vph6 Mutants of *Saccharomyces cerevisiae* Require Calcineurin for Growth and Are Defective in Vacuolar H^+-ATPase Assembly

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ABSTRACT

We have characterized a *Saccharomyces cerevisiae* mutant strain that is hypersensitive to cyclosporin A (CsA) and FK506, immunosuppressants that inhibit calcineurin, a serine-threonine-specific phosphatase (PP2B). A single nuclear mutation, designated *cev1* for calcineurin essential for viability, is responsible for the CsA-FK506-sensitive phenotype. The peptidyl-prolyl *cis-trans* isomerases cyclophilin A and FKBP12, respectively, mediate CsA and FK506 toxicity in the *cev1* mutant strain. We demonstrate that *cev1* is an allele of the *VPH6* gene and that *vph6* mutant strains fail to assemble the vacuolar H^+-ATPase (V-ATPase). The *VPH6* gene was mapped on chromosome *VIII* and is predicted to encode a 181-amino acid (21 kD) protein with no identity to other known proteins. We find that calcineurin is essential for viability in many mutant strains with defects in V-ATPase function or vacuolar acidification. In addition, we find that calcineurin modulates extracellular acidification in response to glucose, which we propose occurs via calcineurin regulation of the plasma membrane H^+-ATPase PMA1. Taken together, our findings suggest calcineurin plays a general role in the regulation of cation transport and homeostasis.

CALCINEURIN is a Ca^{2+}-calmodulin-regulated serine-threonine-specific protein phosphatase, also known as PP2B (KLEE *et al.* 1988). The functional enzyme is a heterotrimer composed of a catalytic A subunit, a regulatory B subunit, and calmodulin. The calcineurin B subunit and calmodulin are structurally similar Ca^{2+}-binding proteins that are together responsible for the Ca^{2+} dependence of calcineurin yet play different roles in enzyme activation (STEMMER and KLEE 1994). In complex with the immunophilins, cyclophilin A and FKBP12, the immunosuppressive drugs cyclosporin A (CsA) and FK506 inhibit calcineurin (LIU *et al.* 1991a). In T-cells, calcineurin is activated by a rise in intracellular Ca^{2+} that occurs upon stimulation of the antigen receptor pathway (reviewed in CARDENAS *et al.* 1994b). A target of calcineurin in T cells is the cytoplasmic subunit of the transcription factor NFAT. Phosphorylated NFAT is localized to the cytoplasm. After dephosphorylation NFAT translocates to the nucleus, associates with members of the Fos and Jun family of DNA-binding proteins, and stimulates transcription of genes required for T-cell activation (FLANAGAN *et al.* 1991; CLIPSTONE and CRABTREE 1992; O'KEEFE *et al.* 1992; JAIN *et al.* 1993). Calcineurin is widely distributed in mammalian tissues and inhibition of calcineurin may account for some of the adverse effects, such as nephrotoxicity and neurotoxicity, associated with clinical use of CsA and FK506. Calcineurin is conserved from unicellular eukaryotes to vertebrates, suggesting an important regulatory role in all cells.

While the roles of calcineurin other than in T-cell activation are less well understood, cation channels have emerged as a common target of calcineurin in diverse organisms. For example, calcineurin regulates the renal Na^+/K^+ pump in mammals and a guard cell K^+ channel in the plant *Vicia faba* and functionally interacts with a Ca^{2+}-ATPase in vacuoles of the yeast *Saccharomyces cerevisiae* (APERIA *et al.* 1992; LUAN *et al.* 1993; CUNNINGHAM and FINK 1994). Calcineurin is also essential for viability of *S. cerevisiae* under conditions of high salt stress (NAKAMURA *et al.* 1993; BREUDER *et al.* 1994; MENDOZA *et al.* 1994).

Calcineurin is not essential in most laboratory strains of *S. cerevisiae*. Among mutants resistant to lithium plus neomycin, reagents that perturb phosphatidylinositol metabolism (CARNEY *et al.* 1985; BERRIDGE 1989), we identified a mutation, *cev1*, that renders calcineurin essential for viability. We demonstrate that *cev1* is allelic with *VPH6*, a gene required for normal vacuolar acidification and morphology (PRESTON *et al.* 1992). We report the cloning and sequencing of the *VPH6* gene, genetic and physical mapping of the *VPH6* locus on chromosome *VIII*, and characterization of *vph6* mutant phenotypes. We demonstrate that a *vph6* mutant yeast strain fails to assemble the vacuolar H^+-ATPase (V-ATPase). Many mutations that impair acidification of the

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yeast vacuole, some of which prevent either synthesis of specific subunits or assembly of the V-ATPase, similarly render calcineurin essential. Finally, we present evidence that calcineurin may regulate the plasma membrane H^+-ATPase PMA1. We propose that calcineurin plays a central role in cation homeostasis in *S. cerevisiae* and possibly other organisms.

MATERIALS AND METHODS

Materials: Zymolyase 20T was from ICN Biochemicals. Restriction enzymes were from New England Biolabs. Cyclosporin A in vehicle (30% v/v EtOH/70% v/v cremophor EL) was obtained commercially (Sandoz). FK506 was provided by FUJISAWA and dissolved in EtOH. Neomycin sulfate was from Sigma. 5-fluororootic acid (5-FOA) was obtained from the Genetics Society of America consortium. Mouse anti-VPH1 and anti-CPY monoclonal antibodies were from Molecular Probes. A mouse monoclonal antibody against VMA1 was provided by M. MANOLSON. Rabbit polyclonal antiserum against yeast cyclophilin A was as described (CARDENAS *et al.* 1995). The ECL Western blot detection system was from Amersham.

Strains and media: Strains and their sources are listed in Table 1. YPD, synthetic, sporulation, and 5-FOA media were prepared as described (ROSE *et al.* 1990). Sterile stock solutions of $CaCl_2$, LiCl, $ZnCl_2$, CsA, FK506, or neomycin were added to medium before pouring.

Cloning: The *VPH6* gene was cloned by transformation of *vph6–1* mutant strain BJ6756 with a genomic library (ROSE *et al.* 1987). Twenty thousand uracil prototrophic transformants were replica-plated to YPD medium containing 4 mM $ZnCl_2$ to select for complementation of the *vph6* mutant phenotype. The original complementing plasmid contained an 11.4-kb insert and was subcloned to determine the minimal complementing region as a 1.05-kb *Spe*I-*Nar*I fragment that was obtained by releasing a *Nar*I-*Cla*I (*Cla*I in the polylinker and *Nar*I in the insert) fragment and religating the cohesive ends to generate plasmid pKDcVPH6. To genetically mark the *VPH6* locus and confirm that the authentic gene had been cloned, a 3.5-kb *Hind*III-*Sal*I *vph6-1* complementing insert was ligated into the *Hind*III and *Sal*I sites of YIp5. The resulting YIp5-VPH6 plasmid was linearized with *Xho*I and used to transform the *vph6-1* mutant strain with selection on synthetic medium lacking uracil.

Transformations and gene disruptions: Yeast transformations were as described (GIETZ *et al.* 1992). *cmp1::LEU2* and *cmp2::URA3* one-step gene disruptions were as described (CYERT *et al.* 1991; LIU *et al.* 1991b; BREUDER *et al.* 1994).

