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Calcineurin, the Ca^{2+}/Calmodulin-Dependent Protein Phosphatase, Is Essential in Yeast Mutants with Cell Integrity Defects and in Mutants That Lack a Functional Vacuolar H^+-ATPase

PHILIP GARRETT-ENGELE, BRITA MOILANEN, AND MARTHA S. CYERT*

Department of Biological Sciences, Stanford University, Stanford, California 94305-5020

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Calcineurin is a conserved Ca^{2+}/calmodulin-dependent protein phosphatase that plays a critical role in Ca^{2+}-mediated signaling in many cells. Yeast cells lacking functional calcineurin (*cna1 cna2* or *cnb1* mutants) display growth defects under specific environmental conditions, for example, in the presence of high concentrations of Na^+, Li^+, Mn^{2+}, or OH^- but are indistinguishable from wild-type cells under standard culture conditions. To characterize regulatory pathways that may overlap with calcineurin, we performed a synthetic lethal screen to identify mutants that require calcineurin on standard growth media. The characterization of one such mutant, *cnd1-8*, is presented. The *CND1* gene was cloned, and sequence analysis predicts that it encodes a novel protein 1,876 amino acids in length with multiple membrane-spanning domains. *CND1* is identical to the gene identified previously as *FKS1*, *ETG1*, and *CWH53*. *cnd1* mutants are sensitive to FK506 and cyclosporin A and exhibit slow growth that is improved by the addition of osmotic stabilizing agents. This osmotic agent-remedial growth defect and microscopic evidence of spontaneous cell lysis in *cnd1* cultures suggest that cell integrity is compromised in these mutants. Mutations in the genes for yeast protein kinase C (*pkc1*) and a MAP kinase (*mpk1*/*slt2*) disrupt a Ca^{2+}-dependent signaling pathway required to maintain a normal cell wall and cell integrity. We show that *pkc1* and *mpk1*/*slt2* growth defects are more severe in the absence of calcineurin function and less severe in the presence of a constitutively active form of calcineurin. These observations suggest that calcineurin and protein kinase C perform independent but physiologically related functions in yeast cells. We show that several mutants that lack a functional vacuolar H^+-ATPase (*vma*) require calcineurin for vegetative growth. We discuss possible roles for calcineurin in regulating intracellular ion homeostasis and in maintaining cell integrity.

The role of Ca^{2+} as a second messenger has been well established (9). A change in the intracellular concentration of this ion acts as a signal that can effect a variety of physiological changes in different cell types. One of the mechanisms by which Ca^{2+} acts is by binding to and activating calmodulin, enabling this intracellular calcium receptor to activate a number of target enzymes. Among these target enzymes are kinases and phosphatases that alter cell physiology by modulating the phosphorylation state of many proteins.

Several components of Ca^{2+}-mediated signaling pathways in higher eukaryotes have been identified in *Saccharomyces cerevisiae*, in which they also function in a regulatory capacity. Protein kinase C, for example, encoded by the *PKC1* gene, regulates a mitogen-activated protein (MAP) kinase-containing signal transduction pathway that is required to maintain the integrity of the yeast cell (reviewed in reference 19). Yeast mutants lacking phospholipase C, encoded by the *PLC1* gene, display pleiotropic phenotypes, including osmotic sensitivity, inability to grow on some carbon sources, and increased chromosome loss (19a, 51, 63). Yeast cells also possess calmodulin, and this protein is essential for growth (15). However, the Ca^{2+}-dependent signaling properties of calmodulin appear to be dispensable for vegetative growth, as mutants that contain a calmodulin defective for all Ca^{2+} binding are viable (23). We have focused on trying to understand the role of calmodulin-mediated Ca^{2+} signaling in yeast cells by establishing the functions of one particular calmodulin-regulated enzyme, the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (or PP2B).

In many cell types, including mammalian T cells and plant guard cells, this enzyme plays a critical role in mediating Ca^{2+}-dependent signaling (41, 43). Although some proteins have been proposed as candidate substrates for calcineurin in vivo, in no case has a calcineurin-containing signal transduction pathway been defined. In part, the functions of calcineurin in different cell types has been assessed by examining the effects of the drugs FK506 and cyclosporin A. In the presence of FK506 and cyclosporin A, FK506-binding proteins and cyclophilins (the intracellular receptors for these drugs) bind to and inactivate calcineurin; thus, these compounds are specific inhibitors of calcineurin both in vivo and in vitro (reviewed in reference 41).

In yeast cells, it is possible to use genetic approaches to examine calcineurin function. Calcineurin has been purified from yeast extracts (14), and the genes encoding its components have been identified. Calcineurin is a heterodimer; two genes, *CNA1*/*CMP1* and *CNA2*/*CMP2*, encode functionally redundant catalytic subunits of the enzyme, and the *CNB1* gene encodes a necessary regulatory subunit (13, 14, 33, 42). Cells lacking functional calcineurin (i.e., *cna1 cna2* mutants and *cnb1* mutants) are viable, indicating that this phosphatase is not required for vegetative growth under most conditions. However, calcineurin-deficient cells do display specific growth defects. Calcineurin mutants fail to grow when high concentrations of any of a number of different ions (Na^+, Li^+, Mn^{2+}, and OH^-) are included in the growth medium (6, 12, 45, 46). Also, calcineurin mutants die during continuous exposure to pheromone, as do wild-type cells that are deprived of Ca^{2+} (12,

* Corresponding author. Mailing address: Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020. Phone: (415) 723-9970. Fax: (415) 725-8309.

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype[a]	Source or reference
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1*	59
YPH500	*MAT*α *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1*	59
MCY300	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3*	13
MCY3-1D	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 cnb1Δ1::LEU2*	14
PGY1	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3 ade3Δ*	This work
PGY2	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3 ade3Δ* + pCNA2-29	This work
PGY2-8	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3 ade3Δ cnd1-8* + pCNA2-29	This work
PGY4	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 ade3Δ cnd1-8*	This work
PGY5	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 ade3Δ cnd1Δ1::URA3 hisG*	This work
PGY6	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 ade3Δ CND1::TRP1*	This work
EG123	*MAT*α *leu2-3,112 ura3-52 trp1-1 his4 can1*^{r}	39
FL102	*MAT***a** *ura3-52 trp1-1 his4 leu2-3,112 can1*^{R} *pkc1Δ::LEU2* + pGAL1::PKC1	39
DL454	*MAT***a** *ura3-52 trp1-1 his4 leu2-3,112 can1*^{R} *mpk1Δ::TRP1*	35
PGY12	*MAT***a**/α EG123 × YPH499	This work
PGY13	*MAT***a**/α PGY12 *cnb1Δ1::URA3/CNB1 PKC1/pkc1 Δ::LEU2*	This work
PGY14	*MAT***a**/α PGY12 *cnb1 Δ1::LEU2/CNB1 MPK1/mpk1Δ::TRP1*	This work
PGY15	*MAT***a**/α PGY12 *cnd1-8/CND1 PKC1/pkc1Δ::LEU2*	This work
PGY16	*MAT***a**/α PGY12 *cnd1Δ1::URA3/CND1 MPK1/mpk1Δ::TRP1*	This work
RH104	*MAT***a** *ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 vma1::TRP1*	Y. Anraku laboratory
YOV503	*MAT*α *ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 vma2::TRP1*	Y. Anraku laboratory
DV3T-1B	*MAT*α *ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 vma3::TRP1*	Y. Anraku laboratory
YRH11	*MAT*α *ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 vma11::TRP1*	Y. Anraku laboratory
RH205	*MAT*α *ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 vma12::TRP1*	Y. Anraku laboratory

[a] In the *cnd1Δ1::URA3 hisG* allele, *URA3* is flanked by the *Salmonella hisG* sequence (see text).

29). These phenotypes suggest that calcineurin participates in regulating the intracellular concentration of several ions.

