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Calcineurin Inhibits *VCX1*-Dependent H^+/Ca^{2+} Exchange and Induces Ca^{2+} ATPases in *Saccharomyces cerevisiae*

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The *PMC1* gene in *Saccharomyces cerevisiae* encodes a vacuolar Ca^{2+} ATPase required for growth in high-Ca^{2+} conditions. Previous work showed that Ca^{2+} tolerance can be restored to *pmc1* mutants by inactivation of calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase sensitive to the immunosuppressive drug FK506. We now report that calcineurin decreases Ca^{2+} tolerance of *pmc1* mutants by inhibiting the function of *VCX1*, which encodes a vacuolar H^+/Ca^{2+} exchanger related to vertebrate Na^+/Ca^{2+} exchangers. The contribution of *VCX1* in Ca^{2+} tolerance is low in strains with a functional calcineurin and is high in strains which lack calcineurin activity. In contrast, the contribution of *PMC1* to Ca^{2+} tolerance is augmented by calcineurin activation. Consistent with these positive and negative roles of calcineurin, expression of a *vcx1*::*lacZ* reporter was slightly diminished and a *pmc1*::*lacZ* reporter was induced up to 500-fold by processes dependent on calcineurin, calmodulin, and Ca^{2+}. It is likely that calcineurin inhibits *VCX1* function mainly by posttranslational mechanisms. Activities of *VCX1* and *PMC1* help to control cytosolic free Ca^{2+} concentrations because their function can decrease *pmc1*::*lacZ* induction by calcineurin. Additional studies with reporter genes and mutants indicate that *PMR1* and *PMR2A*, encoding P-type ion pumps required for Mn^{2+} and Na^+ tolerance, may also be induced physiologically in response to high-Mn^{2+} and -Na^+ conditions through calcineurin-dependent mechanisms. In these situations, inhibition of *VCX1* function may be important for the production of Ca^{2+} signals. We propose that elevated cytosolic free Ca^{2+} concentrations, calmodulin, and calcineurin regulate at least four ion transporters in *S. cerevisiae* in response to several environmental conditions.

The immunosuppressive drugs cyclosporin A and FK506 block activation of human T cells by specifically inactivating calcineurin, which is necessary for induction of interleukin-2 and other genes (for a review, see reference 59). Though these drugs are structurally unrelated, they both require binding to cytosolic receptor proteins, cyclophilin and FKBP-12, respectively, to form inactive complexes with calcineurin. Calcineurin may have additional functions in other cell types, and the loss of these functions may contribute to the side effects of these drugs, which include nephrotoxicity, neurotoxicity, and osteoporosis (13). Recent studies have implicated calcineurin as part of a regulatory cascade leading to control of the Na^+/H^+ exchanger in T cells (56), the Na^+/K^+ ATPase in kidney cells (5), and the *N*-methyl-D-aspartate receptor in neurons (42, 63). A better understanding of the biological roles for calcineurin in different cell types may promote the development of improved strategies for immunosuppression.

In the budding yeast *Saccharomyces cerevisiae*, homologs of calcineurin catalytic (15, 39, 68) and regulatory (16, 35) subunits have been cloned and characterized. The protein phosphatase activity of yeast calcineurin is stimulated by binding Ca^{2+}/calmodulin and inhibited by binding FK506 and cyclosporin A complexes with the yeast FKBP-12 and cyclophilin A homologs (18, 51), which are encoded by *FPR1* (11, 29) and *CPR1* (27, 64), respectively. Mutants lacking calcineurin function are partially defective in the recovery from prolonged exposure to mating pheromones (15, 16, 18). Calcineurin-deficient mutants also exhibit decreased tolerance to Na^+, Li^+, and other ions (40, 45). Recently, inactivation of calcineurin has been shown to increase Ca^{2+} tolerance of at least one Ca^{2+}-sensitive mutant (14) and to decrease Ca^{2+} tolerance of others (21, 62). In none of these situations has the target(s) for calcineurin been identified.

The yeast genes *PMC1* and *PMR1* encode membrane proteins related to mammalian plasma membrane Ca^{2+} ATPases and sarcoendoplasmic reticulum Ca^{2+} pumps, respectively (4, 14, 57). Although the Pmc1p and Pmr1p proteins are localized to different organelles (Pmc1p to the vacuole and Pmr1p to the Golgi complex), they both function in Ca^{2+} sequestration and promote growth in media containing high Ca^{2+} concentrations. Mutants lacking *PMC1* function grow poorly in media containing 200 mM Ca^{2+}, and *pmc1 pmr1* double mutants are inviable even at low Ca^{2+} concentrations. Both of these effects are reversed by the action of FK506/FKBP-12 and cyclosporin A/cyclophilin A, by null mutations in the calcineurin A or B subunit, or by point mutations in calmodulin (14) which destroy its high-affinity Ca^{2+} binding sites (22). One interpretation of these results is that although calcineurin activation by Ca^{2+}/calmodulin is not essential for vegetative growth, it may inhibit the function of another factor that is necessary for growth under high-Ca^{2+} conditions.

This report describes the cloning and characterization of a low-affinity vacuolar H^+/Ca^{2+} exchanger (17, 47, 50), whose function in vivo is inhibited by calcineurin activation. *VCX1* functions in Ca^{2+} tolerance and Ca^{2+} sequestration much more efficiently when calcineurin is inactivated that when it is activated. Studies of *vcx1* mutants suggest that calcineurin also promotes the expression of *PMC1*, *PMR1*, and *PMR2A*, which increases their contribution to tolerance of Ca^{2+}, Mn^{2+}, and Na^+. The intracellular Ca^{2+} transporters encoded by *VCX1*, *PMC1*, and *PMR1* help to control cytosolic free Ca^{2+} con-

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centrations ($[Ca^{2+}]c$) in high-Ca^{2+} conditions and thereby modulate calcineurin function. We suggest that this feedback control mechanism is important to promote efficient Ca^{2+} signaling in yeast cells exposed to a wide range of environmental conditions.

MATERIALS AND METHODS

Yeast culture media. Standard yeast culture media (60) were prepared from reagents supplied by Difco or BBL. YPD pH 5.5 medium was prepared and supplemented with $CaCl_2$ (Sigma catalog no. C 2536) and either Difco Noble Agar or BBL granulated agar as described previously (14). Samples of FK506 were generously provided by Vertex Pharmaceuticals, Inc. (Cambridge, Mass.) and Fujisawa Corp. (Tokyo, Japan) and added where indicated from 0.2-mg/ml stock solutions in dimethyl sulfoxide.

