confirming CsA resistance is attributable to the cyclophilin mutation and not to reversion of the mutations that confer CsA sensitivity.

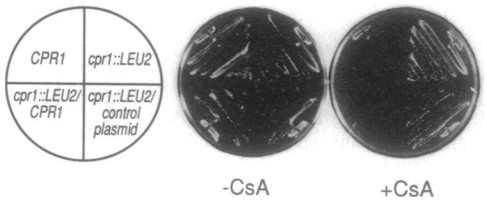

FIG. 1. Cyclophilin A mediates CsA toxicity in yeast. Yeast strain IL993/5c (*CPR1*) is CsA-sensitive; an isogenic mutant lacking cyclophilin A (*cpr1::LEU2* = TB27) is not. A centromeric plasmid expressing cyclophilin A complements the cyclophilin A mutation and restores CsA sensitivity (*cpr1::LEU2/CPR1* = TB26/pTB4a); a control plasmid (*cpr1::LEU2*/control plasmid = TB26/pRS316) does not. Strains were grown (72 hr, 30°C) on SD-URA medium with or without CsA at 200 μg/ml.

To identify other CsA-sensitive yeast strains, we screened mutants hypersensitive to other toxins (35, 36). Growth of one such strain, K42, was partially inhibited by CsA at 100 μg/ml. When strain K42 was crossed to the CsA-resistant strain JK9-3dα (Table 1), sporulation yielded haploid meiotic segregants with increased CsA sensitivity compared to the K42 parent. Disruption of the cyclophilin A gene in one segregant (JHY31-11D) conferred CsA resistance (*cpr1::LEU2* strain TB25), and introduction of the *CPR1* gene restored CsA sensitivity (data not shown). Thus, the cyclophilin A–CsA complex is toxic in two CsA-sensitive strains.

CsA-Resistant Mutants. To identify other proteins involved in CsA toxicity, we isolated 32 independent spontaneous mutants resistant to CsA at 100 μg/ml, 16 in the original IL993/5c strain (*MATα ilv5 URA*$^+$) and 16 in an isogenic derivative of opposite mating type with complementing auxotrophic mutations (TB24; *MATa ILV*$^+$ *ura3*). CsA-resistant mutants were designated TOC, for targets of cyclosporin, and were characterized by genetic crosses to wild type, to a cyclophilin A mutant (*cpr1::LEU2*; TB27), and to each other and by complementation tests with the cloned cyclophilin A gene (plasmid pTB4a). While a cyclophilin A null mutation (*cpr1::LEU2*) confers recessive CsA resistance, only 1 of our CsA-resistant mutants was recessive (*toc8-1*) (Table 2). This mutant was complemented by the cloned cyclophilin A gene and failed to complement a cyclophilin A mutation. The remainder of the mutants conferred semidominant (30 mutants) or dominant (1 mutant; *TOC6-1*) CsA resistance. The one dominant mutation (*TOC6-1*) is not a cyclophilin A mutation because disruption of the cyclophilin A gene did not alter the *TOC6-1* dominant CsA-resistance phenotype when a *cpr1::LEU2 TOC6-1* haploid strain (TB55) was crossed to wild type. Based on genetic behavior and other nonselected phenotypes (slow growth; poor growth at 37°C), the 32 mutants can be placed in five groups (Table 2).

We considered several mechanisms by which TOC mutations might confer CsA resistance. First, if yeast strains were CsA sensitive because mutations render the cell permeable, mutations that confer CsA resistance might alter permeability. However, sensitivity to other toxins (1.5 M KCl, cycloheximide, or nalidixic acid) was largely unaltered in CsA-resistant mutants (data not shown). Moreover, [^{3}H]CsA import did not differ between CsA-resistant strain JK9-3da, CsA-sensitive strain IL993/5c, and CsA-resistant IL993/5c mutants (data not shown). Thus CsA sensitivity does not result from mutations that permeabilize the cell, and mutations that confer CsA resistance do not prevent CsA entry.