Calcineurin mutants may display no dramatic phenotypes under optimal growth conditions because other regulatory pathways in the cell exist that perform similar functions. To address this possibility, we have identified mutants that depend on calcineurin for vegetative growth under standard laboratory growth conditions. The characterization of some of those mutants is presented here. First, we describe the isolation of *CND1*, a novel gene encoding a putative integral membrane protein that is essential in calcineurin mutants. *cnd1* mutants are sensitive to FK506 and cyclosporin A, have an increased tendency to lyse, and exhibit poor growth that is improved by the addition of osmotic stabilizing agents. These observations suggest that the *CND1* gene product is required to maintain cell integrity. Spontaneous lysis and osmotic agent-remedial growth defects are characteristic of mutations that disrupt the *PKC1*-regulated MAP kinase signal transduction pathway. Mutants with mutations in this pathway, such as *pkc1*, *bck1*/*slk1*, and *mpk1*/*slt2*, display alterations in cell wall structure and composition and defects in polarized growth (10, 30, 35–37, 44, 49, 58, 61). We show that, like *cnd1* mutants, *pkc1* and *mpk1*/*slt2* mutants depend on calcineurin for vegetative growth. Furthermore, a constitutively active form of calcineurin partially suppresses the spontaneous lysis of *pkc1* and *mpk1*/*slt2* mutants. These findings suggest that calcineurin defines a second Ca^{2+}-dependent pathway which acts together with the *PKC1* pathway to maintain the integrity of the cell. Finally, we report that mutations in several *VMA* genes, known to encode necessary subunits of the vacuolar H^{+}-ATPase (reviewed in reference 2), are lethal in combination with calcineurin mutations. We discuss the possible implications of these genetic interactions for understanding the physiological functions of calcineurin.

MATERIALS AND METHODS

Yeast strains, media, and general methods. All yeast strains used in this study are listed in Table 1. Standard procedures were used for genetic manipulation of yeast strains (57), and the media and culture conditions were those recommended (57) except that nutritional supplements in synthetic media were added at twice the specified level. All galactose-containing media also contained 0.2% sucrose. For sectoring assays, synthetic medium containing a reduced concentration of adenine was used (6 mg/liter [26]). Medium containing 5-fluoroorotic acid (5FOA) was made as described before (5). When noted, salts or sorbitol was added to YP medium at the specified level. FK506 was obtained from Fugisawa, Inc. A stock solution of FK506 (5 mg/ml in 90% ethanol–10% Tween 20) was added to liquid or solid media to the final concentration specified. Cyclosporin A was obtained from Sandoz. A stock solution of cyclosporin A (30 mg/ml in 90% ethanol–10% Tween 20) was added to liquid or solid media to the final concentration specified. DNA was introduced into yeast cells by lithium acetate transformation (3) and into bacteria by electroporation (3). Yeast genomic and plasmid DNAs were isolated as described before (3). The location of *CND1* on the physical map of the yeast genome was determined by hybridization of a radiolabeled 2.2-kb *Hin*dIII-*Cla*I fragment to a set of λ phages carrying ordered segments of *S. cerevisiae* chromosomal DNA (ATCC 77284 [40]). Hybridization analyses of DNA transferred to Hybond N+ membranes (Amersham) were performed by standard procedures (3). Radioactive probes were synthesized by using random primers (Pharmacia) and [α-^{32}P]dCTP (NEN Dupont).

Plasmids. Plasmid pCNA2-29 (used in the sectoring screen; see below) was constructed by inserting a 2.1-kb *Bam*HI-*Xho*I fragment containing the *CNA2* open reading frame (ORF) from pCNA2-R (27) into pMW29 (62). The construction of pCNA2-R will be described in a future manuscript. It contains the entire *CNA2* ORF, modified by site-directed mutagenesis to introduce a *Bam*HI site just upstream of the *CNA2* initiator ATG, inserted into pBluescript (Stratagene). Low-copy-number plasmids expressing *CNA1* (pCNA1-314) and *CNA2* (pCNA2-314) were constructed by using pRS314 (59). A high-copy-number plasmid expressing *CNA2* was constructed by using YEp352 (25). A similar plasmid containing *CNA2*Δ, a truncated version of *CNA2*, was created by changing amino acid 505 (a leucine) to a stop codon by site-directed mutagenesis (3). *CNA2*Δ lacks the calmodulin-binding domain and the putative C-terminal inhibitory domain of *CNA2* (13). pCND1-315, a low-copy (cen) plasmid containing the *CND1* ORF and the *LEU2* gene, was constructed by inserting a 9-kb genomic *Spe*I-*Pst*I fragment into pRS315. pCND1-YEp, a high-copy plasmid containing the *CND1* ORF and the *LEU2* gene, was constructed by inserting the same fragment into YEp351 (25). A plasmid containing a dominant activated form of *BCK1*, pRS314[BCK1-20], has been described (36).

Isolation of mutants that require calcineurin for growth. Strain PGY2, used to screen for calcineurin-dependent mutants, was constructed as follows. First, the

ADE3 gene of MCY300 was disrupted to generate strain PGY1. This was done by using pMW22 (62), which introduces an *ade3* deletion by a two-step pop-in/pop-out replacement procedure (56). This strain was then transformed with pCNA2-29 to generate PGY2. Mutagenesis with ethyl methyl sulfonate was performed (57). The mutagenized culture was plated (approx. 200 colonies per plate), and 74,000 colonies were screened visually to identify those that failed to sector. Colonies without sectors were retested for nonsectoring colony morphology and the inability to grow on dextrose-containing media and on galactose media containing 5FOA. A total of six strains that both failed to sector and failed to grow on dextrose-containing media and 5FOA-galactose media were identified.

Genetic analysis of calcineurin-dependent mutants. All six mutants were mated to YPH500 and/or MCY 3-1D, and the resulting diploids were tested for growth on SCGal solid medium containing FK506 (5 μg/ml). Growth on this medium indicated that the mutation was recessive. These diploids were sporulated, and the haploid segregants of individual tetrads were tested for FK506 sensitivity. A 2:2 segregation of FK506 sensitivity indicated that it resulted from a single mutation. The *cnd1-8* mutant was backcrossed to a nonmutagenized strain for two generations to generate the FK506-sensitive strain PGY4. Complementation tests were performed as follows. Calcineurin-dependent strains were mated to each other (strains of the α mating type were generated from the crosses described above), and the diploids were tested for FK506 sensitivity, with lack of sensitivity indicating complementation.

Isolation and characterization of clones complementing calcineurin synthetic lethal mutation. A genomic library in p366, a low-copy cen plasmid containing *LEU2* (ATCC 77162 [60]), was transformed into strain PGY2-8 (which cannot grow on dextrose), transformants were selected on plates containing dextrose and lacking leucine and purified, and Ura$^-$ derivatives were isolated on 5FOA medium (to cure pCNA2-29). To eliminate transformants that contained plasmids encoding *CNA1* or *CNA2*, all transformants were screened for FK506 sensitivity. Only FK506-insensitive transformants were selected for further analysis. Plasmid DNA was isolated from these transformants and analyzed by restriction analysis. Plasmids containing an insert were retransformed into PGY2-8 to test for their ability to support growth of this strain on medium containing dextrose and lacking leucine. Three plasmids containing the identical insert were identified by this analysis. Determination of the minimal complementing region was assessed by the ability of low-copy (cen) plasmids containing DNA fragments from the *CND1* region to support growth of PGY2-8 on dextrose medium.

Construction of *cnd1* alleles. The *cnd1Δ1*::*URA3 hisG* allele was assembled in pBluescript from a 1.2-kb *Pst*I-*Eco*RI fragment (from *CND1*), a 3.8-kb *Eco*RI-*Bam*HI fragment containing *URA3* flanked by the *Salmonella hisG* sequence (derived from pNKY51 [1] cloned into pBluescript), and a 500-bp *Bgl*II-*Spe*I fragment (from *CND1*). The *cnd1Δ1*::*URA3 hisG* allele was then excised as a 5.5-kb *Sph*I-*Spe*I linear fragment and introduced into yeast cells by transformation. The genotype of *cnd1Δ1*::*URA3 hisG* strains was confirmed by Southern blotting. *TRP1* was inserted upstream but tightly linked to a functional *CND1* gene as follows. The 2.7-kb *Pst*I-*Eco*RV fragment that includes the *CND1* ATG and upstream region was subcloned into pBluescript and digested with *Stu*I and *Eco*RI to insert an 830-bp *Stu*I-*Eco*RI fragment encoding *TRP1* (from pJJ281 [31]). This region was characterized by DNA sequence analysis and does not contain an ORF. A 2.5-kb fragment containing upstream *CND1* flanking sequence and *TRP1* was excised from this plasmid by digesting with *Bam*HI and *Cla*I and transformed into YPH500 to create strain PGY6. The *TRP1* insertion was confirmed by Southern blotting.