Cloning of *VCX1* and recombinant DNA. All procedures involving recombinant DNA in *S. cerevisiae* and *Escherichia coli* were performed by using standard techniques (58) as follows. The *pmc1*::*LEU2* strain K473 was transformed with a high-copy-number library of yeast genomic DNA carried on the vector pRS202 (13a) and plated on synthetic complete (SC)-Ura agar medium at a density of approximately 5,000 colonies per plate. After 2 days of incubation at 30°C, the colonies were replica plated to YPD pH 5.5 medium supplemented with 200 mM $CaCl_2$ and incubated at 30°C for 3 days. Ca^{2+}-tolerant clones were recovered, and plasmid DNA was isolated and characterized by restriction mapping. Of 24 plasmids isolated independently by this approach, 1 plasmid contained *PMC1*, 1 plasmid contained *PMR1*, 2 plasmids contained overlapping inserts spanning *VCX1*, and 20 plasmids contained overlapping inserts spanning *CRP1*, a previously undescribed gene locus currently under evaluation. Deletion mapping revealed that the functional *VCX1* gene was carried on a 1.7-kb minimal fragment which hybridized to lambda-prime clone 2439 representing a new locus on chromosome IV (55). The 1.7-kb fragment in pRS202 (pKC159) was sequenced on both strands with full overlap, using a nested deletion strategy (Amersham). Sequencing and restriction mapping of adjacent sequences indicate that *VCX1* is adjacent to *CDC48* and *ORF-D* (19). To construct pKC72 used for creating *vcx1* null mutants, the 1.7-kb fragment containing *VCX1* was first subcloned into plasmid pBSII (Stratagene), and then the 559-bp segment between the *Stu*I and *Msc*I sites in *VCX1* was replaced with a 5.0-kbp fragment of pSE1076 containing *URA3* and kanamycin resistance flanked by 1.1-kbp direct repeats of *hisG* (1). The *VCX1*::hemagglutinin (HA) epitope-tagged variant (*VCX1*::*HA*) on pKC142 was constructed by a multistep process. A 7.5-kbp genomic DNA fragment spanning *VCX1* was first inserted into low-copy-number centromere-based plasmid pRS316 (61), forming pKC98. Site-directed mutagenesis of pKC98 was performed to create a unique *Nhe*I cleavage site at codon +3 (forming pKC140), into which was inserted a 96-bp *Xba*I fragment of pKC54 (14) coding for three repeats of the HA epitope (forming pKC142). Sequencing and complementation assays of pKC140 and pKC142 confirmed that the expected modifications were successful and functional in yeast strains. To rescue the *VCX1-D1* allele, a 0.78-kbp *Sac*I-to-*Cla*I fragment of pKC159 containing the 5′ end of *VCX1* was inserted into pRS303 (forming pKC106), which was digested with *Stu*I and integrated into the *VCX1* locus by transformation of strains K482 and K482-1 (14). Genomic DNA prepared from these transformed strains was digested with *Sal*I and treated with T4 DNA ligase, and plasmids pKC129 (*VCX1*) and pKC130 (*VCX1-D1*) were recovered by transformation into *E. coli* DH5α and then sequenced by using complementary oligonucleotides. Reintegration of pKC130 into *pmc1* mutants produced the original Ca^{2+}-tolerant phenotype associated with *VCX1-D1*. Plasmid pKC151 was isolated from a cDNA library (38) and contained a functional *PMC1* cDNA under control of the *GAL1* promoter in pRS316-*gal*.

A series of *lacZ* reporter genes was constructed from the well-characterized *cyc1*::*lacZ* reporters pLGΔ312 and pLGΔ178 (24). The *vcx1*::*lacZ* reporter plasmid pKC200 was constructed by first removing the *cyc1* sequences from pLGΔ178 with *Xho*I and *Bam*HI and then inserting a 2.0-kbp *Hin*dIII-to-*Nhe*I segment of pKC142 along with flanking polylinker sequences from pBSII and YEp356R (44). The *pmr1*::*lacZ* reporter plasmid pKC199 was constructed by inserting a 1,180-bp *Xho*I-*Bam*HI fragment of pKC11 (37) into *Xho*I-plus-*Bam*HI-digested pLGΔ178. The *pmr2A*::*lacZ* reporter plasmid pKC201 contained a 1.4-kbp *Sal*I-*Eco*RI fragment from plasmid B1999 (57) inserted into *Xho*I-plus-*Bam*HI-digested pLGΔ178 along with flanking polylinker sequences from pBSII. The reporter plasmid pKC190 containing *pmc1*::*lacZ* was constructed by inserting a 585-bp *Hin*dIII-*Xba*I fragment of pKC45 (14) plus flanking polylinker sequences from YEp356R into *Xho*I-plus-*Bam*HI-digested pLGΔ178. pKC211 is a derivative of pKC190 in which 413 bp of *PMC1* untranslated DNA (from *Hin*dIII at −579 to *Afl*II at −166 relative to the initiation codon) is deleted, leaving 172 bp of *PMC1* 5′ untranslated DNA containing putative transcriptional and translational initiation sequences. pKC191 is a derivative of pKC190 and pLGΔ178 in which *cyc1* transcriptional and translational initiation sequences (from *Xho*I to *Bam*HI) replace the 172-bp *Afl*II-*Bam*HI fragment of pKC190, leaving 413 bp of *PMC1* 5′ untranslated DNA (from *Hin*dIII to *Afl*II). pLB178-43 contains three copies of oligonucleotide TCCACGAAAA, which confers cell cycle regulation on *HO*, inserted into the *Xho*I site of pLGΔ178 (10).

Construction of yeast strains. All yeast strains listed in Table 1 are derivatives of strain W303-1A (65) which were constructed through transformation or isogenic crosses by using standard techniques (26). The *vcx1*::*URA3* null allele was originally created by transformation of diploid strain KCX178 (*MAT***a**/α *cnb1*::*LEU2*/+ *pmc1*::*TRP1*/+*pmr2*::*HIS3*/+) with the 5.7-kbp *Kpn*I-to-*Sac*I fragment of pKC72. Uracil prototrophs were screened by Southern blotting to confirm replacement of one *VCX1* allele with *vcx1*::*URA3* and then sporulated and subjected to tetrad analysis. The *vcx1*Δ null allele was recovered by selection against *vcx1*::*URA3* on 5-fluoro-orotic acid medium (9). The *pmr2*::*HIS3* null mutation was first introduced into strain K473 by using plasmid B2000 (57) and confirmed by Southern blotting. A Ca^{2+}-tolerant and 5-fluoro-orotic acid-resistant transformant of K619 with a 7.5-kbp *Kpn*I-to-*Sac*I fragment of pKC142 was isolated to obtain strain K697 in which *vcx1*::*URA3* is replaced with *VCX1*::*HA*. Strain K699 was constructed by selecting for uracil auxotrophs of K470 and screening for retention of the triple HA epitopes by Western blotting (immunoblotting). All other strains were derived from crosses with other strains in the W303-1A background, and in each case no independently segregating suppressors or enhancers were evident.