The *VPH6* gene was disrupted as follows: a *VPH6* disruption fragment was produced by inserting the *VPH6*-containing *Spe*I-*Sal*I fragment of plasmid pKDcVPH6 into the *Xba*I-*Sal*I sites of pUC18. The 354-bp *Mun*I fragment within the the *VPH6* gene-coding region was replaced by a 953-bp *Eco*RI fragment with the *TRP1* gene from plasmid pJJ246 (JONES and PRAKASH 1990). This *vph6Δ::TRP1* construct, pvph6d, was linearized with *Eco*RI and *Sal*I to yield a 1.6-kb fragment used to transform haploid yeast strain 9933-20C. Tryptophan prototrophic transformants were selected, and disruption of the chromosomal copy of *VPH6* was confirmed by PCR using sequence-specific primers 219-235 and 849-833 (see below). The resulting *vph6Δ::TRP1* mutant strain was designated CHY251.

Sequencing: Sequencing reactions were performed with Sequenase version 2.0 (USB) with T7 DNA polymerase and T3, T7, and custom-made primers. The *Spe*I-*Nar*I insert in pRS316 served as sequencing templates for the *VPH6* gene. In addition to the T3 and T7 primers, the following oligonucleotides were employed for internal sequencing: 5′-TACAGATGAACAGTACC [nucleotides (nt) 219-235], 5′-TCTTTTTCAAACCATGG (nt 849-833), 5′-CAAGACTACGACCCAATTTTG (nt 575-596), 5′-CCACTGTTGCCATTAGTTGACC (nt 423-401), 5′-GCGATTGTCTCAATGAAGAAGG (nt 641-619). Both strands of the *VPH6* gene were sequenced. The Genbank accession number for the cosmid containing *VPH6* (YHR060w) is #U00061.

Vacuole isolation: Vacuoles were prepared essentially as described (CONRADT *et al.* 1992) with minor modifications as described (CARDENAS and HEITMAN 1995). For protein extracts, samples from the total spheroplast lysate or from the vacuolar fraction were adjusted to 20% trichloroacetic acid and incubated for 1.5 hr at 4°. Protein was recovered by centrifugation at 15,000 rpm in a microfuge at 4°. Pellets were directly resuspended in 100 mM Tris-HCl pH 11, 3% SDS, 100 mM dithiothreitol, 15% glycerol, and 0.02% bromophenol blue, and further neutralized by the addition of Tris-HCl pH 8.8 as required.

Immunoblotting: For immunoblotting, equal amounts of protein from the total and vacuolar fraction preparation were fractionated by SDS-PAGE and transferred to nitrocellulose filters. The filters were probed with monoclonal antibodies against VPH1, CPY, VMA1, and an anti-cyclophilin A polyclonal antiserum.

Extracellular acidification assays: Glucose-induced extracellular acidification was measured according to the method of SERRANO (1980) with some modifications. Yeast cells were grown in YPD medium to mid-log phase and then washed twice in distilled water. Washed cells were resuspended in distilled water at a concentration of 100–200 mg cells (wet weight) per ml and stored on ice. A stirred suspension containing 4 ml washed cells in 14 ml of distilled water was adjusted with dilute HCl to pH 4 at 25°. Glucose was added to the suspension at time zero to achieve a glucose concentration of 2% and a final volume of 20 ml. pH of the medium was measured every 60 sec for 25 min. For experiments utilizing CsA- or FK506-treated cells, control experiments contained drug vehicle alone (cremophor EL/ethanol 70%/30% v/v or ethanol, respectively).

RESULTS

***cev1* mutation confers CsA-FK506 sensitivity:** Many yeast strains tolerate high concentrations of either Li^+ (up to 1.3 M) (ASENSIO *et al.* 1976) or neomycin (up to 100 mM) (SHIMMA and UNO 1990). Thus, strain JK93d (HEITMAN *et al.* 1991) is viable on YPD medium containing 200 mM LiCl or 5 mM neomycin. However, when combined, 200 mM LiCl and 1 mM neomycin are toxic to JK93d (Figure 1). Twenty one spontaneous mutants resistant to LiCl plus neomycin were isolated and designated Lnr^+ (lithium plus neomycin resistant). Their complete characterization will be described elsewhere.

The resistance of the Lnr^+ strains to cation stress was further assessed by testing growth on YPD medium containing 200 mM LiCl plus 100 μg/ml CsA. CsA inhibits calcineurin and results in Li^+ sensitivity in wild-type yeast strains (NAKAMURA *et al.* 1993; BREUDER *et al.* 1994). In fact, four of the Lnr^+ mutants are resistant to LiCl plus CsA, indicating that calcineurin is not required under conditions of cation stress in these mutants. Unexpectedly, growth of Lnr^+ mutant CHY204 is inhibited by CsA alone; growth of the isogenic Lnr^- parental strain is CsA-resistant (Figure 1). When