Second, because cyclophilin A is required for CsA toxicity, mutations in genes required for cyclophilin A expression or function might confer resistance. This was tested by measuring [^{3}H]CsA-binding activity in cell extracts by LH-20

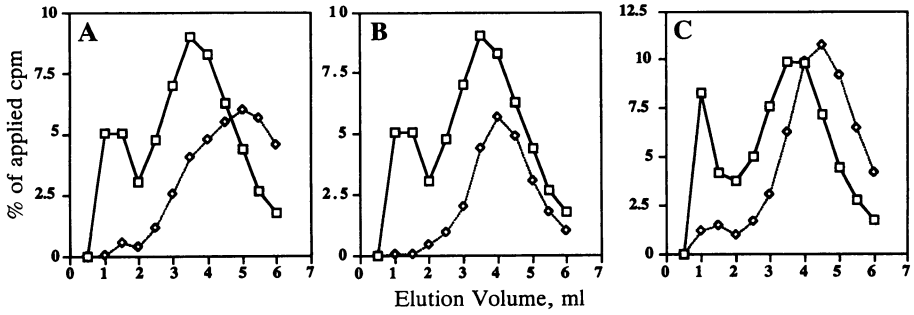

FIG. 2. CsA-binding activity in CsA-sensitive and -resistant strains. Soluble CsA-binding activity in extracts was determined by LH-20 [^{3}H]CsA-binding assay. Free CsA elution varied slightly with minor column differences. (*A*) CsA-binding activity in wild-type strain IL993/5c (□) and an isogenic cyclophilin A mutant (TB26, *cpr1::LEU2*) (◇). (*B*)CsA-binding activity in the CsA-resistant *toc8-1* mutant (◇) and wild-type IL993/5c (□). (*C*) CsA-binding activity in CsA-resistant mutant *TOC17-1* cultured in yeast extract/peptone/dextrose (YPD) medium without CsA (□) or with CsA at 50 μg/ml (◇).

assay, in which cyclophilin–CsA complexes elute early from an LH-20 column, whereas free CsA is retained and elutes late (4) (Fig. 2). Wild-type IL993/5c contains prominent CsA-binding activity; an isogenic mutant lacking cyclophilin A (*cpr1::LEU2* strain TB26) contains <10% of wild-type binding activity (Fig. 2*A*). Residual CsA binding in extracts lacking cyclophilin A is probably attributable to minor cyclophilins CPR2, CPR3, and CPR4. In contrast, extracts from many (9 out of 10) of the spontaneous CsA-resistant mutant strains contained readily detectable CsA-binding activity (Fig. 2 and Table 2). Western analysis confirmed that the *TOC6-1, -9-1, -11-1, -14-1, -15-1, -16-1, -17-1, -23-1, -24-1, -25-1, -26-1,* and *-31-1* mutants express cyclophilin A (Table 2). Thus, in the majority of cases, CsA resistance did not result from altered cyclophilin A activity.

Only the *toc8-1* mutant, which our genetic results argue bears a cyclophilin A mutation, lacked CsA-binding activity (Fig. 2*B*) and failed to express cyclophilin A by Western (Table 2). The gene encoding cyclophilin A, *CPR1*, was PCR amplified and sequenced from wild-type IL993/5c and CsA-resistant mutants. In the *toc8-1* mutant, a UAG stop codon replaces the Tyr-23 codon in the *CPR1* gene. Sequence analysis revealed no mutations in the *CPR1* gene in *TOC6-1, -9-1, -11-1, -14-1, -17-1, -18-1, -19-1,* or *-20-1*. Thus, in contrast to a previous report (13), most of our CsA-resistant mutants express wild-type cyclophilin A.