TABLE 1. Yeast strains used in this study

Strain	Genotype[a]	Reference
W303-1A	+	65
JGY41	*cmd1-3*	22
K470	*PMC1*::*HA* (*pmc1*::*URA3*)	14
K473	*pmc1*::*LEU2*	14
K603	*cnb1*::*LEU2*	14
K605	*pmc1*::*TRP1*	14
K607	*cnb1*::*LEU2 pmc1*::*TRP1*	14
K609	*pmr1*::*HIS3*	14
K617	*vcx1*::*URA3*	
K619	*cnb1*::*LEU2 vcx1*::*URA3*	
K625	*pmr1*::*HIS3 vcx1*::*URA3*	
K633	*pmr2*::*HIS3*	
K635	*cnb1*::*LEU2 pmr2*::*HIS3*	
K641	*pmr2*::*HIS3 vcx1*::*URA3*	
K643	*cnb1*::*LEU2 pmr2*::*HIS3 vcx1*::*URA3*	
K661	*vcx1*Δ	
K663	*cnb1*::*LEU2 vcx1*Δ	
K665	*pmc1*::*TRP1 vcx1*Δ	
K667	*cnb1*::*LEU2 pmc1*::*TRP1 vcx1*Δ	
K669	*cmd1-3 vcx1*Δ	
K673	*cmd1-3 pmc1*::*TRP1 vcx1*Δ	
K685	*cmd1-3*	
K689	*cmd1-3 pmc1*::*TRP1*	
K697	*VCX1*::*HA cnb1*::*LEU2*	
K698	*VCX1*::*HA*	
K699	*PMC1*::*HA*	
K737	*pmc1*::*TRP1 VCX1-D1*	

[a] All strains contain additional mutations (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and are isogenic to W303-1A.

Immunological techniques. Immunofluorescence microscopy was performed on log-phase yeast cells as described previously (14) except that ascites fluid containing anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim) was used at a dilution of 1:400 and cyanine-3-conjugated goat anti-mouse immunoglobulin G (Rockland, Inc.) was used at a dilution of 1:250. For Western blot analysis (58), log-phase cultures were harvested, lysed with NaOH plus β-mercaptoethanol, and then precipitated with trichloroacetic acid (53). Precipitates were solubilized at 65°C for 10 min in 0.2 ml of sodium dodecyl sulfate (SDS) sample buffer, separated on polyacrylamide gels, transferred to Hybond Super-C (Amersham), stained with Ponceau S (Sigma), and imaged using with an enhanced chemiluminescence detection kit (Amersham) after probing with monoclonal antibody 12CA5 at a dilution of 1:4,000.

$^{45}Ca^{2+}$ transport in cell lysates. Uptake of $^{45}Ca^{2+}$ into particles of crude spheroplast lysates was quantitated by using a previously developed method (17), with slight modifications. Briefly, yeast strains were grown to mid-log phase in YPD medium or SCGal-Ura medium and then harvested, washed once in buffer TS (10 mM Tris-Cl [pH 8.0], 1 M sorbitol), and digested with 0.1 mg of Zymolyase 100T (Seikagaku) per ml for 45 min at 37°C in buffer TS plus 0.1% β-mercaptoethanol. The resulting spheroplasts were washed twice with 1 ml of buffer TS, resuspended in 1 ml of YPD medium supplemented with 0.6 M sorbitol and 50 mM potassium phosphate (pH 7.5), and incubated for 1 h at 37°C. The partially regenerated spheroplasts were cooled on ice, washed once with ice-cold buffer A [10 mM sodium piperazine-*N*,*N*′-bis(ethanesulfonic acid) (Na-PIPES), 10 mM sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Na-HEPES), 2 mM $MgCl_2$, 100 mM KCl, 0.6 M sorbitol (pH 7.0)], and cell

pellets were resuspended with 20 μl of buffer A on ice. Three minutes before the reactions were initiated, each cell suspension was lysed osmotically by diluting 10 μl into 1.0 ml of prewarmed buffer B (10 mM Na-PIPES, 10 mM Na-HEPES, 2 mM $MgCl_2$, 100 mM KCl [30°C, pH 7.0]) supplemented with 10 or 100 μM $CaCl_2$, 1 to 2 μCi of [^{45}Ca]Cl_2 (New England Nuclear) per ml, and where indicated 10 μM concanamycin B (generous gift of H. L. Ploegh, Massachusetts Institute of Technology). Reactions were initiated at time zero by the addition of ATP to 1 mM from a 100 mM pH 7.0 stock solution. At the indicated time intervals, 100- or 200-μl aliquots were removed, diluted into 4 ml of ice-cold 20 mM $MgCl_2$, rapidly filtered onto 2.4-cm GFF filters (Whatman), and washed three times with the same solution. Filters were dried, and radioactivity was quantitated by liquid scintillation counting. Ca^{2+} uptake per 10^9 lysed cells was calculated from the measured counts, the specific activity, and culture turbidity.

Measurement of nonexchangeable Ca^{2+} pools in living cells. Yeast strains were grown to mid-log phase (optical density at 600 nm [OD_{600}] = 0.7 to 1.8) at 30°C in YPD medium, harvested, and then resuspended to a final OD_{600} of 0.1 in 1.5 ml of fresh YPD pH 5.5 medium supplemented with 5 mM $CaCl_2$ and ~20 μCi of [^{45}Ca]Cl_2 per ml. After growth at 30°C for an additional 7.25 h (four to five doublings in cell density), cultures were harvested, washed twice with medium lacking $^{45}Ca^{2+}$, resuspended in an equal volume, and incubated for an additional 40 min at 30°C to allow efflux of the exchangeable Ca^{2+} pool. Total cell-associated radioactivity was determined by rapid filtration and liquid scintillation counting (14), and total cell number was determined by turbidity measurements. The average of duplicate samples was used to calculate the amount of Ca^{2+} remaining in the nonexchangeable pool. The levels of Ca^{2+} in the exchangeable pool was relatively constant in these strains at 2.6 ± 0.7 (standard deviation) nmol/10^9 cells.

Ion tolerance assays. Yeast strains were inoculated into YPD pH 5.5 medium and grown overnight at 30°C. Saturated cell suspensions were then diluted 500-fold into 8 or 12 0.2-ml cultures in 96-well flat-bottom dishes containing YPD pH 5.5 medium supplemented with a wide range of $CaCl_2$, $MnCl_2$, or NaCl. Suspensions were mixed and incubated 16 to 20 h at 30°C without shaking, and the OD_{570} was measured for each resuspended culture, using a microplate spectrophotometer (Dynatech Laboratories). All added salts were completely soluble under these conditions. The amount of each cation causing a 50% decrease in cell growth relative to the growth of unsupplemented cultures was interpolated from linear plots of the data.

Miscellaneous procedures. Total cellular β-galactosidase was determined at room temperature in cells permeabilized with chloroform and SDS (23), using the substrate *o*-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co.).

Nucleotide sequence accession number. The DNA sequences of *VCX1* and *VCX1-D1* are listed in the GenBank and EMBL databases under accession number U36603.