TABLE 1

Yeast strains

Strain	Genotype
JK93da	*MAT***a** *his4 HML***a** *leu2-3,112 rme1 trp1 ura3-52*
CHY204α	*MATα cev1 his4 HML***a** *leu2-3,112 rme1 ura3-52*
CHY204a	*MAT***a** *cev1 his4 HML***a** *leu2-3,112 rme1 ura3-52*
CHY204-S	*MATα cev1 his4 HML***a** *leu2-3,112* Lnr$^-$ *rme1 trp1 ura3-52*
CHY204-D1	*MAT***a**/*α cev1*/*vph6 his4*/*HIS4 leu2-3,112*/*leu2 rme1*/*RME1 ura3-52*/*ura3*
TB68	*MAT***a** *cmp1::LEU2 cmp2::URA3 his4 HML***a** *leu2-3,112 rme1 trp1 ura3-52*
TB85	*MAT***a** *cnb1Δ1::LEU2 his4 HML***a** *leu2-3,112 rme1 trp1 ura3-52*
JHY58	*MAT***a** *ade2 cnb1::ADE2 his4 HML***a** *leu2-3,112 rme1 trp1 ura3-52*
CHY504	*MAT***a** *ena1::kan*^{r} *his4 HML***a** *leu2-3,112 rme1 trp1 ura3-52*
MH250-2C	*MAT***a** *cpr1::LEU2 his4 HML***a** *leu2-3,112 rme1 trp1 ura3-52*
JHY2-1C	*MAT***a** *ade2 fpr1::ADE2 his4 HML***a** *leu2-3,112 rme1 trp1 ura3-52*
TB138-1A	*MAT***a** *cev1 cpr1::LEU2 his4 HML***a** *leu2-3,112 rme1 ura3-52*
TB139-8D	*MATα cev1 fpr1::ADE2 his4 HML***a** *leu2-3,112 rme1 ura3-52*
KDY21	*MATα cev1 VPH6::URA3 his4 HML***a** *leu2-3,112* Lnr$^-$ *rme1 trp1 ura3-52*
KDY22	*MAT***a**/*α cev1*/*CEV1 cpr1::LEU2*/*CPR1 cpr2::TRP1*/*CPR2 cpr3::HIS3*/*CPR3 fpr1::URA3*/*FPR1 his4*/*his4 HML***a**/*HML***a** *leu2-3,112*/*leu2-3,112 rme1*/*rme1 trp1*/*trp1 ura3-52*/*ura3-52*
KDY23	*MAT***a**/*α cev1*/*CEV1 cpr2::TRP1*/*CPR2 cpr3::HIS3*/*CPR3 his4*/*his4 HML***a**/*HML***a** *leu2-3, 112*/*leu2-3,112 rme1*/*rme1 ste12::LEU2*/*STE12 trp1*/*trp1 ura3-52*/*ura3-52*
9933-20C	*MATα his3 leu2-3,112 trp1-Δ101 ura3-52*
CHY251	*MATα his3 leu2-3,112 trp1-Δ101 ura3-52 vph6Δ::TRP1*
BJ1983	*MAT***a** *trp1-Δ101*
BJ4304	*MAT***a** *pep14-5 trp1-Δ101 ura3-52*
BJ4387	*MATα leu2-1 pep21*
BJ5113	*MATα ade6 his3 leu2 pep12::LEU2 ura3*
BJ5194	*MAT***a** *leu2 ura3 vph5-1*^{d}
BJ5195	*MAT***a** *leu2 ura3 vph6-1*^{d}
BJ5200	*MAT***a** *leu2 vph2-1*^{d}
BJ5205	*MATα trp1 vph7-1*^{d}
BJ5207	*MATα trp1 vph8-1*^{d}
BJ5208	*MATα trp1 vph9*^{d}
BJ5555	*MATα leu2-1 pep3::LEU2 trp1-Δ101 ura3-52*
BJ6756	*MAT***a** *leu2-3,112 ura3-52 vph6-1*
BJ6757	*MATα trp1-Δ101 ura3-52 vph6-1*
BJ7244	*MATα lys2-B011 trp1-Δ101 stv1::LYS2*
BJ2793	*MATα his3-Δ200 leu2-Δ1 lys2-801 ura3-52 stv1::LYS2 vph1::LEU2*
BJ7287	*MAT***a** *his3 leu2 lys2 pep8::URA3 trp1 ura3-52*
BJ7964	*MAT***a** *his3 leu2 pep5::TRP1 trp1-Δ101 ura3*
BJ8081	*MAT***a** *leu2-1 pep7::LEU2 ura3-52*
MM53	*MATα ura3-52 vph1::LEU2*^{a,d}
MM106	*MATα ura3 vat2Δ*^{a,d}
MM108	*MATα his4 tfp1::URA3 ura3-52*^{a,d}
MM110	*MATα ade6 gal2 leu2-3,112 vma4::URA3*^{a,d}
MM111	*MATα ade6 gal2 leu2-3,112 vma3::URA3*^{a,d}
SF838-1D	*MATα ade6 his4-519 leu2-3,112 pep4-3 ura3-52*^{b}
SF838-1D Δ*vma5*	*MATα ade6 his4-519 leu2-3,112 pep4-3 ura3-52*^{b} *vma5::LEU2*^{b,d}
SF838-1D Δ*vma6*	*MATα ade6 his4-519 leu2-3,112 pep4-3 ura3-52 vma6::LEU2*^{b,d}
SF838-1D Δ*vma7*	*MATα ade6 his4-519 leu2-3,112 pep4-3 ura 3-52 vma7::LEU2*^{b,d}
KHY2	*MAT***a** *ade2-101 his3-Δ200 leu2-3,112 suc2Δ-9 trp1-901 ura3-52 vma21::LEU2*^{b}
YPH499	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*^{c}
RH304	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 vma13::TRP1*^{c}
YRH11a	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 vma11::TRP1*^{c}

[a] Strains provided by MORRIS MANOLSON.
[b] Strains provided by TOM STEVENS.
[c] Strains provided by RYOGO HIRATA.
[d] Strains used for complementation testing of *cev1* mutant strain.

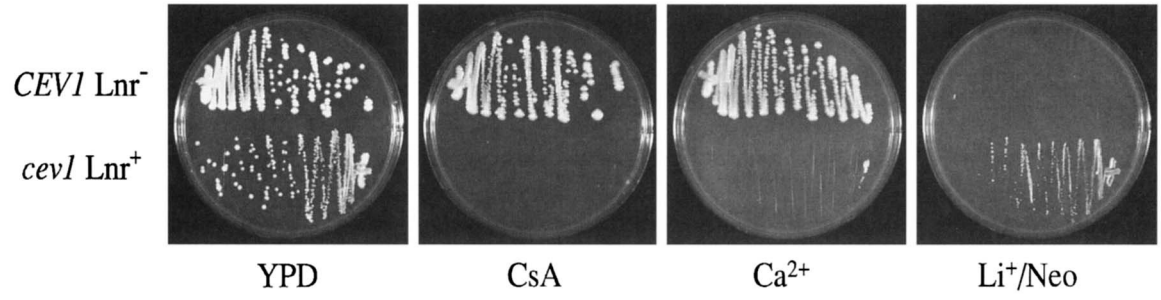

FIGURE 1.—Growth phenotypes of the *cev1* mutant strain. Wild-type *CEV1* Lnr$^-$ strain JK93da and the isogenic *cev1* Lnr$^+$ mutant strain CHY204a were grown at 30° for 72 hr on YPD medium, YPD containing 100 μg/ml CsA or 100 mM $CaCl_2$ or for 120 hr on YPD medium containing 1 mM neomycin plus 200 mM LiCl.

CHY204α was crossed to the isogenic wild-type strain, the resulting diploid was CsA-resistant and lithium/neomycin-sensitive, demonstrating that the mutant phenotypes are recessive. After sporulation, each of 11 tetrads yielded 2 CsA-sensitive:2 CsA-resistant meiotic segregants; therefore, a single nuclear mutation confers CsA sensitivity. In contrast, meiotic segregation of the lithium/neomycin-resistant phenotype consisted of two 2 Lnr$^+$:2 Lnr$^-$, eight 1 Lnr$^+$:3 Lnr$^-$, and one 0 Lnr$^+$:4 Lnr$^-$ segregations. Of note, all of the Lnr$^+$ segregants were also CsA-sensitive. Thus, at least two mutations are necessary to confer lithium/neomycin-resistance, one of which suffices to confer CsA sensitivity. Here we have characterized the mutation conferring CsA sensitivity.

Calcineurin is essential in the *cev1* mutant: To determine if calcineurin is the target of CsA in strain CHY204, we tested the effects of FK506, a calcineurin inhibitor structurally distinct from CsA (LIU *et al.* 1991a). Unlike the wild-type strain JK93d, which is FK506-resistant, growth of strain CHY204 was completely inhibited by 1 μg/ml FK506 (Figure 2). When CHY204 was crossed to the isogenic wild-type strain and sporulated, each of 11 tetrads yielded 2 CsA- and FK506-resistant:2 CsA- and FK506-sensitive segregants; thus, a single nuclear mutation confers both phenotypes. Strain CHY204 was crossed to an isogenic *cnb1::LEU2* strain, which lacks the calcineurin B regulatory subunit, and the resulting diploid was sporulated and dissected. Of 20 tetrads analyzed, the segregation pattern was as follows: one tetrad with 2 CsA-FK506^S Leu$^-$:2 CsA-FK506^R Leu$^+$ segregants, 13 tetrads with 1 CsA-FK506^S Leu$^-$:1 CsA-FK506^R Leu$^-$:1 CsA-FK506^R Leu$^+$:1 inviable segregants, and six tetrads with 2 CsA-FK506^R Leu$^-$:2 inviable segregants. Importantly, no *cnb1::LEU2* CsA-FK506-sensitive segregants were obtained. Thus, the *cnb1::LEU2* and CsA-FK506-sensitive mutations are synthetically lethal. We conclude that calcineurin is essential for viability in the Lnr$^+$ strain CHY204 and designate the responsible mutation *cev1*. Disruption of either redundant gene encoding calcineurin A, *CMP1* or *CMP2* had no effect on *cev1* mutant viability, hence either subunit suffices (data not shown).