DNA sequencing. Restriction fragments derived from library plasmids were subcloned into Bluescript vectors (Stratagene), and nested deletions were created (24). Double-stranded DNA templates were prepared according to the manufacturer's instructions (Promega Wizard prep). The nucleotide sequences of both strands were determined by using the deletions and, where necessary, custom-synthesized oligonucleotide primers. The Sequenase version of phage T7 DNA polymerase and nonradioactive nucleotides were obtained from U.S. Biochemicals, and sequencing reactions were conducted according to the manufacturer's instructions. [α-^{35}S]dATP was purchased from Amersham.

FK506 and cyclosporin A sensitivity. Sensitivity to FK506 and cyclosporin A was assessed in two ways.

(i) Liquid culture assay. Each strain tested was grown to mid-log phase in either YPDex or YPGal and diluted in duplicate to a final optical density (OD) of 0.05 with the appropriate final concentration of either FK506 or cyclosporin A. All cultures were then incubated at 30°C in a shaking water bath until the culture without drug reached an OD at 600 nm (OD_{600}) of 0.9 to 1.1. At this time, all cultures were harvested, and their OD_{600} was measured. The relative sensitivities of each strain to each drug were quantitated by comparing the 50% inhibitory concentrations (IC_{50}s) (as determined from the data graphed in Fig. 5).

(ii) Solid-phase culture assay (halo assay). Plates (either synthetic or YP medium) were inoculated with 2×10^6 cells in top agar (0.75% final concentration). A sterile disk saturated with 5 μg of FK506 or 50 μg of cyclosporin A was immediately placed on the plate, and they were incubated at the appropriate temperature (25 or 30°C) for 2 to 4 days.

Microscopic analysis of cultures. Yeast cultures were observed by phase-contrast microscopy at a final magnification of 400×. Methylene blue staining to determine the number of viable cells was performed by adding equal volumes of culture and 0.02% methylene blue–4% sodium citrate solution (29). The numbers of methylene blue-negative and -positive cells were determined immediately by bright-field microscopy. More than 300 cells of each strain were counted.

Nucleotide sequence accession number. The *CND1* sequence was submitted to GenBank and appears there under accession number L35923.

RESULTS

Identification and characterization of mutants that require calcineurin for vegetative growth. Under standard laboratory conditions, the growth and morphology of calcineurin mutants is indistinguishable from that of wild-type cells. Only under specific environmental conditions, for example, in the presence of pheromone, high pH, or high concentrations of Na^+ or some other cations, do calcineurin mutants display growth and viability defects relative to wild-type cells (6, 12, 45, 47).

To characterize other regulatory pathways in the cell that may compensate for the lack of calcineurin activity, we have identified mutants that depend on functional calcineurin for vegetative growth on standard growth media. We performed a screen for such synthetic lethal mutations that makes use of a simple colony color or sectoring assay. A haploid yeast strain (PGY1) that contains deletions of both calcineurin regulatory subunits, *cna1Δ1*::*hisG* and *cna2Δ1*::*HIS3*, *ade2*, and *ade3* was constructed. This strain is adenine requiring, grows on medium containing both dextrose and galactose, and produces white colonies. A centromeric plasmid containing *CNA2* driven by the regulated *GAL1*, *10* promoter, *URA3*, and *ADE3* was transformed into this strain to create PGY2. On galactose-containing medium, cells containing this plasmid express functional calcineurin and produce colonies that are red because of the presence of the *ade2* mutation. However, under nonselective conditions, this plasmid is lost at a relatively high frequency, giving rise to red colonies with many white sectors. The white sectors are produced by cells that have lost the plasmid and therefore lack calcineurin.

To isolate mutants that require calcineurin for growth, PGY2 was mutagenized, and potential calcineurin-dependent mutants were identified as solid red colonies that failed to give rise to white sectors. These mutants were further examined to identify those that failed to grow on dextrose-containing medium (when the expression of calcineurin from the plasmid is repressed) and on galactose-containing 5FOA medium (which selects for loss of the *URA3*-bearing calcineurin expression plasmid). By this screening procedure, we identified five strains whose growth was calcineurin dependent (isolates 6, 8, 112, 116, and 126). We also identified one strain whose growth was significantly decreased in the absence of calcineurin (isolate 106). Each calcineurin-dependent strain was shown, by genetic analysis, to contain a single recessive mutation (see Materials and Methods). Each strain, when transformed with a second centromeric plasmid containing either *CNA1* or *CNA2*, expressed from their endogenous promoters, became able to sector and grow on dextrose-containing medium (data not shown). Thus, each strain contained a single recessive mutation that rendered its growth dependent on either of two possible calcineurin isoforms, Cna1p-containing calcineurin or Cna2p-containing calcineurin. Furthermore, although the strains were originally identified under conditions of calcineurin overexpression (from the *GAL1*,*10* promoter), low levels of calcineurin are sufficient to support their growth.

Calcineurin-dependent mutants are hypersensitive to FK506 and cyclosporin A. The immunosuppressant drugs FK506 and cyclosporin A have been shown to specifically inhibit calcineurin in many different cell types, including yeast cells (reviewed in references 34 and 41). Wild-type yeast cells exposed to relatively low concentrations of FK506 and cyclosporin A grow normally on standard growth media but acquire

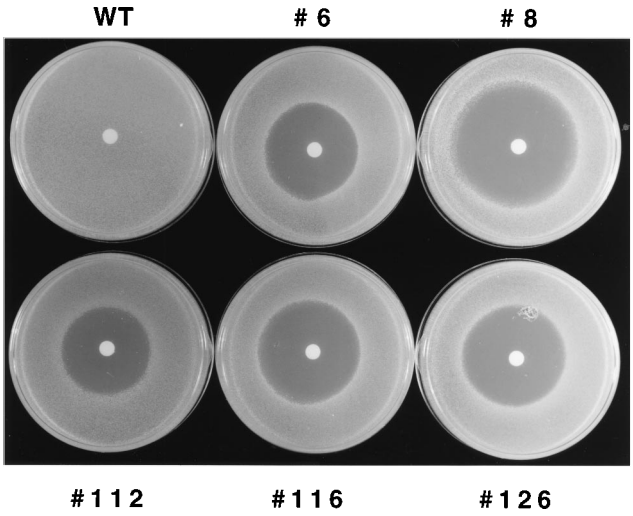

FIG. 1. Calcineurin-dependent mutants are sensitive to FK506. PGY2 (labeled WT for wild type) and mutagenized derivatives of PGY2 identified in the synthetic lethal mutation screen (isolates 6, 8, 112, 116, and 126) were plated on SCGal medium with a sterile disk saturated with FK506 and incubated at room temperature for 4 days (see Materials and Methods).

all the phenotypes of calcineurin mutants (6, 12, 20, 46). However, strains such as those described above that require calcineurin for vegetative growth should fail to grow when exposed to the same concentrations of these drugs (6, 18, 50). We found that the five calcineurin-dependent mutants identified in our screen (isolates 6, 8, 112, 116, and 126) did exhibit various degrees of hypersensitivity to FK506 (see Fig. 1) and cyclosporin A (data not shown). Isolate 8 was the most sensitive to both drugs. Isolate 106, which had a decreased growth rate in the absence of calcineurin, also showed slow growth in the presence of FK506 and cyclosporin A (data not shown).

Screen for calcineurin-dependent mutants identifies three complementation groups. Further genetic analysis revealed that the five calcineurin-dependent mutations identified two different complementation groups (see Materials and Methods). Four mutants fell into a single complementation group (isolates 6, 8, 116, and 126), and the other mutant (isolate 112) identified an additional complementation group. Isolate 106, which showed slow growth in the absence of calcineurin, defined a third complementation group. We have designated the gene identified by the mutants in the first complementation group as *CND1* (for calcineurin dependent) and have used the isolate number to name the *cnd1* allele contained within each strain (i.e., *cnd1-6*, *cnd1-8*, *cnd1-116*, and *cnd1-126*). We describe here a characterization of the wild-type *CND1* gene and of strains containing two different *cnd1* alleles.