RESULTS

Isolation and analysis of *VCX1*. Calcineurin appears to inhibit a factor required for growth of *pmc1* mutants in media containing high Ca^{2+} concentrations (14). To identify this target, we isolated genes from a high-dosage library which, when overexpressed, restored growth to *pmc1* mutants in YPD pH 5.5 medium containing 200 mM $CaCl_2$ (see Materials and Methods). Analysis of 24 independent clones yielded plasmids containing four different genes: *PMC1*, *PMR1*, and two novel genes termed *CRP1* and *VCX1*. The predicted 411-amino-acid product of *VCX1* (Fig. 1) indicated that it might be an appropriate candidate for inhibition by calcineurin. The Vcx1p protein contains 11 putative transmembrane domains, as determined from hydropathy plots (Fig. 2B), and shows the highest sequence similarity to the retinal rod $Na^+/Ca^{2+},K^+$ exchanger (54) and to the cardiac Na^+/Ca^{2+} exchanger (46) (Fig. 2C). Vcx1p and these mammalian exchangers are predicted to be similar in transmembrane organization, although the mammalian enzymes also contain cleaved leader peptides and two large hydrophilic insertions. The regions with the greatest homology between these gene products encompass putative membrane spanning domains in the N- and C-terminal halves of the proteins. In a BLAST search of GenBank release 90 (2), open reading frames with unknown functions in the genomes of *E. coli* (accession number U18997) and *S. cerevisiae* (accession number P42839) were found to be significantly related to that of *VCX1*. The *VCX1* gene product exhibits little or no sequence similarity to a putative H^+/Ca^{2+} exchanger of *E. coli* (32). Thus, *VCX1* overexpression may prevent Ca^{2+}-dependent growth inhibition of *pmc1* mutants by increasing Ca^{2+} export from the cytosol.

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GATCAAAATTCCCGAAAAATAAAAATTTCCCTCACGAGAATATGGAAATTACTATCGCCC      60
AGCATTATTTTATCTCCTCAGAGAGTAAAAGAAGTTTCCTCTTTCCTAAACTTAAAAAGC     120
AGTCCTAAATATATAGTTGACTTCGACGCATATCATTCATCGGCTGCTGATAGCAAATAA     180
AACAACATAGATACAATGGATGCAACTACCCCACTATTAACTGTTGCGAACAGTCATCCC     240
1                   M  D  A  T  T  P  L  L  T  V  A  N  S  H  P
GCCCGCAATCCAAAGCACACTGCATGGAGAGCAGCTGTGTATGATTTACAGTATATTTTG     300
16  A  R  N  P  K  H  T  A  W  R  A  A  V  Y  D  L  Q  Y  I  L
AAAGCGTCACCCCTGAATTTCCTATTGGTATTTGTTCCTTTAGGTCTGATTTGGGGACAC     360
36  K  A  S  P  L  N  F  L  L  V  F  V  P  L  G  L  I  W  G  H
TTCCAACTATCTCATACACTGACATTTCTTTTTAATTTCTTGGCAATTATACCGTTGGCA     420
56  F  Q  L  S  H  T  L  T  F  L  F  N  F  L  A  I  I  P  L  A
GCTATCTTGGCTAATGCCACGGAAGAGTTGGCTGATAAGGCTGGTAACACCATTGGGGGA     480
76  A  I  L  A  N  A  T  E  E  L  A  D  K  A  G  N  T  I  G  G
CTGCTAAATGCTACTTTTGGTAACGCTGTGGAACTAATTGTTTCTATCATTGCCCTGAAA     540
96  L  L  N  A  T  F  G  N  A  V  E  L  I  V  S  I  I  A  L  K
AAAGGTCAAGTGAGAATTGTGCAGGCCTCGATGCTAGGTAGTCTTCTTTCTAATTTGCTG     600
116 K  G  Q  V  R  I  V  Q  A  S  M  L  G  S  L  L  S  N  L  L
TTAGTGCTTGGATTATGCTTCATATTCGGTGGATACAATAGAGTCCAACAGACATTCAAC     660
136 L  V  L  G  L  C  F  I  F  G  G  Y  N  R  V  Q  Q  T  F  N
CAAACCGCCGCTCAAACAATGTCCTCATTACTTGCCATTGCGTGTGCATCCCTACTGATT     720
156 Q  T  A  A  Q  T  M  S  S  L  L  A  I  A  C  A  S  L  L  I
CCCGCTGCCTTTAGAGCCACCCTACCTCATGGCAAGGAAGACCACTTCATCGATGGAAAA     780
176 P  A  A  F  R  A  T  L  P  H  G  K  E  D  H  F  I  D  G  K
ATATTGGAGTTATCCAGAGGCACCTCTATTGTTATTCTCATCGTTTACGTTTGTTCTTA     840
196 I  L  E  L  S  R  G  T  S  I  V  I  L  I  V  Y  V  L  F  L
TATTTCCAGCTAGGGAGCCATCACGCCTTGTTTGAGCAACAAGAAGAAGAGACCGATGAA     900
216 Y  F  Q  L  G  S  H  H  A  L  F  E  Q  Q  E  E  E  T  D  E
GTTATGAGCACCATTTCCAGGAATCCACATCACTCTTTGAGTGTCAAGTCATCATTGGTG     960
236 V  M  S  T  I  S  R  N  P  H  H  S  L  S  V  K  S  S  L  V
ATACTTCTAGGTACAACTGTGATCATCTCTTTTTGTGCGGATTTTCTAGTCGGTACGATA    1020
256 I  L  L  G  T  T  V  I  I  S  F  C  A  D  F  L  V  G  T  I
GACAACGTTGTTGAATCTACCGGGCTATCTAAAACATTTATAGGTTTGATTGTCATTCCT    1080
276 D  N  V  V  E  S  T  G  L  S  K  T  F  I  G  L  I  V  I  P
ATTGTGGGTAATGCCGCAGAGCATGTCACTTCAGTCTTGGTGGCCATGAAGGATAAGATG    1140
296 I  V  G  N  A  A  E  H  V  T  S  V  L  V  A  M  K  D  K  M
GATCTGGCGCTAGGTGTTGCCATCGGTTCCTCTTTACAAGTTGCCTTATTTGTTACACCA    1200
316 D  L  A  L  G  V  A  I  G  S  S  L  Q  V  A  L  F  V  T  P
TTCATGGTTCTTGTGGGCTGGATGATCGATGTTCCAATGACGCTAAATTTCTCCACTTTT    1260
336 F  M  V  L  V  G  W  M  I  D  V  P  M  T  L  N  F  S  T  F
GAAACCGCTACTCTTTTTATTGCTGTTTTCTTATCCAATTACTTAATTCTCGATGGTGAG    1320
356 E  T  A  T  L  F  I  A  V  F  L  S  N  Y  L  I  L  D  G  E
TCAAACTGGTTGGAGGGTGTC ATG TCTCTAGCTATGTATATTTTGATTGCAATGGCATTT    1380
376 S  N  W  L  E  G  V  M  S  L  A  M  Y  I  L  I  A  M  A  F
TTCTATTATCCAGACGAAAAAACCCTTGACTCTATTGGAAATAGTTTATGAGTAAGAACA    1440
396 F  Y  Y  P  D  E  K  T  L  D  S  I  G  N  S  L  .
GTAGCGCAGAAATTCACCATTCTCTTTTTTTTTATATTTTATCTTCTTCGTAATTCTATC    1500
GATGGACCAATACAAAGATGATTATGACTAGCAAGTCATTATTATGATCGTTATATTATA    1560
CATATTATGTTTACGCAACTAATTTTTATATCGAATTTTATCTGAAAAAAAGTTTTCAAA    1620
ACCAACAAAATATTTTGTACACTCTTCGAAACTCAAATTCTGCACCATTTCCATTCTGAT    1680
AAAAGGTTTAATATTTCGAAGCTT                                        1704
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FIG. 1. DNA sequence of the *VCX1* locus and predicted gene product. The DNA sequence (residues numbered at the right) and deduced protein sequence (residues numbered at the left) of the *VCX1* locus were determined as described in Materials and Methods and deposited in GenBank (accession number U36603). Sequencing of adjacent DNA showed that residues 1699 through 1704 are contiguous with residues 6485 through 6480 of a previously sequenced locus encoding *CDC48* (19). Sequencing of *VCX1-D1*, a spontaneous mutation which confers a dominant Ca^{2+} tolerance phenotype in *pmc1* mutants, revealed only a single nucleotide substitution (underlined) in which the ATG (Met) codon at position 383 is converted to ATA (Ile).