Cyclophilin A-CsA and FKBP12-FK506 complexes are toxic in *cev1* mutants: CsA and FK506 bind cyclophilin A and FKBP12 to form protein-drug complexes that inhibit calcineurin (LIU *et al.* 1991a). To test if this occurs in the *cev1* mutant, *cev1* mutants lacking cyclophilin A (*cpr1*) and FKBP12 (*fpr1*) were constructed by genetic crosses. The *cev1 cpr1* mutant strain is CsA-resistant and FK506-sensitive, while the *cev1 fpr1* mutant strain is CsA-sensitive and FK506-resistant (Figure 2). Plasmid expression of yeast FKBP12 complemented the *cev1 fpr1* mutant and restored FK506 sensitivity (not shown). Thus, CsA and FK506 toxicity are mediated by cyclophilin A-CsA and FKBP12-FK506 complexes in the *cev1* mutant strain, as in other CsA-FK506-sensitive

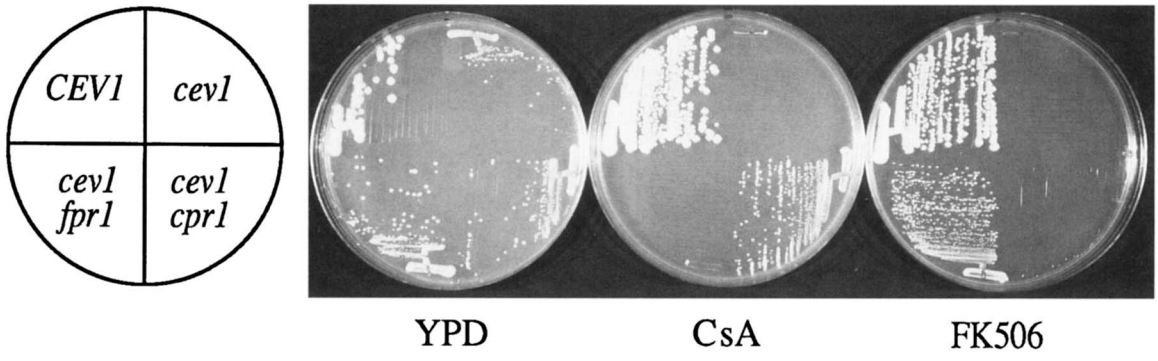

FIGURE 2.—Cyclophilin A-CsA and FKBP12-FK506 complexes are toxic in *cev1* mutants. Isogenic wild-type *CEV1* strain JK93da and isogenic *cev1* mutant strain CHY204, *cev1 cpr1* mutant strain lacking cyclophilin A (TB138-1A), and *cev1 fpr1* mutant strain lacking FKBP12 (TB139-8D) were grown on YPD medium, YPD with 100 μg/ml CsA, or YPD with 1 μg/ml FK506 at 30° for 72 hr.

strains (PARENT *et al.* 1993; BREUDER *et al.* 1994; ENG *et al.* 1994).

Characterization of the *cev1* phenotype: The growth rate and colony size of the *cev1* mutant are decreased relative to the isogenic *CEV1* parent. In liquid YPD medium at 30°, the doubling time of the *cev1* mutant was 180 min compared to 110 min for the *CEV1* wild type. The *cev1* mutant is not overtly cold- or heat-sensitive but does grow poorly on medium containing the nonfermentable carbon source glycerol. The *cev1* mutant phenotype is unlike that of *FKS1*, which encodes 1,3-β-D-glucan synthase (DOUGLAS *et al.* 1994) and when mutated confers CsA-FK506-sensitive growth (PARENT *et al.* 1993; ENG *et al.* 1994), because the growth defect of *fks1* mutants is Ca^{2+}-remediable whereas that of the *cev1* mutant is not (Figure 1).

A number of vacuolar mutants confer a similar phenotype of slow growth, poor utilization of nonfermentable carbon sources, and Ca^{2+} sensitivity (KLIONSKY *et al.* 1990; PRESTON *et al.* 1992). The *cev1* mutant was thus crossed with a collection of mutants (Table 1) that fail to acidify the vacuole or that have defects in vacuolar H^+-ATPase assembly or function (PRESTON *et al.* 1992; BAUERLE *et al.* 1993; HO *et al.* 1993a; MANOLSON *et al.* 1994; GRAHAM *et al.* 1994). The *cev1* mutant strain complemented all of the vacuolar mutant strains except one. In this case, the *cev1* × *vph6* diploid retained the *cev1* mutant phenotypes of CsA, FK506, and Ca^{2+} sensitivity, suggesting *cev1* and *vph6* are allelic, which was confirmed with the cloned *VPH6* gene, as described below.

Isolation, sequence, and chromosomal mapping of the *VPH6* gene: The *VPH6* gene was cloned by complementing the $ZnCl_2$ sensitivity of a *vph6-1* mutant strain (MATERIALS AND METHODS). Two overlapping clones containing an 11.4-kb region were identified. They restored growth in the presence of 4 mM $ZnCl_2$ after rescue in *E. coli* and reintroduction into a *vph6-1* mutant yeast strain.

To determine whether these complementing clones bore the *VPH6* gene, a 3.5-kb *Hind*III-*Sal*I-complementing DNA fragment was cloned in YIp5, linearized with *Xho*I, and used to transform the *vph6-1* mutant strain, resulting in $ZnCl_2$-resistant growth (see MATERIALS AND METHODS). This integrant was crossed to two isogenic strains: BJ1983, a *VPH6* strain, and BJ6757, a *vph6-1* mutant strain. Twenty-seven of 28 tetrads from the cross to the *VPH6* strain yielded 4 $ZnCl_2$-resistant (Vph^+):0 $ZnCl_2$-sensitive (Vph^-) meiotic segregants. All eight tetrads from the cross to the *vph6-1* mutant were parental ditypes, with 2 $ZnCl_2$-resistant:2 $ZnCl_2$-sensitive meiotic segregants. These results prove by linkage analysis that the complementing clone bears the *VPH6* gene.

The *VPH6* gene was localized to a 1.05-kb *Nar*I-*Spe*I fragment within the 11.4-kb insert by subcloning. Sequence analysis revealed a single open reading frame (ORF) of 545 nucleotides, which is predicted to encode a 181-amino acid, 21-kD protein with no significant similarity to other proteins in the current databases. The *VPH6* DNA sequence was identical to YHR060w, a putative ORF identified on the right arm of chromosome *VIII* (JOHNSTON *et al.* 1994), using the NCBI BLAST network service (data not shown).