Cloning the *CND1* gene by complementation. The *CND1* gene was cloned by complementing the inability of the *cnd1-8* strain to grow on dextrose (see Materials and Methods for details). Three plasmids that complemented the growth defects of the *cnd1-8* strain and did not encode *CNA1* or *CNA2* were identified. Restriction mapping analysis revealed that these plasmids contained identical inserts of 11 kb of yeast genomic DNA (Fig. 2). These plasmids restored the ability of all *cnd1* strains identified to sector and grow on dextrose and failed to restore these properties to strains in the other complementation groups.

Characterization of the *CND1* gene product by DNA sequencing. The *cnd1-8*-complementing region was localized to a 9-kb *Pst*I-*Spe*I fragment (Fig. 2), and the complete DNA sequence of 8 kb of this fragment was determined (see Materials and Methods). This fragment contains a single long ORF that is predicted to encode a protein 1,876 amino acids in length and with a molecular weight (MW) of 215,110 (Fig. 3). Comparison of this ORF with the database initially indicated that *CND1* encodes a novel protein with no significant homology to previously characterized proteins. However, while the manu-

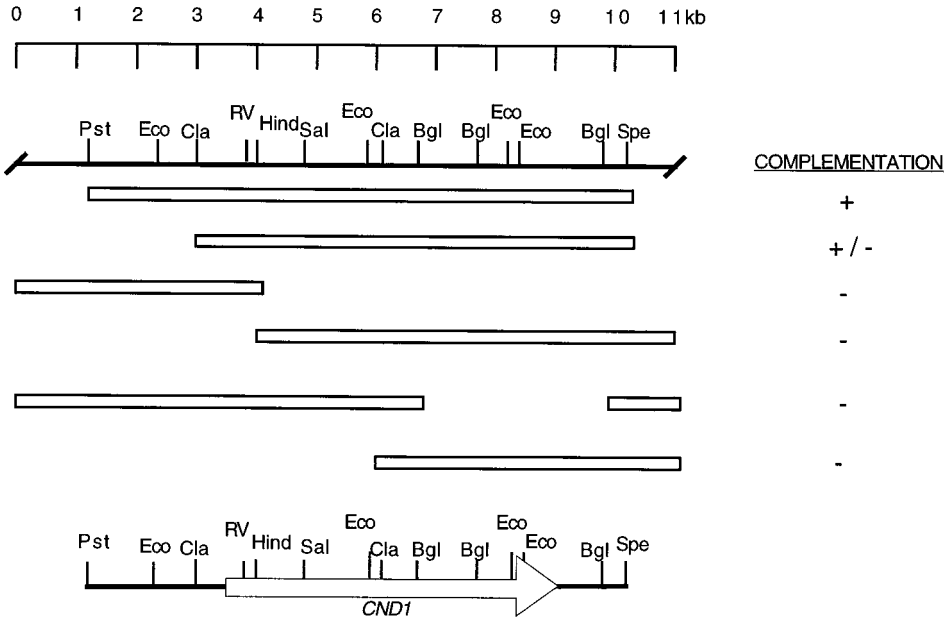

FIG. 2. Restriction map and complementation analysis of the *CND1* region of chromosome XII. Complementation was assayed by testing the ability of fragments inserted into pRS315 to support the growth of strain PGY2-8 on SCD medium. The large open arrow denotes the position and direction of the *CND1* ORF. Pst, *Pst*I; Eco, *Eco*RI; Cla, *Cla*I; Hind, *Hin*dIII; Sal, *Sal*I; Bgl, *Bgl*II; Spe, *Spe*I; RV, *Eco*RV.

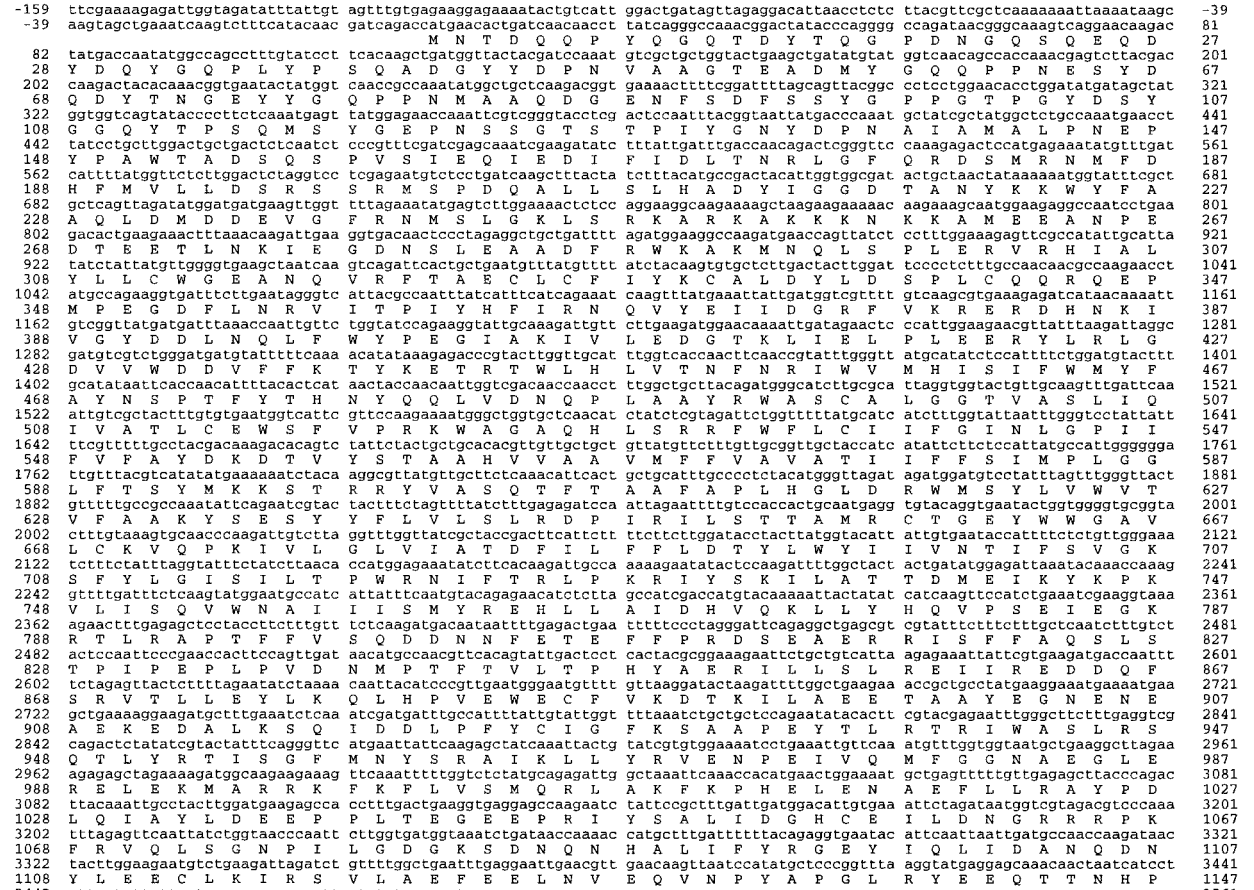

FIG. 3. Sequence of the *CND1* region. The nucleotide sequence is depicted above the predicted *CND1* protein sequence. Numbers indicate the positions of nucleic acid and amino acid residues.

script for this article was under review, we determined that *CND1* is identical to the gene characterized previously as *FKS1*, *ETG1*, and *CWH53* (16, 18, 53) (see Discussion). Hydropathy analysis of the predicted gene product encoded by *CND1* revealed that, while the N terminus of the protein is quite hydrophilic, the rest of the protein contains highly hydrophobic domains that are likely to span a membrane (Fig. 4). Thus, the protein is predicted to be an integral membrane protein that crosses an intracellular membrane at least 10 times. The 10 putative transmembrane domains are clustered in two groups, with a large intervening hydrophilic region, predicted to form an intracellular or luminal domain.

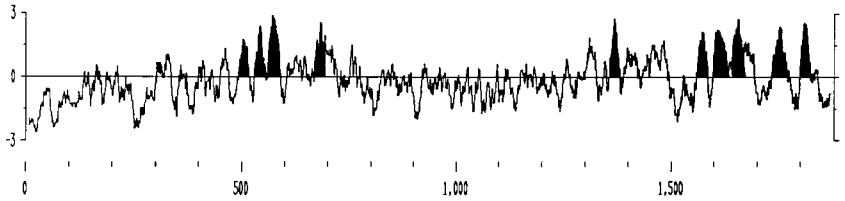

FIG. 4. Hydropathy analysis of the predicted *CND1* protein product. Hydrophobicity, on a scale from −3 to 3, was determined with the Kyte and Doolittle algorithm and a window size of 15 residues. The regions most likely to encode membrane-spanning domains are depicted as solid black. Amino acid residue numbers are indicated on the lower line.