CsA-Sensitive Strains Are FK506 and FK520 Hypersensitive. Although our findings confirm that cyclophilin A is required for CsA toxicity in yeast, most spontaneous mutations that confer CsA resistance are semidominant and do not alter cyclophilin A. One possibility is that CsA-sensitive strains lack some function that normally overlaps with calcineurin. If so, calcineurin, a known target of cyclophilin–CsA (10), would be essential in these strains. Accordingly, yeast strains sensitive to CsA might also be sensitive to FK506 or FK520, which in complex with FKBP12 inhibits calcineurin (10). As shown in Fig. 3, growth of strain IL993/5c was markedly inhibited by FK520 (or by FK506) at concentrations as low as 1 μg/ml. Thus, FK520 and FK506 are roughly 100-fold more potent than CsA in yeast, as in T cells. In addition, the CsA-sensitive strain JHY31-11D is also FK506/FK520 hypersensitive, and most spontaneous CsA-resistant mutants of IL993/5c and JHY31-11D are cross-resistant to FK506 and FK520 (Table 2).

To determine whether yeast FKBP12 mediates FK506/FK520 hypersensitivity, an FKBP12 disruption mutation (*fpr1::URA3*) was introduced into wild type and a cyclophilin A mutant strain to produce isogenic strains lacking neither, either, or both cyclophilin A and FKBP12 (Table 1). As shown in Fig. 3, the parental *CPR1 FPR1* strain is CsA sensitive and FK520 hypersensitive, the *cpr1 FPR1* mutant is CsA resistant and FK520 hypersensitive, the *CPR1 fpr1* mutant is CsA sensitive and FK520 resistant, and the *cpr1 fpr1* mutant is both CsA and FK520 resistant. Strains lacking FKBP12 are also FK506 resistant (data not shown). Thus, FKBP12–FK520 and FKBP12–FK506 complexes are toxic.

Calcineurin Is Essential in CsA/FK506-Sensitive Strain IL993/5c. That growth of strain IL993/5c is sensitive to both immunophilin–drug complexes suggests that calcineurin is essential in this strain. The calcineurin A catalytic subunits are encoded by two homologous redundant yeast genes, *CMP1* and *CMP2* (22, 23, 37). Disruption of either *CMP1* or *CMP2* was tolerated in the IL993/5c background (yielding strains TB70 and TB71), but disruption of both *CMP1* and *CMP2* was not. When the *CMP2* gene was disrupted in a haploid *cmp1::hisG* strain containing the *CMP1* gene on a plasmid, the plasmid-borne *CMP1* gene became essential for viability, as indicated by retention of the plasmid during nonselective growth conditions (Fig. 4). These findings confirm calcineurin is essential in strain IL993/5c.

Lithium Ions, Sodium Ions, or Neomycin Render Calcineurin Essential. Why is calcineurin essential in CsA/FK506-sensitive strains but not in other strains? Calcineurin enables cells to recover from pheromone cell cycle arrest (20, 23, 30). In addition, strains lacking both calcineurin catalytic subunits (*cmp1 cmp2*) have been noted to be sensitive to 100 mM LiCl,

Table 2. CsA-resistant mutants

Isolate	Parent	CsA	FK	D or r*	30°C†	37°C†	Bind CsA‡	Western	LiCl	LiCl+CsA	*CPR1*
Wild type	IL993/5c, TB24	S	S	—	+	+/−	+	+	S	S	WT
TOC6-1	TB24	R	S	D	+	+/−	+	+	S	S/R	WT
toc8-1	TB24	R	S	r	+	+/−	−	−	S	R	Y23UAG
TOC14-1§	TB24	R	R	semiD	+/−	+/−	+	+	R	S	WT
TOC9-1, -11-1	IL993/5c	R	R	semiD	++	−/+	+	+	S/R	S	WT
TOC1-1¶	IL993/5c, TB24	R	R	semiD	+	+	+	+	R	S	WT

Where indicated, media contained CsA at 100 μg/ml, FK506 or FK520 at 1 μg/ml (FK), 100 mM LiCl, or 100 mM LiCl plus CsA at 1 μg/ml. WT, wild type; S, sensitive; R, resistant.

*Dominant (D) or recessive (r). semiD, semi-dominant.

†Growth on YPD control medium (1% EtOH/Tween 20) at 30°C and 37°C is indicated as follows: ++, larger than wild type; +, wild-type growth; +/−, small colonies; −/+, poor growth.

‡See Fig. 2 for LH-20 assays.

§This class includes *TOC15-1* and *TOC16-1*.