Location of *VPH6* between *CPR2* and *STE12* on chromosome *VIII* (JOHNSTON *et al.* 1994) was confirmed by genetic mapping. Analysis of 50 tetrads from a *vph6-101/VPH6 ste12::LEU2/STE12* heterozygous diploid (KDY23) revealed that the *VPH6* and *STE12* loci are 15 cM apart, based on 35 parental ditypes:0 nonparental ditype:15 tetratypes. All 35 tetrads from a *vph6-101/VPH6 cpr2::TRP1/CPR2* diploid (KDY22) were parental ditypes, indicating the distance between *VPH6* and *CPR2* is ≤1.4 cM. These findings are consistent with the gene order *CEN (VIII)-CPR2-VPH6-STE12.*

***CEV1* and *VPH6* are allelic:** The cloned *VPH6* gene was shown to complement the *cev1* mutation by transforming strain CHY204 (*cev1*). All nine Ura^+ transformants tested were resistant to 100 μg/ml CsA. Loss of the *VPH6* plasmid by selection of uracil auxotrophs on 5-FOA (BOEKE *et al.* 1984) restored CsA sensitivity. Thus, the *cev1* mutation is complemented by the cloned *VPH6* gene.

CEV1 and *VPH6* were proved allelic by transforming a *cev1* mutant strain with the *VPH6*-integrating vector and crossing the integrant (KDY21) to isogenic *CEV1* and *cev1* strains. All of the 18 tetrads from the cross to wild type yielded 4 $ZnCl_2$-, CsA-resistant:0 $ZnCl_2$-, CsA-sensitive meiotic segregants. The *URA3* marker segregated 2 Ura^+:2 Ura^- in all tetrads as expected. All 10 tetrads from the cross to the *cev1* mutant were parental ditypes, showing 2 $ZnCl_2$-, CsA-resistant Ura^+:2 $ZnCl_2$-, CsA-sensitive Ura^- meiotic segregants. These data demonstrate by linkage analysis that *CEV1* and *VPH6* are allelic. The *cev1* mutation was assigned the *VPH6* allele designation *vph6-101.*

Disruption of the *VPH6* gene results in CsA and FK506 sensitivity: The *VPH6* gene was disrupted by deletion and replacement of a large portion of the ORF with the *TRP1*-selectable marker. Like the *vph6-1* and *vph6-101* mutant strains, the *vph6Δ::TRP1* mutant strain exhibited slow growth, calcium sensitivity, and poor utilization of glycerol as a carbon source compared to the isogenic wild-type parental strain. Furthermore, growth of the *vph6Δ::TRP1* disruption mutant was completely inhibited by 100 μg/ml CsA or 1 μg/ml FK506, while the isogenic wild-type parental strain grew normally in the presence of either drug.

***vph6* mutant cells fail to assemble the vacuolar H^+-ATPase:** Because mutants that fail to assemble the vacuolar H^+-ATPase also fail to acidify the vacuole (HIRATA *et al.* 1993; HILL and STEVENS 1994), we tested whether the *vph6-101* mutant cells are defective in V-ATPase assembly. Total protein extracts and vacuolar fractions from wild-type and *vph6-101* mutant cells were examined by Western blot analysis with antibodies directed against the V-ATPase 95-kD integral membrane subunit

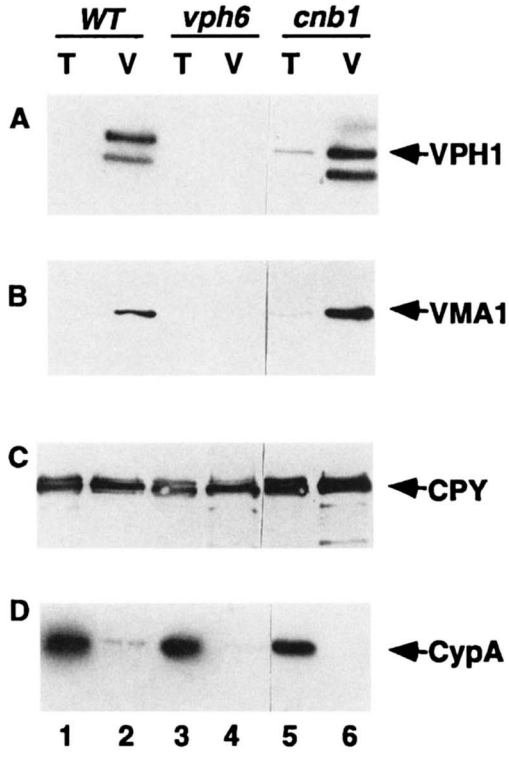

FIGURE 3.—Assembly of the V-ATPase in wild-type and *vph6* strains. Equal amounts of protein from the total cell extracts and vacuolar membrane fractions, prepared as previously described (CONRADT *et al.* 1992), were separated on a 12% (A, C, and D) or 15% (B) SDS-PAGE gel, transferred to nitrocellulose filters, and probed with the following antibodies: A, anti-VPH1; B, anti-VMA1; C, anti-carboxypeptidase Y (CPY); and D, anti-yeast cyclophilin A (CypA). Further experimental details are described in MATERIALS AND METHODS. VPH1 is the 95-kD integral membrane V-ATPase subunit, and VMA1 is the 69-kD peripheral V-ATPase subunit. Carboxypeptidase Y (CPY) and cyclophilin A (CypA) served as vacuolar and cytoplasmic markers, respectively.

VPH1 and the 69-kD peripheral subunit VMA1. In the wild-type strain, the integral membrane subunit VPH1 (Figure 3A, lane 2) is readily detected in the vacuolar fraction and cofractionates with the vacuolar marker carboxypeptidase Y (Figure 3C, lane 2). VPH1 was detected in the total cell lysate, albeit to a lesser extent than in the vacuolar fraction (Figure 3A, lanes 1 and 5). In contrast, VPH1 is virtually undetectable in the vacuolar fraction or total lysates from isogenic *vph6-101* mutant cells (Figure 3A, lanes 3 and 4). This finding agrees with previous reports that demonstrate the integral membrane subunit is promptly degraded and disappears from the cell when not properly assembled into the V-ATPase (KANE 1992; BAUERLE *et al.* 1993; HILL and STEVENS 1994). The peripheral subunit VMA1 is detected in the vacuolar fraction from wild-type cells (Figure 3B, lane 2), but not in the vacuolar fraction from the *vph6-101* mutant strain (Figure 3B, lane 4), indicating that this subunit does not associate with the vacuole in the *vph6-101* mutant. Importantly, the absence of the calcineurin B regulatory subunit had no effect on the total levels or multimeric assembly of these V-ATPase subunits (Figure 3, lanes 5 and 6). These results suggest that the VPH6 protein plays a role in vacuolar H^+-ATPase assembly.