Genomic location of *CND1*. Hybridization of a probe from the *CND1*-encoding plasmid to a collection of ordered lambda phage representing the yeast genome (40) revealed that the *CND1* locus resides on chromosome XII, between *CDC42* and *ILV5*. The *CND1* locus is closely linked to the *cnd1-8* mutation, confirming that the plasmids identified by complementation encode the same gene identified by the synthetic lethal mutations. Strain PGY6, which contains the *TRP1* gene integrated upstream of the *CND1* ORF (see Materials and Methods), was mated to a *cnd1-8* strain (PGY2-8) and sporulated. Analysis of the resulting haploid meiotic segregants revealed that the *TRP1* gene and the *cnd1-8* mutation were closely linked (≤2.4 centimorgans, 83 spores analyzed).

***CND1* is not essential.** We constructed a *cnd1* null mutation that deleted the *CND1* ORF (see Materials and Methods). The *cnd1Δ1::URA3 hisG* mutation was introduced into wild-type diploid yeast strains. Several heterozygous diploid transformants were sporulated, and haploid segregants from individual tetrads were analyzed. All haploid segregants from this cross, i.e., both *CND1* and *cnd1Δ1::URA3 hisG*, were viable on YPD at 20, 30, and 37°C, demonstrating that *CND1* is not an essential gene under these growth conditions (data not shown).

***cnd1* mutants exhibit growth defects and sensitivity to FK506 and cyclosporin A.** *cnd1Δ1::URA3 hisG* and *cnd1-8* strains display two characteristic phenotypes: they are sensitive to FK506 and cyclosporin A (Fig. 5), and they are slower

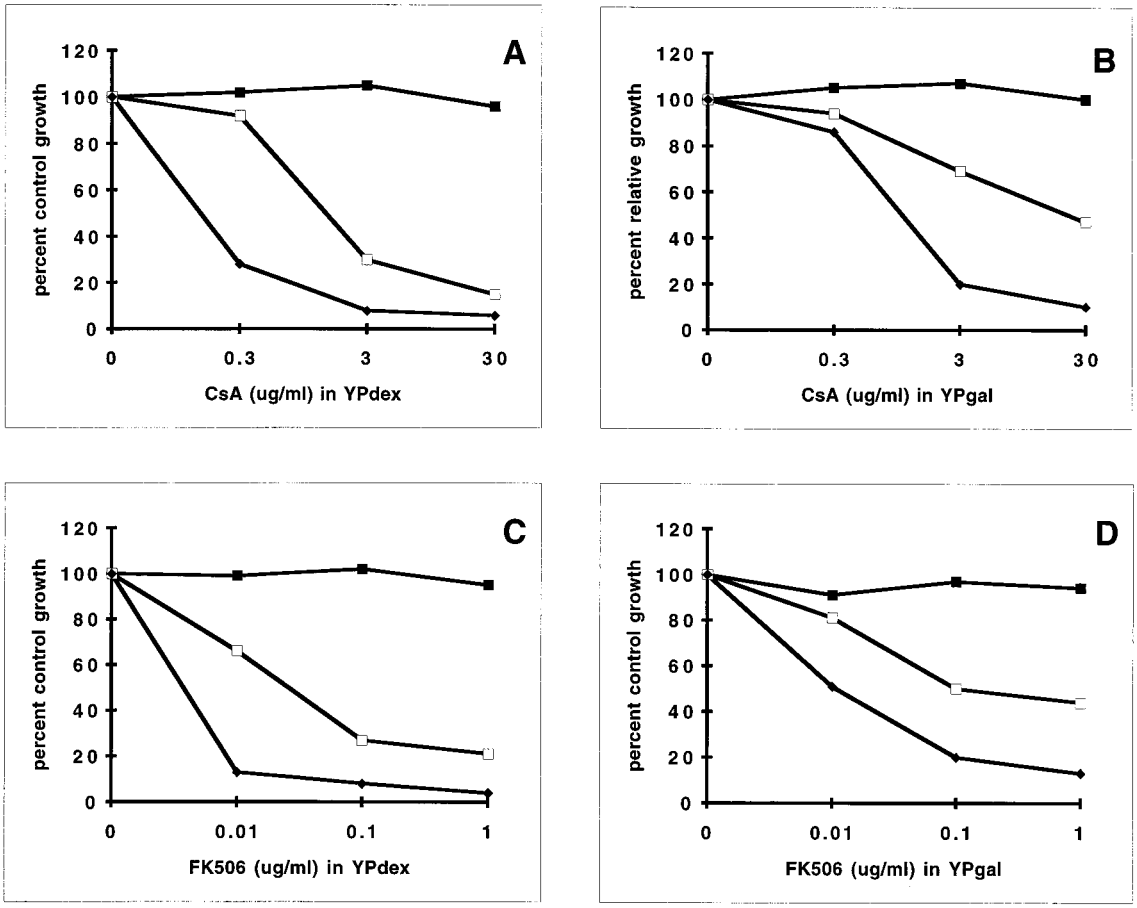

FIG. 5. Sensitivity of wild-type (YPH499, solid squares), *cnd1Δ1::URA3 hisG* (PGY5, open squares), and *cnd1-8* (PGY4, solid diamonds) strains in liquid culture. See Materials and Methods for experimental details. Growth inhibition is plotted as a function of drug concentration, plotted on a log scale. (A and C) Cultures grown in YPDex with cyclosporin A (CsA) and FK506, respectively; (B and D) cultures grown in YPGal with cyclosporin A and FK506, respectively.

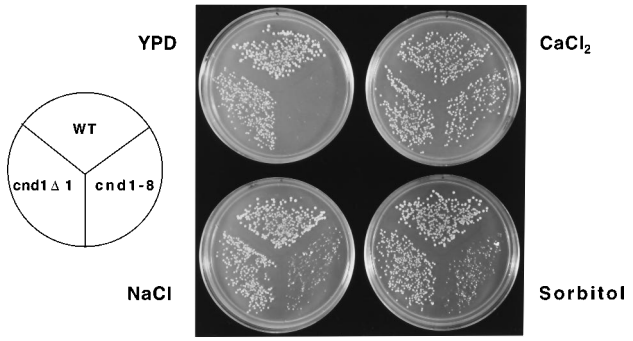

FIG. 6. Growth of wild-type (YPH499), *cnd1-8* (PGY4), and *cnd1Δ1*::*URA3 hisG* (PGY5) strains on different media. Plates contained no additions (YPD), 100 mM $CaCl_2$, 100 mM NaCl, or 1 M sorbitol. Approximately equal numbers of cells (as determined by OD_{600}) were spread on each quadrant, and the plates were incubated at 30°C for 2 days.

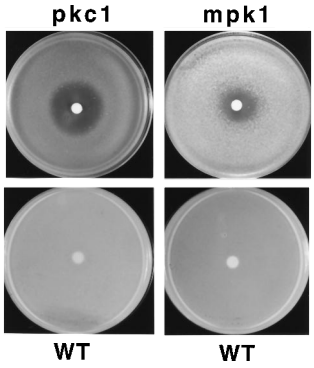

FIG. 7. Sensitivity of *pkc1* and *mpk1* mutants to FK506. Strains FL102 (*pkc1*) and EG123 (wild type [WT]) were grown on YPD containing 1 M sorbitol (left panels). Strains DL454 (*mpk1*) and EG123 (WT) were grown on YPD (right panels). A sterile disk saturated with 10 μg of FK506 was placed on each plate, and they were incubated at 30°C for 2 days.

growing than isogenic wild-type strains (Fig. 6). Both of these phenotypes are more pronounced in *cnd1-8* mutants than in the *cnd1* deletion mutant. The sensitivities of isogenic wild-type, *cnd1Δ1*::*URA3 hisG*, and *cnd1-8* strains to FK506 and cyclosporin A were compared with either dextrose or galactose as the carbon source. Under all conditions, the *cnd1-8* strain was significantly more sensitive (6- to 20-fold) than the *cnd1Δ1*::*URA3 hisG* strain to either drug (see Materials and Methods). Similarly, the *cnd1-8* strain exhibits a more severe growth defect than the *cnd1Δ1*::*URA3 hisG* strain (Fig. 6). In YPD medium at 30°C, the *cnd1-8* strain grows with a doubling time of 450 min, compared with 270 min for the *cnd1Δ1*::*URA3 hisG* strain and 95 min for the wild-type strain (data not shown). The weaker phenotype of the *cnd1Δ1* strain than of the *cnd1-8* strain suggests that the *cnd1-8* mutation, rather than being a simple loss-of-function allele, produces a protein that interferes to some extent with a cellular function (see Discussion).