***VCX1* encodes a vacuolar H^+/Ca^{2+} exchanger.** An oligonucleotide encoding three tandem repeats of the HA epitope was fused in frame near the predicted translational start site of *VCX1* (see Materials and Methods) in order to visualize the gene product in situ. The resulting *VCX1*::*HA* derivative was fully functional in assays for high-dosage suppression of *pmc1* and low-copy-number complementation of *vcx1* mutant phenotypes (see Fig. 6). Cells carrying *VCX1*::*HA* displayed epitopes exclusively near the vacuole membrane (Fig. 3A), whereas a control *VCX1* strain lacking the epitope tag displayed only weak background staining (Fig. 3B). The distribution of Vcx1p by immunofluorescence was similar to but much brighter than that of Pmc1p (14).

The major Ca^{2+} uptake activity observed in crude cell ex-

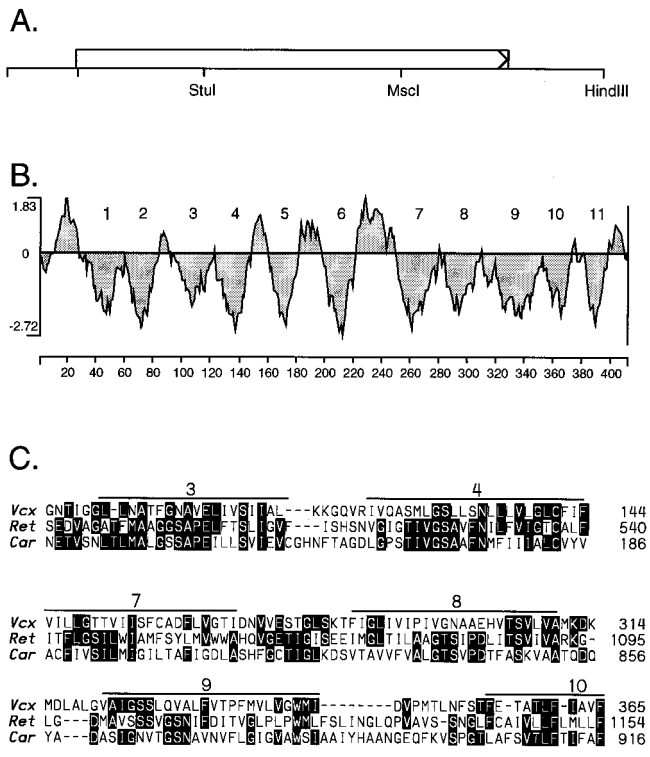

FIG. 2. Predicted features of Vcx1p. (A) Predicted open reading frame and partial restriction map of the functional *VCX1* locus. (B) Kyte-Doolittle hydropathy plot (window = 15 residues) of the predicted Vcx1p sequence. Putative transmembrane domains are numbered 1 to 11. (C) Multiple sequence alignment of Vcx1p, bovine retinal $Na^+/Ca^{2+},K^+$ exchanger (54), and canine Na^+/Ca^{2+} exchanger (46). Highlighted residues indicate identity in at least two of three sequences, and overlines correspond to predicted transmembrane domains. Numbers at the right refer to amino acid positions. No significant similarities were detected outside the aligned regions.

tracts and in purified vacuole membrane vesicles is a low-affinity H^+/Ca^{2+} exchange which utilizes a pH gradient formed by the vacuolar H^+ ATPase (17, 47, 50). To test whether *VCX1* function is necessary for this activity, we assayed for $^{45}Ca^{2+}$ uptake in total cell lysates (17) prepared from a wild-type strain (W303-1A) and a *vcx1* null mutant (K661) in which the chromosomal *VCX1* open reading frame was partially deleted and disrupted with foreign sequences (see Materials and Methods). Prior to the addition of ATP, the levels of $^{45}Ca^{2+}$ accumulation in the particulate fraction were similar in the two lysates (Fig. 4A, time zero). However, after ATP was added, the *VCX1* lysate accumulated much more $^{45}Ca^{2+}$ than the *vcx1* lysate, and the former activity was inhibited by addition of 10 μM concanamycin B, a compound related to bafilomycin A that specifically inhibits vacuolar H^+ ATPases (33). Both *VCX1* and *vcx1* strains accumulate the weak base quinacrine in their vacuoles in vivo, as detected by fluorescence microscopy (66), and this accumulation was blocked by 10 μM concanamycin B (data not shown). These results show that vacuolar H^+/Ca^{2+} exchange activity is markedly reduced in *vcx1* mutants, but vacuole morphology and vacuolar acidification are not noticeably affected by the mutation.

The residual concanamycin B-resistant Ca^{2+} transport detected in *vcx1* mutants was not detected in *pmc1 vcx1* mutants (data not shown), suggesting that *PMC1* may encode a functional Ca^{2+} pump. To test this point directly, a *vcx1 pmc1* double mutant (K665) was transformed with an empty plasmid or pKC151, which expresses *PMC1* in galactose medium through the strongly inducible *GAL1* promoter. After growth in galactose medium, cell lysates were prepared and assayed for $^{45}Ca^{2+}$ uptake as described above except that $CaCl_2$ in the buffer was reduced from 100 to 10 μM and the ATP-independent transport activity in parallel samples was subtracted for each time point (Fig. 4B). Lysates of the strain expressing *PMC1* contained significant levels of ATP-dependent Ca^{2+} transport activity (Fig. 4B) that was resistant to concanamycin B (data not shown), whereas lysates of the strain lacking *PMC1* contained very little of this activity (Fig. 4B). These data show that *VCX1* encodes a vacuolar H^+/Ca^{2+} exchanger and *PMC1* encodes a vacuolar Ca^{2+} ATPase. Additionally, the cell-free Ca^{2+} transport assay shows that *VCX1*-dependent activity is much greater than residual *PMC1*-dependent activity (Fig. 4A) under these optimized assay conditions (containing 100 μM Ca^{2+}). However, the following results indicate that in growing cells, *PMC1* functions are greater than *VCX1* functions as a result of both lower $[Ca^{2+}]c$ and regulation by calcineurin.