Yeast strains with defects in vacuolar acidification are CsA-FK506-sensitive: We sought to determine whether the CsA-FK506-sensitive phenotype of *vph6* mutant strains is a general characteristic of yeast strains with defects in vacuolar acidification. Mutant strains previously identified as having defects in the V-ATPase or defects in vacuolar acidification (Table 2) were tested for growth on YPD media containing 100 μg/ml CsA or 1 μg/ml FK506. The results are summarized in Table 2. The majority of mutant strains with vacuolar acidification defects were found to be sensitive to both CsA and to FK506. This included strains with mutations affecting structural subunits of the V-ATPase (VMA1, VMA2, VMA5, VMA6, VMA4, VMA7), proteins required for V-ATPase assembly (VMA12 and VMA21), or other proteins required for vacuolar acidification (VPH4, VPH5, VPH8, VPH9). One notable exception is that mutations in *VPH1* or *STV1,* which encode partially redundant 95/100 kD V-ATPAse subunits, do not confer CsA or FK506 sensitivity. Because *VPH1* and *STV1* encode functional homologues (MANOLSON *et al.* 1994), we reasoned that in the *vph1* and *stv1* single mutants, the activity of the remaining subunit might suffice to render growth calcineurin independent. In fact, a *vph1 stv1* double mutant strain is CsA- and FK506-sensitive (Table 2). Based on these results, we conclude that calcineurin is essential in most yeast strains with impaired vacuolar acidification, regardless of the specific mutation causing the defect.

Calcineurin regulates glucose-stimulated extracellular acidification: Because the V-ATPase is a proton pump, other mutations affecting the regulation of cellular pH might impose a requirement for intact V-ATPase and, by extension, the *VPH6* gene. Calcineurin might modulate the activity of proteins that regulate proton transport, thereby accounting for the calcineurin dependence of *vph6* mutant strains. Based on such a model, the *PMA1* gene encoding the yeast plasma membrane proton ATPase is a candidate for regulation by calcineurin. We assessed activity of *PMA1* by measuring extracellular acidification in response to glucose stimulation (SERRANO 1980, 1983). Glucose-stimulated wild-type yeast cells exhibited a progressive acidification of the medium. In contrast, calcineurin mutant strains lacking either the calcineurin B subunit CNB1 or both catalytic subunits CMP1 and CMP2, or wild-type calcineurin strains treated with CsA or FK506, all exhibited an initial glucose-stimulated extracellular acidification that, after ~10 minutes, reversed to extracellular alkalinization (Figure 4A) (data not shown for FK506). As expected, cyclophilin A and FKBP12 were required for CsA- and FK506-dependent inhibition of calcineurin under these conditions (Figure 4B) (data not shown for FK506). The full effect of CsA on extracellular acidification required exposure to the drug for significant

TABLE 2

Effects of CsA and FK506 on yeast vacuolar mutants

Gene	Protein function	Growth of mutant on		Reference
		CsA	FK506	
VPH1	95 kDa V-ATPase subunit	+	+	MANOLSON *et al.* (1992)
STV1	102 kDa homologue of VPH1	+	+	MANOLSON *et al.* (1994)
vph1 stv1 mutant		−	−	
TFP1/VMA1	69 kDa V-ATPase subunit	−	−	HIRATA *et al.* (1990), SHIH *et al.* (1988)
VAT2/VMA2	60 kDa V-ATPase subunit	−	−	NELSON and NELSON (1989); YAMASHIRO *et al.* (1990)
VMA13	54 kDa V-ATPase subunit	+	−	HO *et al.* (1993b)
VMA5	42 kDA V-ATPase subunit	−	−	HO *et al.* (1993a)
VMA6	36 kDa V-ATPase subunit	−	−	BAUERLE *et al.* (1993)
VMA8	32 kDa V-ATPase subunit	nt	nt	NELSON *et al.* (1995)
VMA4	27 kDa V-ATPase subunit	−	−	HO *et al.* (1993a)
VMA3	17 kDa V-ATPase subunit	−	−	NELSON and NELSON (1989)
VMA11	17 kDa V-ATPase subunit	+	−	UMEMOTO *et al.* (1991)
VMA7	14 kDa V-ATPase subunit	−	−	GRAHAM *et al.* (1994)
CEV1/VPH6	This study, predicted 21 kDa protein involved in V-ATPase assembly	−	−	
VMA12/VPH2	25 kDa protein required for V-ATPase assembly	−	−	BACHHAWAT *et al.* (1993); HIRATA *et al.* (1993)
VMA21	8.5 kDa protein required for V-ATPase assembly	−	−	HILL and STEVENS (1994)
VPH4	Vacuolar acidification	±	±	PRESTON *et al.* (1992)
VPH5	Vacuolar acidification	−	−	PRESTON (1992)
VPH7	Vacuolar acidification	+	+	PRESTON (1992)
VPH8	Vacuolar acidification	−	−	PRESTON (1992)
VPH9	Vacuolar acidification	±	±	PRESTON (1992)
PEP3	Vacuolar membrane protein	±	±	PRESTON (1992)
PEP5	Vacuolar membrane protein	±	±	PRESTON (1992)

+, drug resistant; ±, partially drug-sensitive; −, drug-sensitive; nt, not tested. Growth was tested on YPD medium containing 100 μg/ml CsA or 1 μg/ml FK506. *pep7, pep8, pep12, pep14, pep21* mutant strains (PRESTON *et al.* 1992) were not CsA- or FK506-sensitive.

amounts of time (throughout the entire growth phase of the cells), and little effect was observed when CsA was added to washed cells 5 hr before the assay (Figure 4C). Thus, calcineurin modulates the process of glucose-stimulated proton efflux and may participate in the regulation of intracellular pH.

A *vph6Δ::TRP1* mutant strain also exhibited abnormalities in extracellular acidification in response to glucose stimulation (Figure 4D). Both the rate and degree of extracellular acidification were decreased compared to the isogenic wild-type parental strain. In contrast to calcineurin mutant strains, however, no increase in the pH of the medium was observed once the minimum pH was attained. To confirm that this finding was a general feature of V-ATPase mutants, the response of a *vma5::LEU2* mutant strain was tested. Again, the rate and degree of glucose-stimulated extracellular acidification was diminished relative to the wild-type parental strain and no late increase in pH was observed (data not shown).

DISCUSSION

Calcineurin is not essential for viability of wild-type strains of *S. cerevisiae*. In fact, the phenotypes of mutants lacking either the two genes encoding the catalytic subunit *CMP1* and *CMP2*, or the regulatory subunit *CNB1* are subtle. Such mutants display normal vegetative growth but cannot grow in the presence of high concentrations of Na^+ or Li^+ salts and are unable to recover from pheromone-induced G1 arrest (CYERT *et al.* 1991; CYERT and THORNER 1992; FOOR *et al.* 1992; NAKAMURA *et al.* 1993; BREUDER *et al.* 1994; CARDENAS *et al.* 1994a). *S. cerevisiae* mutants in which calcineurin is essential have recently been described (PARENT *et al.* 1993; BREUDER *et al.* 1994). Additionally, calcineurin is essential in the fungus *Aspergillus nidulans* (RASMUSSEN *et al.* 1994). Due to the highly conserved nature of calcineurin, analysis of calcineurin-dependent yeast mutants should provide insight into the functions of this protein phosphatase in yeast and possibly other organisms.