Phenotypes of *cnd1* mutants vary with carbon source. The degree of drug sensitivity of both the *cnd1-8* and *cnd1Δ1*::*URA3 hisG* strains varied depending on the carbon source. Both strains were more sensitive to the effects of either FK506 or cyclosporin A when grown with dextrose than with galactose (Fig. 5), with the difference in sensitivity ranging from 2.5- to 16-fold (see Materials and Methods). *cnd1* mutants also displayed a greater growth defect relative to wild-type cells in dextrose than in galactose (data not shown). This difference was especially striking for the *cnd1-8* mutant, which, with a doubling time of 300 min on galactose, actually grew faster than on dextrose (see above). The reason for this variation in the drug sensitivity and growth rate of *cnd1* mutants depending on carbon source is not clear.

Growth of *cnd1-8* mutants is improved by osmotic stabilizing agents. The *CND1* gene product may perform a similar function to calcineurin in the cell; therefore, we examined *cnd1-8* and *cnd1Δ1*::*URA3 hisG* mutants to see if they displayed any phenotypes that were similar to calcineurin mutants. Calcineurin mutants are sensitive to several ions, including Na^+ and OH^- (45, 46). In contrast, *cnd1* mutants showed no sensitivity to Na^+ or OH^-, and their growth actually improved in media containing cations. Adding up to 100 mM KCl, NaCl, $CaCl_2$, or $MgCl_2$ visibly improved the growth of *cnd1-8* mutants (Fig. 6). Sorbitol, a nonionic agent, also improved the growth of this strain at concentrations of up to 1 M (Fig. 6). With the exception of $CaCl_2$, which had the greatest effect on growth, all of these treatments were equivalent (Fig. 6 and data not shown). The growth defect of the *cnd1Δ1*::*URA3 hisG* strain, although less severe than that of the *cnd1-8* mutant (see above), was also improved by osmotic stabilizing agents (data not shown). Although the addition of osmotic stabilizers to the medium improves their growth, *cnd1* mutants retain their sensitivity to FK506 and cyclosporin A under these conditions and are therefore still dependent on calcineurin for growth (data not shown).

Microscopic analysis of *cnd1-8* mutant cultures reveals evidence of cell lysis. Some mutants are thought to display osmotic agent-remedial growth defects because they have fragile cell walls. In the absence of osmotic support (at either 30 or 37°C), these mutants undergo spontaneous cell lysis (4, 30, 35–37, 49, 58, 61). Microscopic examination revealed evidence of lysis in *cnd1* cultures. By phase-contrast microscopy, we observed significant numbers of nonrefractile ghosts in *cnd1-8* cultures that were absent in wild-type cultures grown in YPD. The percentage of viable cells in liquid cultures of these strains was determined by staining with methylene blue, a blue dye that is reduced to a colorless form by viable cells. In cultures of wild-type cells growing in YPD, we observed no methylene blue-positive (i.e., dead) cells. In contrast, 25% of *cnd1-8* cells were positive, and this number decreased to 10% in cultures containing an osmotic stabilizer. YPD cultures of *cnd1Δ1*::*URA3 hisG* cells contained 4% inviable cells (data not shown). Taken together, evidence of lysis and osmotic agent-remedial growth indicate that cell integrity is compromised in *cnd1-8* mutants.

***pkc1* and *mpk1* mutants require calcineurin for growth.** A Ca^{2+}-regulated kinase, encoded by the *PKC1* gene, is required to maintain yeast cell integrity (37–39, 49, 58). *pkc1* mutants exhibit a 30% reduction in total cell wall β-glucan (54, 58) and undergo a lethal level of spontaneous lysis, so that a *pkc1Δ* mutant can grow only when an osmotic stabilizing agent is included in the medium. This lysis phenotype is similar to but more extreme than that observed for *cnd1-8* mutants. Thus, we were prompted to look for interactions between *PKC1*-regulated pathways and calcineurin-regulated pathways. We examined the sensitivity of a *pkc1Δ* strain to FK506 and cyclosporin A. We found, as previously reported, that the *pkc1* mutant grew in the presence of 1 M sorbitol. However, when either FK506 or cyclosporin A was added, *pkc1* mutants were no longer able to grow even in the presence of 1 M sorbitol (Fig. 7 and data not shown). Isogenic *PKC1* strains displayed no increased sensitivity to either drug (Fig. 7 and data not shown).

DIPLOID GENOTYPE		SPORE GENOTYPE			
		WT	*cnb1::URA3*	*pkc1::LEU2*	*cnb1::URA3 pkc1::LEU2*
cnb1::URA3	*PKC1*	13	24	9	1
CNB1	*pkc1::LEU2*				
		WT	*cnb1::LEU2*	*mpk1::TRP1*	*cnb1::LEU2 mpk1::TRP1*
cnb1::LEU2	*MPK1*	29	18	10	0
CNB1	*mpk1::TRP1*				
		WT	*cnd1-8*	*pkc1::LEU2*	*cnd1-8 pkc1::LEU2*
cnd1-8	*PKC1*	8	15	9	0
CND1	*pkc1::LEU2*				
		WT	*cnd1::URA*	*mpk1::TRP1*	*cnd1::URA3 mpk1::TRP1*
cnd1::URA3	*MPK1*	12	15	6	0
CND1	*mpk1::TRP1*				
		WT	*cnb1::LEU2*	*vma::TRP1*	*cnb1:LEU2 vma::TRP1*
cnb1::LEU2	*VMA3*	14	9	7	0
CNB1	*vma3::TRP1*				
cnb1::LEU2	*VMA12*	6	13	15	0
CNB1	*vma12::TRP1*				

FIG. 8. Analysis of viable spores from heterozygous diploids. The number of spores of each genotype is indicated.

These observations indicate that *pkc1* mutants are inviable in the absence of calcineurin. To confirm this conclusion, we characterized the meitoic segregants from strain PGY13 (heterozygous for *pkc1* and *cnb1*); only 1 *pkc1 cnb1* double mutant was recovered out of a total of 47 viable progeny (Fig. 8). The underrepresentation of the double mutant class was statistically significant ($P < 0.005$), and the one double mutant strain observed was most likely due to the acquisition of a spontaneous mutation that suppressed its growth defect.

The *mpk1*/*slt2* gene encodes a MAP kinase that acts downstream of *PKC1* in the same signal transduction pathway (35). *mpk1*/*slt2* null mutants are viable at 30°C but at 37°C are dependent on osmotic stabilizers for growth because of spontaneous lysis. We found that *mpk1*/*slt2* mutants were hypersensitive to FK506 and cyclosporin A at 30°C, i.e., they were inviable when calcineurin was inhibited (Fig. 7 and data not shown). In contrast, the growth of an isogenic *MPK1* strain was unaffected by the addition of either drug (Fig. 7 and data not shown). Consistent with these observations, when a diploid strain heterozygous for *cnb1* and *mpk1* (PGY14) was induced to sporulate and its haploid progeny were examined, no *cnb1 mpk1* double mutant spores were recovered ($P < 0.005$; see Fig. 8). Thus, yeast cells whose integrity is compromised by mutations in the *PKC1* signal transduction pathway depend on calcineurin for growth.