***VCX1*-dependent Ca^{2+} sequestration is enhanced when calcineurin is inactive.** To examine the role of *VCX1* in vivo, Ca^{2+} sequestration into a nonexchangeable intracellular pool was quantitated in log-phase cells containing various combinations of the *vcx1*, *pmc1*, and *cnb1* null mutations (Fig. 5). Each strain was labeled uniformly with $^{45}Ca^{2+}$ in duplicate cultures during four to five generations of logarithmic growth in YPD pH 5.5 medium supplemented with 5 mM $CaCl_2$. The cells were collected, washed, and subjected to a 30-min chase period in medium lacking radioactivity to allow efflux of the exchangeable Ca^{2+} pool. The cells were then rapidly collected on filters, washed, and counted to estimate the nonexchangeable Ca^{2+} pool. The wild-type cells accumulated approximately four times more Ca^{2+} in the nonexchangeable pool than *pmc1* mutants (Fig. 5, bars 1 and 3) as observed previously (14), and these levels were only slightly affected by the additional *vcx1* null mutations (bars 2 and 4). Thus, relative to *PMC1*, *VCX1* plays only a small role in vacuolar Ca^{2+} sequestration in proliferating cells. However, the large increase in Ca^{2+} sequestration observed in *pmc1 cnb1* double mutants (bar 5) relative to *pmc1* mutants is completely dependent on *VCX1* function because *vcx1 pmc1 cnb1* triple mutants accumulate only low levels of nonexchangeable Ca^{2+} (bar 6). Qualitatively similar results were obtained in experiments in which extracellular Ca^{2+} was decreased to about 0.2 mM (YPD medium) or increased to 20 mM (data not shown), although the sizes of exchangeable and nonexchangeable pools varied considerably under these conditions as observed previously (17). Therefore, *VCX1* plays a much larger role in Ca^{2+} sequestration in *cnb1* mutants than in strains in which calcineurin is functional.

***VCX1* functions in Ca^{2+} tolerance when calcineurin is inactive.** The role of *VCX1* in Ca^{2+} tolerance was addressed by monitoring growth of *vcx1* mutants in liquid media supplemented with increasing concentrations of $CaCl_2$. After a 16-h incubation, the levels of added $CaCl_2$ that caused a 50% decrease in relative growth (the IC_{50}s) were 360 and 320 nM for wild-type and *vcx1* strains, whereas the IC_{50}s for *pmc1* mutants and *pmc1 vcx1* double mutants were approximately 65 and 38 mM, respectively (Fig. 6). In five similar experiments, *VCX1* was always found to confer a relatively low level of Ca^{2+} tolerance, although the measured IC_{50}s varied somewhat because of variations in inoculum size and time of incubation.

In contrast to the small role of *VCX1* in strains in which calcineurin is functional, *VCX1* confers a much larger degree of Ca^{2+} tolerance when calcineurin has been inactivated. The high Ca^{2+} tolerance of *cnb1 pmc1* double mutants (IC_{50}, ~320 mM $CaCl_2$) is completely dependent on *VCX1* function because *cnb1 pmc1 vcx1* triple mutants are extremely sensitive to

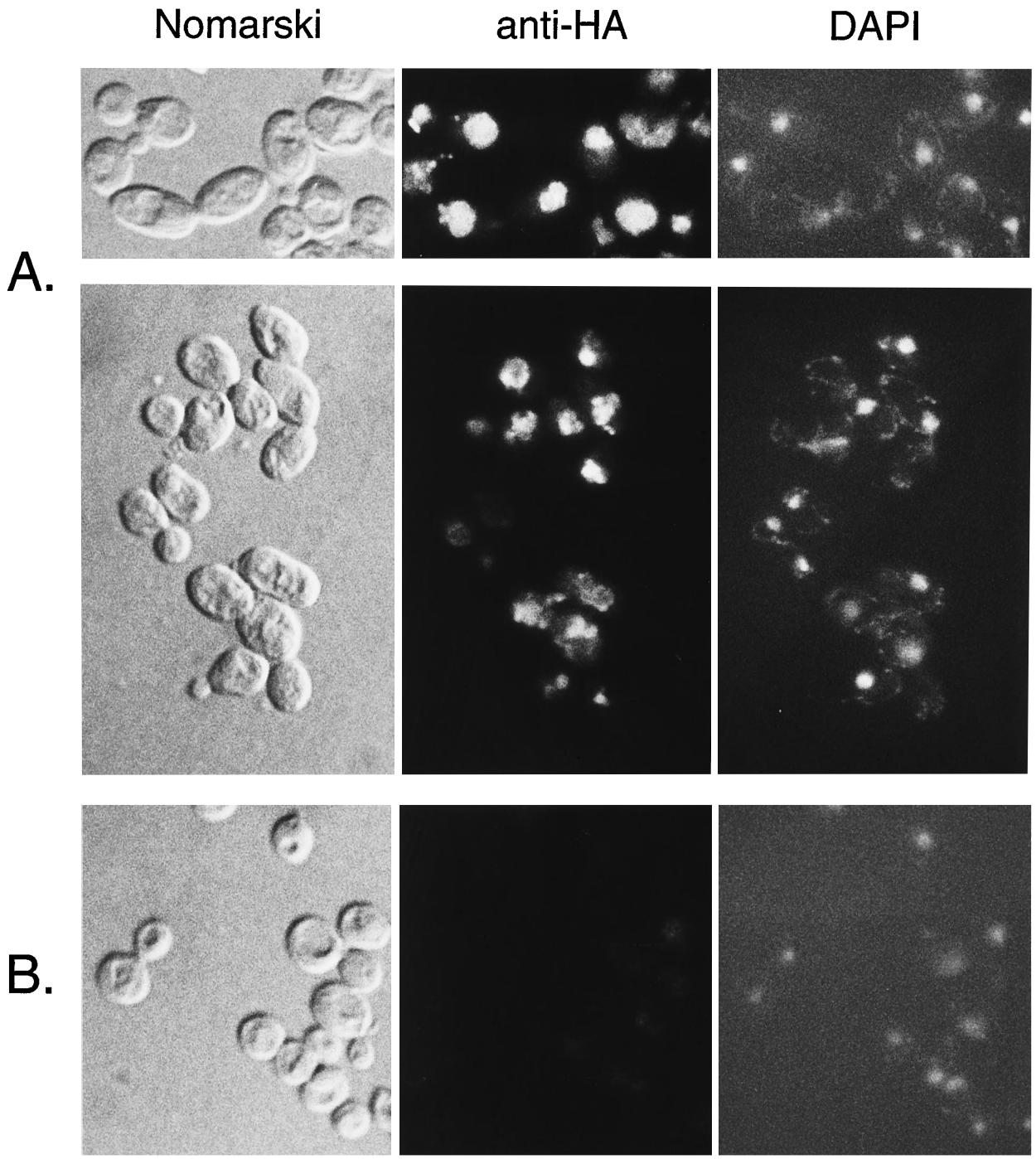

FIG. 3. Immunofluorescence localization of epitope-tagged Vcx1p. A centromere plasmid containing a functional epitope-tagged *VCX1*::*HA* derivative was constructed as described in Materials and Methods. Strain W303-1A harboring *VCX1*::*HA* (A) or *VCX1* (B) was grown to early exponential phase in SC-Ura medium, harvested, fixed with formaldehyde, and processed for immunofluorescence microscopy using the anti-HA monoclonal antibody 12CA5 (Boehringer) and cyanine-9-conjugated goat anti-mouse immunoglobulin G antibody as described in Materials and Methods. The same field of cells was photographed by Nomarski optics (left) and by indirect immunofluorescence to detect Vcx1p (center) and to detect DNA after staining with 4,6′-diamidino-2-phenylindole (DAPI; right).