We identified a mutation that renders calcineurin essential, *cev1*, and demonstrate that it is an allele of the *VPH6* gene. The *vph6* mutation was originally isolated in a screen for yeast mutants that are defective in vacuolar acidification (PRESTON *et al.* 1992). Having established that *CEV1* and *VPH6* are the same gene, the role of the yeast vacuole in maintaining cellular homeostasis

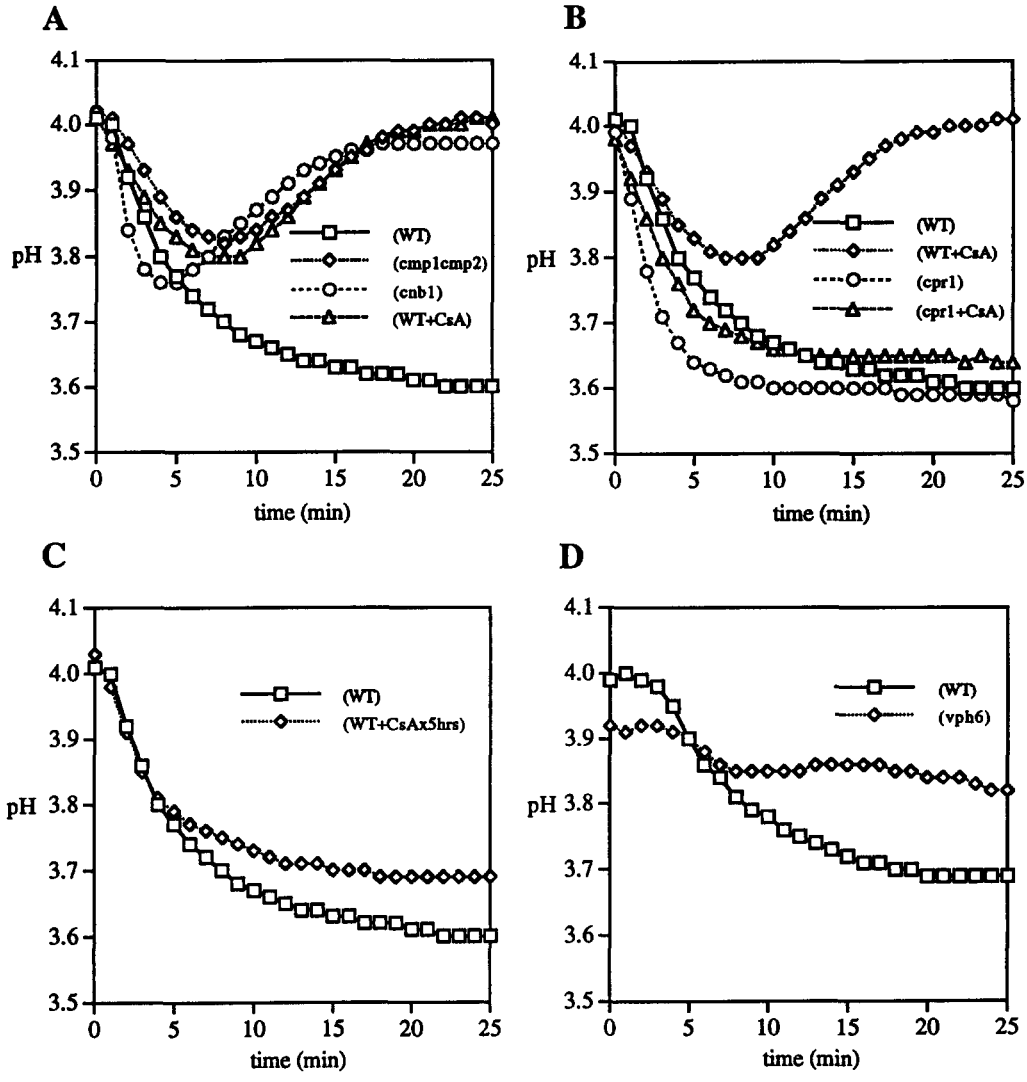

FIGURE 4.—Glucose-stimulated extracellular acidification. Acidification of the extracellular medium in response to glucose was measured as described in MATERIALS AND METHODS. Yeast strains were grown in YPD medium and washed in distilled water or grown in YPD medium containing 100 μg/ml CsA and washed in water with 100 μg/ml CsA (+CsA). (A) Wild-type strain JK93da (WT), isogenic calcineurin *cmp1 cmp2* mutant strain TB68 (cmp1 cmp2) and isogenic calcineurin *cnb1* mutant strain TB85 (cnb1). (B) Wild-type strain JK93da (WT) and isogenic cyclophilin *cpr1* mutant strain MH250–2C (cpr1). (C) Wild-type strain JK93da (WT) was grown in YPD medium and washed in water. Cells were then incubated on ice in water containing CsA 100 μg/ml for 5 hr (CsA × 5hr). (D) Wild-type yeast strain 9933–20C (WT) and isogenic *vph6* mutant strain CHY251 (vph6). Results presented are representative of several similar experiments.

becomes relevant to the discussion of calcineurin function. This is further supported by our finding that most known vacuolar acidification mutants are CsA- and FK506-sensitive and thus also require calcineurin for viability. Like the lysosomes of multicellular eukaryotes, the yeast vacuole contains proteases and hydrolytic enzymes and is also an important storage site for amino acids and inorganic ions. Accumulation of these small molecules against a concentration gradient is coupled to an electrochemical gradient generated by the V-ATPase, a large multimeric complex that pumps protons into and acidifies the vacuole (KLIONSKY *et al.* 1990; KANE 1992). For example, Ca^{2+} is concentrated in the vacuole by a H^+/Ca^{2+} antiporter, which is dependent on the electromotive force established by the proton pump (OHSUMI and ANRAKU 1983). Given that multiple genes contribute to function of the V-ATPase and vacuolar acidification, many CsA-FK506-sensitive yeast strains may harbor mutations in the *VMA* or *VPH* genes.

Our studies reveal that mutations in the *VPH6* gene render cells unable to assemble the vacuolar H^+-ATPase (Figure 3). Previous studies have identified the *VMA21* gene product as an endoplasmic reticulum (ER) protein required for the multimeric assembly of the V-ATPase and have led to the proposal that this complex is assembled in the ER (HILL and STEVENS 1994). The *VMA12/VPH2* gene product is also required for V-ATPase assembly but is not a subunit of the pump (BACHHAWAT *et al.* 1993; HIRATA *et al.* 1993). Although at present we do not know the cellular localization of the VPH6 protein and the VPH6 sequence does not predict any biochemical functions or localization motifs, our

results are most consistent with a role for the VPH6 protein in assembly of the V-ATPase. VPH6 is not likely to be a subunit of the pump because all of the subunits of the V-ATPase have been biochemically identified and their genes cloned; none bear similarities to VPH6.