Constitutively active calcineurin suppresses *pkc1* and *mpk1* growth defects. To further explore the relationship between *PKC1* and calcineurin, we examined the effects of overexpressing a calcineurin subunit in *pkc1* and *mpk1* mutants. Multicopy plasmids encoding a wild-type *CNA2* gene product (YEpCNA2) or a C-terminally truncated *CNA2* (YEpCNA2Δ) were introduced into *pkc1* and *mpk1* strains. C-terminal truncation of the calcineurin catalytic subunit that removes the calmodulin binding site and an inhibitory domain has been shown to produce a Ca^{2+}-independent form of the enzyme (28, 48). We found that overexpression of this Ca^{2+}-independent calcineurin, but not the full-length form, improved the growth of both *pkc1* and *mpk1* mutants, allowing *pkc1* strains to grow on YPD containing 0.5 M sorbitol and *mpk1* strains to grow at 37°C (Fig. 9).

In contrast, activation of the *PKC1*-regulated MAP kinase pathway failed to alter at least two phenotypes of calcineurin mutants. Compared with wild-type strains, calcineurin mutants exhibit increased sensitivity to $MnCl_2$ and an inability to recover from mating pheromone arrest (12–14). Calcineurin mutants containing a plasmid-borne copy of *BCK1-20*, a dominantly activated form of this kinase (pRS314[BCK1-20] [36]) were unaltered in their responses to $MnCl_2$ and pheromone (data not shown).

***cnd1* mutations are synthetically lethal with mutations in the *PKC1* pathway.** We examined the relationship between *cnd1* and elements of the *PKC1* pathway by trying to construct *cnd1 pkc1* and *cnd1 mpk1* strains. However, no such strains were recovered, indicating that these mutations were lethal in combination (Fig. 8). Introduction of *CND1* on a multicopy plasmid into either a *pkc1* or an *mpk1* mutant failed to suppress their growth defects (data not shown).

***vma* mutants require calcineurin for vegetative growth.** We observed, in the course of experiments unrelated to our synthetic lethal mutation screen, that several *vma* mutant strains, i.e., strains lacking a functional vacuolar H^+-ATPase (reviewed in reference 2), were hypersensitive to FK506 and cyclosporin A (Fig. 10). This hypersensitivity indicates that *vma* mutants depend on calcineurin for vegetative growth. We demonstrated this synthetic lethality directly by sporulating diploid

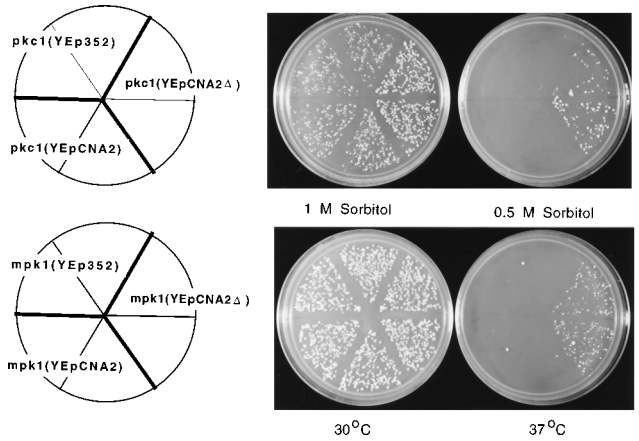

FIG. 9. Constitutively active calcineurin suppresses the growth defects of *pkc1* and *mpk1* strains. (Top panel) Strain FL102 was cured of the pGAL1::PKC1 plasmid and transformed with YEp352, YEpCNA2, or YEpCNA2Δ (see Materials and Methods). Equal numbers of cells from two independent transformants were spread on a YPD plate containing 1 M sorbitol (left) or 0.5 M sorbitol (right). Plates were incubated at 30°C for 3 days. (Bottom panel) Strain DL454 was transformed with YEp352, YEpCNA2, or YEpCNA2Δ. Equal numbers of cells from two independent transformants were spread on a YPD plate and incubated at 30°C (left) or 37°C (right) for 2 days.

strains heterozygous for *vma* mutations (marked with *TRP1*) and *cnb1* (marked with *LEU2*). The genotypes of viable meiotic segregants from this diploid were determined. No Leu^+ Trp^+ haploids, i.e., *vma cnb1* double mutants, were recovered from these crosses, and the absence of this class of spores was statistically significant in each case ($P < 0.005$; see Fig. 8). Thus, this genetic analysis and the FK506/cyclosporin A hypersensitivity of *vma* mutants both indicate that *vma* mutants are inviable in the absence of calcineurin. All *vma* mutants display a distinctive set of phenotypes: they are sensitive to $CaCl_2$, fail to grow at pH 7 or greater, and fail to grow on nonfermentable carbon sources such as glycerol (Pet^-) (2). The two mutants identified in the synthetic lethal mutation screen that did not contain mutations in *CND1* (i.e., isolates 112 and 106) do not display any of these characteristic *vma* phenotypes (data not shown). In fact, it is unlikely that *vma* mutants would be identified by this procedure, because *vma ade2* strains do not develop a strong red colony color.

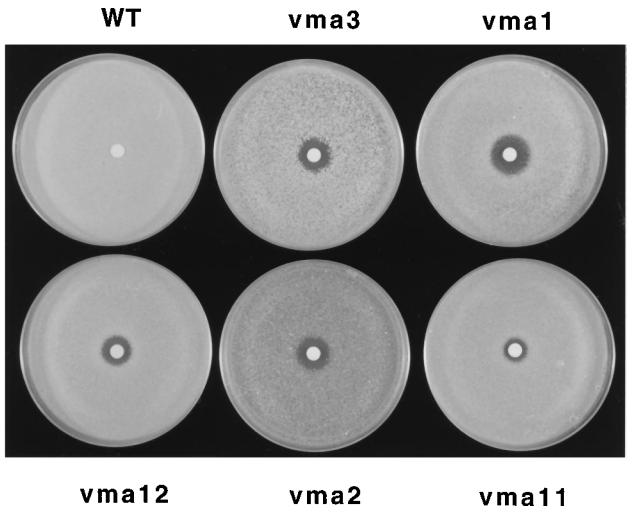

FIG. 10. Sensitivity of *vma* mutants to FK506. Strains YPH500 (WT), DV3T-1B (*vma3*), RH104 (*vma1*), RH205 (*vma12*), YOV503 (*vma2*), and YRH11 (*vma11*) were incubated at 30°C for 2 days on YPD medium in the presence of a sterile disk saturated with FK506 (see Materials and Methods).

***vma2* and *cnd1* mutations are not synthetically lethal.** *vma* mutants and *cnd1* mutants each depend on the presence of calcineurin for vegetative growth on standard laboratory media. Thus, the *VMA* and *CND1* gene products each potentially perform a physiological function that is similar to, or in some way overlaps, that of calcineurin. To further explore the functional relationship between *CND1* and the vacuolar H^+-ATPase, we created a diploid strain that was heterozygous for the *cnd1Δ1*::*URA3 hisG* mutation and the *vma2*::*TRP1* mutation. This strain was sporulated, and the genotype of the haploid segregants of 18 individual tetrads was determined. All possible haploid segregants from this cross were viable, including Ura^+ Trp^+ segregants (i.e., *cnd1 vma2* double mutants). However, these segregants were even more sensitive to FK506 and cyclosporin A than either the *cnd1* or *vma2* single mutant strains (data not shown).

DISCUSSION

In many cells, the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin plays a critical role in Ca^{2+}-mediated signal transduction. The calcineurin inhibitors FK506 and cyclosporin A block a variety of responses, ranging from the activation of mammalian T cells (reviewed in reference 41) to stomatal closure in plant leaves (43). The regulatory pathways through which this phosphatase acts, however, are largely undefined.

In *S. cerevisiae*, genetic analysis of calcineurin function indicates that this phosphatase is required for growth only under particular conditions, for example, in the presence of high concentrations of a number of ions (Na^+, Li^+, Mn^{2+}, and OH^-) (6, 12, 45, 46). Calcineurin mutants are not, however, sensitive to a general increase in osmolarity or ionic content and do not display any increased sensitivity to K^+, Mg^{2+}, Zn^{2+}, Fe^{2+}, or a number of other ions (12). In addition, calcineurin mutants die during continuous exposure to mating pheromone (12–14), as do wild-type cells under these conditions if they are deprived of Ca^{2+} (29). These phenotypes suggest that calcineurin participates in the regulation of intracellular ion homeostasis. One possible explanation for the apparent dispensability of calcineurin under standard laboratory conditions is the existence of other cellular regulatory pathways that execute related functions. To learn more about the physiological functions of calcineurin and how its regulatory role may intersect with other cellular components, we conducted a screen to identify mutants that require calcineurin activity for vegetative growth. Our goal was to identify elements of regulatory pathways that may overlap in function with those regulated by calcineurin.