¶This class includes 25 isolates (*TOC1-1* to *-5-1, -7-1, -10-1, -12-1, -13-1,* and *-17-1* to *-32-1*).

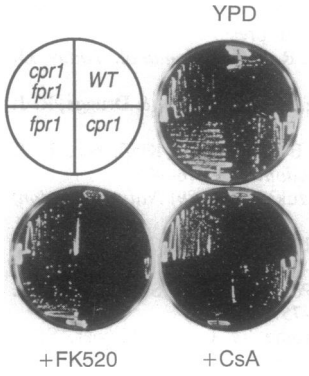

FIG. 3. Cyclophilin A–CsA and FKBP12–FK520 complexes are toxic. Isogenic *CPR1 FPR1* (WT = TB24), *cpr1 FPR1* (*cpr1* = TB40), *CPR1 fpr1* (*fpr1* = TB62), and *cpr1 fpr1* (TB63) strains were grown (72 hr, 30°C) on YPD with solvent alone (YPD), YPD with CsA at 10 μg/ml (+CsA), or YPD with FK520 at 1 μg/ml (+FK520).

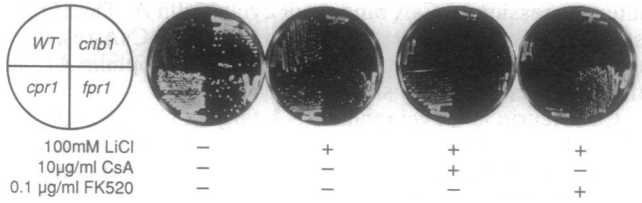

FIG. 5. Mutation or inhibition of calcineurin confers LiCl hypersensitivity. Isogenic derivatives of strain Y190 (WT) lacking calcineurin B (*cnb1* = SMY2), FKBP12 (*fpr1* = SMY1), or cyclophilin A (*cpr1* = CHY1) (Table 1) were grown (96 hr, 30°C) on YPD medium with solvent, 100 mM LiCl, 100 mM LiCl and CsA at 10 μg/ml, or 100 mM LiCl and FK520 at 0.1 μg/ml.

1.5 M NaCl, or 5 mM vanadate (24). We also find that strains lacking the calcineurin B regulatory subunit (*cnb1*::*ADE2*, *SMY2*) recover poorly from α-factor cell cycle arrest in a halo assay (data not shown) and are sensitive to growth inhibition by 100–200 mM LiCl (Fig. 4), 0.5–1 M NaCl, or 2–4 mM vanadate (data not shown), in comparison to isogenic strains expressing calcineurin. In contrast, calcineurin B mutants were not more sensitive to 1 M KCl or 1.5 M sorbitol. Thus, calcineurin mutants are sensitive to Li^+ or Na^+ cations and not to Cl^- ions or increased osmolarity.

Because mutation of calcineurin renders cells LiCl hypersensitive, we tested if inhibition by CsA or FK520 confers a similar effect. As shown in Fig. 5, the CsA/FK520-resistant strain Y190 grows on medium containing 100 mM LiCl but not on medium containing 100 mM LiCl and CsA (10 μg/ml) or containing 100 mM LiCl and FK520 (0.1 μg/ml). CsA or FK520 alone is not toxic (data not shown). The inhibitory effect of CsA requires cyclophilin A, because an isogenic *cpr1* mutant strain grows on LiCl plus CsA (Fig. 5). Inhibition by FK520 requires FKBP12, because an isogenic *fpr1* mutant strain grows normally on LiCl plus FK520 (Fig. 5). An isogenic calcineurin B mutant is sensitive to 100 mM LiCl alone. Thus, calcineurin inhibition by cyclophilin A–CsA or FKBP12–FK520 confers LiCl hypersensitivity.