added Ca^{2+} (IC_{50}, ~8 mM). The *cnb1 vcx1* double mutant is also more sensitive to Ca^{2+} (IC_{50}, ~100 mM) than a *cnb1* single mutant (IC_{50}, ~370 mM), which indicates that *VCX1* performs a significant role in Ca^{2+} tolerance in calcineurin-deficient mutants, independently of *PMC1*. Similar results were obtained in cultures in which calcineurin is inhibited by addition of 0.2 μg of FK506 per ml (not shown) or inactivated by the *cmd1-3* mutation (Fig. 6), which produces a mutant calmodulin that is both unable to bind Ca^{2+} with high affinity (22) and unable to activate calcineurin in vivo (14). Expression of a calmodulin-independent derivative of the calcineurin catalytic subunit *CNA1ΔC* decreases Ca^{2+} tolerance of *cmd1-3 pmc1* mutants (14) and increases Ca^{2+} tolerance of *cmd1-3 vcx1* mutants (data not shown), suggesting that the major role

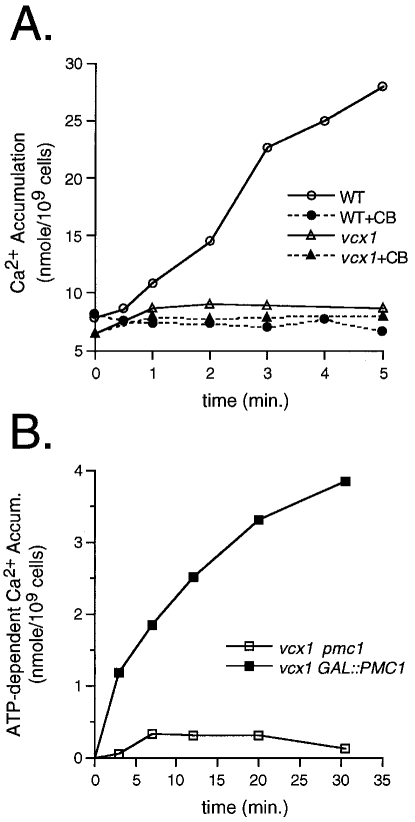

FIG. 4. Ca^{2+} transport assays in yeast cell extracts. (A) Total spheroplast lysates were prepared from mid-log-phase YPD cultures of the wild-type (WT) strain W303-1A and the *vcx1* mutant K661 and assayed for uptake of $^{45}Ca^{2+}$ with and without the inhibitor of vacuolar H^+ ATPase concanamycin B (CB). After 3 min of preincubation at 30°C in buffer containing an optimal level of $^{45}Ca^{2+}$ (100 μM), 1 mM ATP was added at 0 min, and aliquots were then removed at the indicated times, rapidly diluted in cold buffer, filtered, and processed as described in Materials and Methods. Ca^{2+} accumulation in nanomoles per 10^9 cell equivalents was calculated (without subtracting the prior ATP-independent Ca^{2+} accumulation). (B) Total cell lysates were prepared from a *vcx1 pmc1* double mutant (K665) containing plasmid pRS316-*gal* (open squares) or pKC151-*GAL::PMC1* (filled squares). Extracts were preincubated with $^{45}Ca^{2+}$ (10 μM) for 5 min at 30°C and split into two equal portions, and then reactions were initiated at 0 min by addition of 1 mM ATP to one half. Samples were collected and processed as described above, and ATP-dependent Ca^{2+} uptake was calculated by subtracting the ATP-independent Ca^{2+} accumulation from the total Ca^{2+} accumulation.

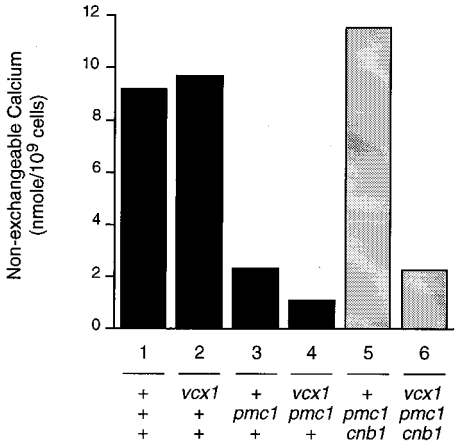

FIG. 5. Nonexchangeable Ca^{2+} pools in yeast mutants. Log-phase yeast cultures were uniformly labeled in YPD pH 5.5 medium supplemented with 5 mM $^{45}Ca^{2+}$ and subjected to a brief chase period in nonradioactive medium to allow efflux of the exchangeable Ca^{2+} pool. The remaining nonexchangeable pool was averaged from duplicate cultures as described in Materials and Methods. Standard deviations from the duplicate samples ranged from 0.1 to 0.3 nmol/10^9 cells. Bars 1 to 6 show data for strains W303-1A, K661, K605, K665, K607, and K667, respectively. +, wild-type alleles.

of calmodulin in Ca^{2+} tolerance is to activate calcineurin. The simplest interpretation of all of these results is that calcineurin activation by Ca^{2+}/calmodulin diminishes the contribution of *VCX1* in Ca^{2+} tolerance and Ca^{2+} sequestration.

Previous studies showed that *pmc1 pmr1* double mutants were inviable at all levels of Ca^{2+} but were viable and very Ca^{2+} tolerant when calcineurin was inactivated by low levels of FK506 or by *cnb1* mutations (14). If calcineurin-dependent inhibition of *VCX1* is responsible for the lethality of *pmc1 pmr1* double mutants, then deletion of *VCX1* should be lethal in the *cnb1 pmc1 pmr1* strain. Tetrad analysis of heterozygous diploids showed that all combinations of these mutations were efficiently recovered as viable clones, except for *pmc1 pmr1* double mutants, *pmc1 pmr1 vcx1* triple mutants, and *cnb1 pmc1 pmr1 vcx1* quadruple mutants. This experiment was reproducible over a wide range of conditions. Thus, *VCX1* function is required for the viability of the *cnb1 pmc1 pmr1* triple mutant, and *VCX1* cannot perform its essential function when calcineurin is active. Attempts to reconstitute calcineurin-dependent inactivation of vacuolar H^+/Ca^{2+} exchange in vitro have not yet succeeded. However, evidence presented below suggests that calcineurin may slightly decrease the expression of *VCX1* as well as the activity of Vcx1p in vivo.