Identification and characterization of *CND1*. The genetic screen described here identified mutations in three complementation groups that cause yeast cells to depend on calcineurin for normal vegetative growth. All the mutants described exhibit increased sensitivity to the calcineurin inhibitors FK506 and cyclosporin A. One of the genes identified, *CND1*, was characterized in detail. It is predicted to encode a novel integral membrane protein of MW 215,110, containing at least 10 potential membrane-spanning domains.

CND1 is not essential for growth; however, we report two observations indicating a role for the *CND1* gene product in maintaining cell integrity. First, microscopic examination of *cnd1* cultures reveals evidence of spontaneous cell lysis. Sec-

ond, the degree of lysis and the slow growth of *cnd1* strains are remedied by the addition of osmotic stabilizing agents to the medium. As other mutations that cause osmotic agent-remedial lysis are known to alter the morphology and composition of the cell wall (4, 30, 35–37, 49, 55, 58, 61), our studies suggest that *cnd1* mutations may also compromise cell wall function. Recently, the *CND1* gene has been independently isolated and characterized by several groups and is identical to *FKS1*, *ETG1*, and *CWH53* (16, 18, 53). Mutations in this gene were identified in screens for mutants exhibiting hypersensitivity to FK506 (18, 50), resistance to echinocandins (17), or sensitivity to Calcofluor White (52). Consistent with our findings, some of these studies demonstrate that *fks1* mutations affect the composition of the cell wall and are lethal in combination with mutations that eliminate calcineurin function (18, 50). Disruptions in this gene reduce the levels of β1,3-glucan in the cell wall (52, 53), and point mutations in the gene can alter the properties of β1,3-glucan synthase activity in vitro (16, 17). Thus, while its exact function is not clear, the *FKS1*/*CND1*/*ETG1*/*CWH53* gene product seems to participate in β1,3-glucan synthesis in vivo.

Differential effects of *cnd1* alleles may reflect interactions between *FKS1*/*CND1* and other cell components. Comparison of two *cnd1* alleles indicates that *cnd1-8* cells, identified by the synthetic lethal mutation screen, are more sensitive to immunosuppressants than cells containing *cnd1Δ1*::*URA3 hisG*, a deletion allele created in vitro. The *cnd1-8* allele also causes more severe growth defects and compromises cell integrity to a greater extent than does *cnd1Δ1*. This ability of mutant Cnd1p to interfere with cell function may indicate that Cnd1p competes with another protein of similar function in the cell for common substrates, regulators, etc. Consistent with this possibility, we have preliminary evidence suggesting the presence of a functional homolog of *FKS1*/*CND1*. We observed that in the collection of ordered clones representing the yeast genome, there was a clone from chromosome VII that displayed a substantial amount of hybridization of *FKS1*/*CND1* probes. Plasmids containing large fragments of yeast DNA (>10 kb) from this lambda clone are capable of complementing *cnd1* mutant phenotypes, suggesting that this other gene can substitute for *FKS1*/*CND1* (22).

Interactions between calcineurin and the *PKC1* signal transduction pathway. Only a few yeast mutants that exhibit osmotic agent-remedial spontaneous cell lysis have been characterized, and most of the mutations disrupt the same signal transduction pathway. This pathway is defined by *PKC1*, a Ca^{2+}-dependent protein kinase related to mammalian protein kinase C. Additional kinases, the *BCK1*/*SLK1*, *MKK1*, *MKK2*, and *MPK1*/*SLT2* products, have been shown by genetic analyses to act downstream of *PKC1*. These downstream kinases are homologous to mammalian MAP kinases and MAP kinase-activating kinases. We examined genetic interactions between *FKS1*/*CND1*, calcineurin, and the *PKC1* pathway. We found that a constitutively active, Ca^{2+}-independent form of calcineurin partially corrects the lysis defects of *pkc1* and *mpk1* mutants. In contrast, overexpression of intact calcineurin failed to suppress the growth defects of these mutants, indicating that the ability of calcineurin to compensate for defects in the *PKC1* pathway in vivo may be limited by the duration of the Ca^{2+} signal itself. We also found that eliminating functional calcineurin in *pkc1* or *mpk1* mutants exacerbates their growth defects. Taken together, these observations suggest that rather than functioning in the same regulatory pathway, calcineurin and *PKC1* perform independent but related functions in determining cell integrity. Clearly, the regulatory targets of each pathway must be established before the relationship between the two can be understood. It is intriguing, however, that in mammalian T cells, calcineurin and protein kinase C have been shown to act synergistically in activating transcription of the interleukin-2 gene (21).

We also determined that *fks1*/*cnd1* is synthetically lethal in combination with *pkc1* or *mpk1*/*slt2*. Thus, *FKS1*/*CND1* can be added to a growing list of genes whose products participate in cell wall synthesis and are required for the viability of *pkc1* mutants. These other genes include *KRE6*, *KRE11*, *KRE2*, and *SKN7*; mutations in these genes are presumably lethal in combination with *pkc1* because cell wall defects are more severe in the double mutants than in either single mutant (7, 54).

Functions of calcineurin as revealed by synthetic lethal mutations. Phenotypic analysis of calcineurin mutants indicates that this enzyme participates in the regulation of intracellular ion homeostasis. The observations reported here, i.e., that cells require either functional calcineurin or a functional vacuolar H^+-ATPase for vegetative growth, are entirely consistent with this idea. This H^+-ATPase plays a crucial role in both pH and Ca^{2+} homeostasis in the cell; VMA-catalyzed acidification of the vacuole is required for correct intracellular targeting of vacuolar proteases and for calcium sequestration in the vacuole via Ca^{2+}/H^+ exchange (reviewed in references 2 and 32). The sensitivity of calcineurin mutants to high pH (>8.5), their loss of viability in pheromone, and their ability to suppress the Ca^{2+}-sensitive *pmc1* mutation (11) all suggest that calcineurin may function in the regulation of pH and Ca^{2+} homeostasis. Although the exact role of calcineurin in the regulation of intracellular pH and Ca^{2+} and other ions is not yet clear, the genetic interactions described here suggest that the levels of several intracellular ions may be altered in calcineurin mutants.

In addition to *VMA* genes, we have characterized three genes, *FKS1*/*CND1*, *PKC1*, and *MPK1*, that are required for cell integrity/cell wall morphogenesis and mutations of which are lethal in combination with calcineurin mutations. The requirement for calcineurin in these cells may be quite indirect. Mutants with fragile cell walls may be more sensitive to changes in intracellular ion homeostasis, especially if those changes also alter osmotic pressure in the cell. In other words, the synthetic lethalities described here between calcineurin and *cnd1*, *pkc1*, and *mpk1* mutations may simply underscore the physiological importance of coordinately regulating ion homeostasis and cell wall morphogenesis. The stimuli that elicit Ca^{2+} signals in yeast cells are largely undefined, but these stimuli may consist of environmental changes that require yeast cells to adjust both their intracellular ionic environment (via calcineurin) and the properties of their cell wall (via *PKC1*).

On the other hand, it is possible that the synthetic lethalities described here are the first indication of a direct role for calcineurin in regulating cell wall structure. This function may be uncovered only in the absence of *FKS1*/*CND1* or the *PKC1* pathway because of genetic redundancy. Redundancy is the rule rather than the exception for cell wall-biosynthetic components. Chitin, for example, is synthesized by three related isozymes, each of which plays a distinct role in chitin deposition and is responsive to different regulatory inputs (8). Similarly, some of the components that participate in β1,6-glucan synthesis and assembly are encoded by multiple, related genes (54, 55). Studies such as those reported here may lead not only to a greater understanding of calcineurin's functions but also to an understanding of the environmental cues that initiate Ca^{2+} signaling pathways in yeast cells. Furthermore, establishing the relevant targets of calcineurin regulation in *S. cerevisiae* may

provide insights into Ca^{2+}-mediated signaling pathways in other eukaryotic cells.

Clarification of nomenclature. In the future, we will use the *FKS1* designation exclusively for this gene. The mutation reported here as *cnd1-8* will be referred to as *fks1-8*, and the mutation reported here as *cnd1Δ1*::*URA3 hisG* will be referred to as *fks1Δ3*::*URA3 hisG*.

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