Lithium ions are known to inhibit lipid phosphatases (38). The aminoglycoside neomycin, which binds phosphoinositols and alters lipid metabolism (39), is often employed with lithium to probe roles of phosphoinositol metabolites. Because calcineurin mutants are LiCl hypersensitive, we tested the effects of neomycin on isogenic wild-type and calcineurin B mutant strains (Y190 and SMY2, respectively). In contrast to wild type, growth of the calcineurin mutant is inhibited by neomycin (1 mM) but not by streptomycin. Calcineurin-deficient strains do not recover from α-factor cell cycle arrest; however, neither 100 mM LiCl nor 1 or 2.5 μM neomycin induced expression of a *FUS1-lacZ* pheromone-inducible reporter gene (plasmid pSB231 or pSB234 in strain JK9-3da) (33); thus, lithium and neomycin sensitivity of calcineurin mutant strains is not attributable to pheromone induction. In sum, calcineurin is required for recovery from α-factor arrest and for growth in the presence of LiCl, NaCl, vanadate, or neomycin.

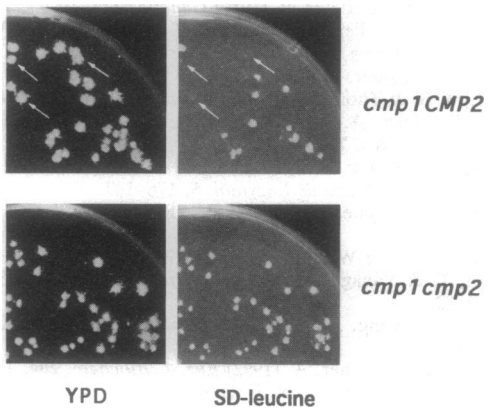

FIG. 4. Calcineurin is essential in CsA/FK506-sensitive yeast. Plasmid pRCAN bearing the *CMP1* and *LEU2* genes was introduced into *cmp1*::*hisG* strain TB88. A *cmp2*::*URA3* disruption was introduced. The resulting *cmp1 CMP2*/*CMP1* (*Upper*) and *cmp1 cmp2*/*CMP1* (*Lower*) strains were replica-plated as patches four times on YPD. Isolated colonies were replica-plated to YPD and SD-leucine medium. *cmp1 CMP2* strain TB88 survives plasmid loss to yield Leu^- segregants (arrows, ≈50% plasmid loss). In contrast, the plasmid-borne *CMP1* gene was essential in *cmp1 cmp2* strain TB121, as indicated by retention of the *LEU2* marker under nonselective conditions.

Because lithium ions render calcineurin essential, we tested the effects of 100 mM LiCl on growth of strain IL993/5c and the CsA-resistant mutants (Table 2). LiCl markedly inhibits growth of the parent strain. Two mutations that confer resistance to only CsA (*toc8-1*, *TOC6-1*) do not alter LiCl hypersensitivity. In contrast, of the CsA/FK506-resistant mutants, *TOC9-1* and *-11-1* are partially resistant to 100 mM LiCl, and the remainder are resistant to 100 mM LiCl (Table 2). Thus strain IL993/5c is CsA-sensitive and FK520- and LiCl-hypersensitive, and most mutations that confer CsA resistance confer cross-resistance to FK520 and LiCl. Strain IL993/5c may have an altered ability to efflux cations that renders calcineurin essential.

DISCUSSION

To determine the mechanism of CsA toxicity in yeast, we studied two CsA-sensitive strains. We confirmed that mutants lacking cyclophilin A are viable and CsA resistant, and introduction of the cyclophilin A gene restored CsA sensitivity (Fig. 1). Thus, a cyclophilin A–CsA complex is toxic.

To identify targets of the cyclophilin–CsA complex, we isolated spontaneous CsA-resistant mutants of strain IL993/5c. In a previous study, all CsA-resistant mutants isolated from this strain were reported to lack cyclophilin or express mutants with reduced CsA binding (13). We isolated additional CsA-resistant mutants and find that, in contrast, the majority (31 out of 32) are not cyclophilin A mutants. Only one mutation (*toc8-1*) conferred recessive CsA resistance that was complemented by the cloned cyclophilin A gene. This strain lacks CsA-binding activity (Fig. 2*B*), bears a nonsense mutation in the cyclophilin A gene, and lacks cyclophilin A by Western analysis (Table 2).