Mutations in *VMA* genes that result in defective assembly of the V-ATPase produce a Ca^{2+}-sensitive phenotype characterized by a sixfold increase in free cytoplasmic Ca^{2+} when grown on YPD (OHYA *et al.* 1991). *vph* mutant strains are also Ca^{2+}-sensitive (PRESTON *et al.* 1992). Is it possible that increased cytoplasmic [Ca^{2+}] renders the catalytic activity of calcineurin essential? In this scenario, a calcineurin-regulated pathway might prevent accumulation of toxic free Ca^{2+} in V-ATPase mutants. Alternatively, calcineurin may activate compensatory pathways that enable survival despite increased free Ca^{2+}. This proposed mechanism of toxicity seems unlikely given findings involving a vacuolar Ca^{2+}-ATPase encoded by the *PMC1* gene (CUNNINGHAM and FINK 1994). Like the *vma* mutants, the *pmc1* mutant is inferred to have increased levels of cytoplasmic Ca^{2+}. This is thought to be due to a defect in the vacuolar Ca^{2+}-ATPase that functions independently of the H^+/Ca^{2+} antiporter. In contrast to the *vph6* mutant, however, the *pmc1* mutant is actually rendered resistant to exogenously added Ca^{2+} when calcineurin is inhibited by CsA or FK506 or inactivated by mutation (CUNNINGHAM and FINK 1994). This observation leads us to conclude that elevated free Ca^{2+} *per se* does not make calcineurin activity essential.

Additional models explaining the calcineurin dependence of the *vph6* mutant strain may also be proposed. Strains with mutations impairing the V-ATPase have recently been shown to require an intact endocytic pathway to maintain viability. Hence, a *vat2 end3* double mutant is inviable (MUNN and RIEZMAN 1994). It has been suggested that fluid phase endocytosis in an acidic environment is sufficient to maintain a functional vacuolar pH gradient in the absence of the V-ATPase (NELSON and NELSON 1990). Calcineurin could therefore be essential for endocytosis; however, this is unlikely. First, not all mutants defective in vacuolar acidification require endocytosis (G. WEBB and E. JONES, unpublished results). Second, calmodulin mutant yeast strains lacking the high affinity Ca^{2+}-binding sites are fully capable of endocytosis whereas calcineurin activation requires calmodulin-Ca^{2+} binding (KUBLER *et al.* 1994; STEMMER and KLEE 1994; PERRINO *et al.* 1995).

More recently, the requirement of calcineurin in yeast grown under conditions of cation stress has been recognized. In an analysis of calcineurin mutants, NAKAMURA *et al.* (1993) found that growth was inhibited by 1.0 M NaCl or by 140 mM LiCl. Moreover, this effect was cation specific as the acetate salts yielded similar results. Neither KCl, $CaCl_2$, $MgCl_2$, nor nonspecific increases in osmolarity of the growth medium affected growth of calcineurin mutants relative to the wild-type strain. A marked diminution in Na^+ export capacity of calcineurin mutants was suggested by measurement of Na^+ efflux from Na^+-loaded cells. The principal Na^+ export channel in *S. cerevisiae* is the *ENA1* gene product (HARO *et al.* 1991). Li^+ is also efficiently transported by this plasma membrane protein, a member of the P-type ATPase family. Mendoza *et al.* (1994) have found that *ENA1* gene transcription and protein expression is reduced twofold in a calcineurin mutant exposed to lithium. The reduction in *ENA1* transcription parallels the twofold reduction in Li^+ efflux noted in this mutant. It thus appears that calcineurin is required for the cell to sense or to respond to the accumulation of intracellular Na^+ or Li^+ and increase *ENA1* expression. We find that the related *ENA2* gene is a multi-copy suppressor of the Li^+-sensitive phenotype of a calcineurin null mutation (unpublished findings), which is consistent with these observations. The *ENA2* gene product differs from that of *ENA1* by only 13 amino acids. However, the *ENA2* gene cannot fully substitute for *ENA1* unless expressed from the *ENA1* promoter region (GARCIADEBLAS *et al.* 1993). Presumably, overexpression of *ENA2* obviates the requirement for calcineurin for adequate *ENA1* expression and function. Nevertheless, *ENA2* overexpression does not suppress the phenotype of a *vph6* mutant strain or eliminate its requirement for calcineurin, and a *vph6 ena1* double mutant strain is viable (data not shown), indicating that ENA1 is not the only critically regulated target of calcineurin in a *vph6* mutant strain.

Our findings that calcineurin participates in glucose-stimulated extracellular acidification may also be relevant to understanding the interaction between calcineurin and the V-ATPase. PMA1, the plasma membrane H^+-ATPase, is responsible for glucose-stimulated proton efflux, suggesting that calcineurin may regulate PMA1. It has recently been demonstrated that H^+, as well as Ca^{2+}, are important in the activation of calcineurin by calmodulin (HUANG and CHEUNG 1994), thus calcineurin may be well suited to regulate intracellular pH. In addition, the PMA1 protein is known to be regulated by phosphorylation. Within 2 min of glucose addition, specific serine and threonine residues of PMA1 are phosphorylated concomitant with enzymatic activation (CHANG and SLAYMAN 1991). Because both an increase in cytoplasmic Ca^{2+} concentration and a fall in pH occur 2–3 min after glucose stimulation (EILAM *et al.* 1990; NAKAJIMA-SHIMADA *et al.* 1991), it is tempting to speculate that activation of calcineurin by a glucose-stimulated increase in H^+ and Ca^{2+} leads to the downstream activation of a protein kinase(s) that phosphorylates PMA1. In this scenario, calcineurin would regulate pH by being activated in response to intracellular acidification. Calcineurin and its targets may thus function in conjunction with the V-ATPase to maintain cytoplasmic pH within narrow limits. However, our findings that calcineurin mutant yeast strains are capable of glucose-stimulated extracellular acidification for up to 10 min and that prolonged preincubation with CsA is required for the drug's effect suggest that the relationship

between calcineurin and PMA1 is more complex than direct activation of a protein kinase acting directly on PMA1. The recent finding that the V_1 and V_0 sectors of the V-ATPase dissociate during glucose starvation and reassemble upon glucose addition (KANE 1995) suggests another means to regulate intracellular pH. It is unknown whether this process is controlled, directly or indirectly, by calcineurin. Finally, we note that our results do not exclude other possible roles for calcineurin, such as regulation of membrane permeability or channels that might also account for abnormal extracellular acidification. Further studies will be required to determine the precise role of calcineurin in PMA1 regulation and the relationship of V-ATPase activity to glucose-stimulated extracellular acidification.

The calcineurin dependence of the *vph6* mutant under all growth conditions must therefore still be explained. Here, the role of the vacuole and other acidified membrane compartments in cation homeostasis must again be considered. The vacuole is capable of sequestering H^+, Na^+, and Li^+ (ORTEGA 1988; PERKINS and GADD 1993). We propose that the PMA1 protein, the ENA1 protein, and perhaps additional, as yet unidentified, calcineurin-modulated plasma membrane channels are the major regulators of intracellular protons and Na^+/Li^+. Nevertheless, the acidified yeast vacuole serves an important protective function by sequestering protons or Na^+/Li^+ should these primary regulators be impaired. H^+ and Na^+ efflux is diminished in calcineurin mutants, and, according to this model, acidified membrane compartments, including the vacuole, assume a critical role in maintaining viability even under normal growth conditions. In combination, the loss of both calcineurin activity and V-ATPase function is lethal.

After this manuscript had been submitted, we learned of similar findings that calcineurin is essential in vacuolar mutants by TANIDA *et al.* (1995) and GARRETT-ENGELE *et al.* (1995).

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