The discrepancy between our results and those of Tropschug *et al.* (13) can be explained by the fact that, in their studies, cell extracts for CsA-binding assays were prepared from CsA-resistant mutants grown in the presence of unlabeled CsA at 50 μg/ml, whereas the CsA-sensitive parental strain was grown without CsA (13). We find that unlabeled CsA in culture medium effectively competes with [^{3}H]CsA for binding and precludes detection of cyclophilin in extracts from CsA-resistant mutants that express cyclophilin A (Fig. 2*C*). We conclude that most of these mutations confer resistance to the toxic cyclophilin A–CsA complex but do not

alter expression or CsA binding of cyclophilin A. The earlier studies of Tropschug *et al.* (13) also described CsA-resistant mutants of *Neurospora crassa* that lacked cyclophilin A, and these results are likely still valid.

Calcineurin is a cyclophilin–CsA target in T cells (10, 16, 17) and yeast (20). Although calcineurin is not essential in several yeast strains (22–24), the cyclophilin A–CsA complex could be toxic to strains in which calcineurin is essential because other overlapping functions are missing. In fact, we find that two CsA-sensitive yeast strains are hypersensitive to a different calcineurin inhibitor, FK506 (or FK520) (Fig. 3). Yeast FKBP12 is required for FK506 and FK520 toxicity (Fig. 3). By gene disruption, we confirmed that calcineurin A is essential in the CsA/FK506-sensitive strain IL993/5c (Fig. 4). Thus, the cyclophilin A–CsA and FKBP12–FK506 complexes poison susceptible yeast by inhibiting calcineurin.

Two of our mutations confer resistance to CsA but not to FK506. One bears a cyclophilin A mutation (*toc8-1*). The other, *TOC6-1*, confers dominant CsA resistance and is not a cyclophilin A mutant. *TOC6-1* could be a calcineurin mutation that prevents inhibition by cyclophilin A–CsA but not by FKBP12–FK506. Alternatively, *TOC6-1* might increase expression of another cyclophilin that sequesters or compartmentalizes CsA. The semidominant CsA/FK520-resistant mutations either render calcineurin resistant to both drugs or render calcineurin dispensable.

Why is calcineurin essential in some yeast strains? Calcineurin is required for recovery from pheromone arrest and might be essential if the pheromone response pathway were constitutively activated. This is not the case. First, the basal expression level of a *FUS1-lacZ* pheromone response reporter (plasmid pSB231) was not increased in strain TB24 compared to a CsA-resistant control strain JK9-3da (data not shown). Second, diploids of strain IL993/5c, in which the pheromone response pathway is inactive, remain CsA and FK506 sensitive.

In addition, strains lacking both calcineurin catalytic subunits (*cmp1 cmp2*) have been noted to be sensitive to 1.5 M NaCl, 100 mM LiCl, and 5 mM vanadate (24). In strain JK9-3d, we find calcineurin B (*cnb1*) or calcineurin A (*cmp1 cmp2*) mutants recover poorly from α-factor arrest and are sensitive to 200 mM LiCl, 1.0 M NaCl, and 3 mM vanadate. We also found that inhibition of calcineurin by either FKBP12–FK520 or cyclophilin A–CsA confers LiCl hypersensitivity (Fig. 5). Thus, FKBP12 and cyclophilin A are the relevant immunophilins that mediate the ability of FK506 and CsA to prevent recovery from pheromone cell cycle arrest (20), to block growth of strain IL993/5c (Fig. 3), and to confer LiCl hypersensitivity (Fig. 5).

That calcineurin-deficient strains are sensitive to NaCl or LiCl suggests that calcineurin regulates cation transport in yeast. Several Na^+ and Li^+ ion selective cation pumps have been identified (ENA1–4), and one or more might be calcineurin regulated (40). Calcineurin is also known to regulate a plant K^+ channel (19) and the renal Na^+,K^+-ATPase (41) and thus might in general regulate cation transport. Because nephrotoxicity is a side effect of both CsA and FK506, these drugs could interfere with calcineurin regulation of renal ion pumps required for electrolyte balance.

While this manuscript was in preparation, a related report (42) was published.

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