Production of Vcx1p and Pmc1p. Our results indicate that calcineurin not only diminishes the role of *VCX1* in Ca^{2+} tolerance but also can promote the role of other Ca^{2+} tolerance factors (for example, *cnb1 vcx1* double mutants are less tolerant of Ca^{2+} than *vcx1* mutants). To determine whether calcineurin differentially modulates expression of *VCX1* and *PMC1*, total cell extracts prepared after growth in different conditions were analyzed by Western blotting. The predicted

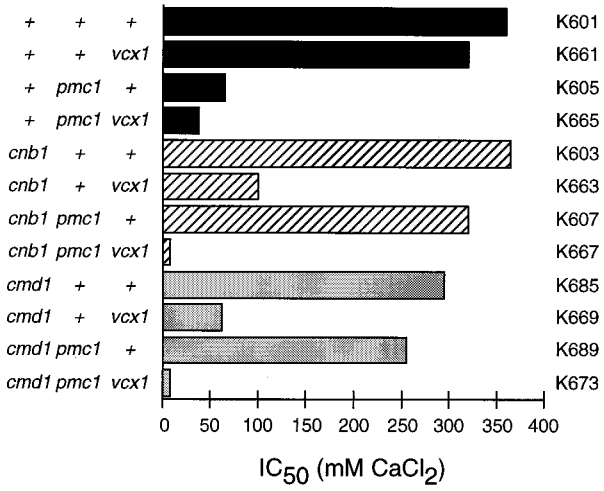

FIG. 6. Ca^{2+} tolerance in yeast mutants. The IC_{50} for each strain was determined after 16 h of growth in supplemented YPD pH 5.5 medium as described in Materials and Methods. Similar results were obtained in independent experiments and when FK506 (0.2 μg/ml) was used in lieu of *cnb1* mutations. All strains listed grew at similar rates in standard unsupplemented media (YPD and SC). Additionally, the epitope-tagged *VCX1::HA* allele integrated at the chromosomal locus was indistinguishable from *VCX1* in similar situations. +, wild-type allele.

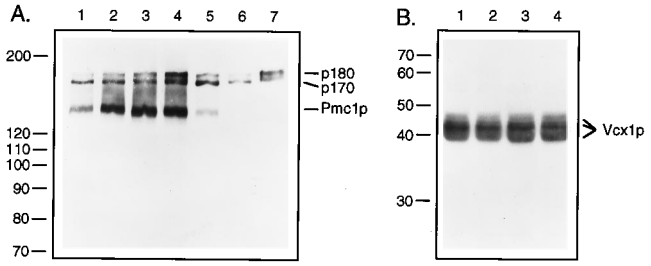

FIG. 7. Expression of Vcx1p and Pmc1p in response to Ca^{2+} and FK506. (A) Western blot analysis of *PMC1*::*HA* (lanes 1 to 5, strain K699) and *PMC1* (lanes 6 and 7, strain W303-1A) grown for 6 h in YPD pH 5.5 medium (lanes 1 and 6) or in medium supplemented with 50 mM $CaCl_2$ (lane 2), 100 mM $CaCl_2$ (lane 3), 200 mM $CaCl_2$ (lane 4), or 200 mM $CaCl_2$ plus 0.4 μg of FK506 per ml (lanes 5 and 7). Total cell extracts were prepared as described in Materials and Methods, and samples corresponding to 1.25 OD_{600} units were analyzed on 8% polyacrylamide gels adjacent to molecular weight markers (positions shown at the left in kilodaltons). Pmc1p and cross-reacting polypeptides p180 and p170 are indicated at the right. (B) Western blot analysis of *VCX1*::*HA* (strain K698) grown for 6 h in YPD pH 5.5 medium (lane 1) or medium supplemented with 200 mM $CaCl_2$ (lane 2), 0.4 μg of FK506 per ml (lane 3), or 200 mM $CaCl_2$ plus 0.4 μg of FK506 per ml (lane 4). Samples corresponding to 0.3 OD_{600} unit were run on 10% polyacrylamide gels as described above. No cross-reacting polypeptides other than Vcx1p were evident under these conditions.

49-kDa product of *VCX1*::*HA* was observed as a series of bands migrating at 39 to 43 kDa during growth in YPD pH 5.5 medium (Fig. 7B, lane 1), which decreased very slightly (two-fold or less) during growth in 200 mM Ca^{2+} (lane 2) but did not noticeably change in abundance or mobility in response to FK506 or Ca^{2+} plus FK506 (lanes 3 and 4). The predicted 135-kDa product of *PMC1*::*HA* increased markedly in abundance as Ca^{2+} in the growth medium was increased from 0 to 200 mM (Fig. 7A, lanes 1 to 4), and this effect was blocked when FK506 was present (lane 5). High Ca^{2+} conditions also caused the FK506-sensitive accumulation of a 180-kDa polypeptide in both *PMC1*::*HA* and wild-type strains, whereas a second cross-reacting polypeptide of ~170 kDa was unchanged (Fig. 7A). High-Ca^{2+} conditions therefore decrease Vcx1p accumulation only slightly and greatly increase Pmc1p accumulation, both through FK506-sensitive mechanisms.

Expression of *VCX1*, *PMC1*, *PMR1*, and *PMR2A* reporter genes is modulated by calcineurin activation in response to elevated $[Ca^{2+}]c$. *PMC1* gene expression was also quantitated by using a series of plasmid-based reporter genes in which *lacZ* coding sequences from *E. coli* were placed downstream of the promoter and predicted N-terminal codons of these genes. All yeast strains containing the *pmc1*::*lacZ* reporter plasmid (pKC190) accumulated very low levels of β-galactosidase activity (0.1 to 0.2 U) when grown in standard media (Fig. 8A). After 4.25 h of incubation in medium supplemented with Ca^{2+}, expression of *pmc1*::*lacZ* increased approximately 150-fold in wild-type strains, 200-fold in *vcx1* mutants, 500-fold in *pmc1* mutants, and 650-fold in *pmc1 vcx1* mutants (Fig. 8A), whereas expression in *cnb1 vcx1* double mutants did not increase during growth in Ca^{2+}-supplemented medium. Similarly, increased β-galactosidase accumulation was blocked by addition of 0.2 μg of FK506 per ml (Table 2). A *cyc1*::*lacZ* reporter used previously to analyze expression of cytochrome *c* (24) was slightly repressed by these conditions (~1.1-fold [Table 2]). To help rule out the possibility of Ca^{2+} and calcineurin-responsive elements in the parent vector, we analyzed derivatives of *cyc1*::*lacZ* that lack all upstream activation sequences (24) or have those sequences replaced with a cell cycle-regulated upstream activation sequence (UAS) derived from the *HO* gene (10). There was no detectable expression of the UAS-deficient *cyc1*::*lacZ* derivative under any of our conditions, and there was only ~1.3-fold repression of the *ho*::*cyc1*::*lacZ* reporter by added Ca^{2+} (Table 2). Finally, a 413-bp fragment of the *PMC1* upstream region (from −579 to −166 relative to initiator codon) was found to be necessary and sufficient for calcineurin-dependent induction by Ca^{2+} because deletion of this region from *pmc1*::*lacZ* abolished all expression and insertion of this segment upstream of the UAS-deficient *cyc1*::*lacZ* reporter restored full Ca^{2+}- and calcineurin-dependent expression of β-galactosidase (Table 2). These results suggest that the *PMC1* gene can be regulated at the transcriptional level through upstream activation sequences which depend on calcineurin activation.

Expression of *vcx1*::*lacZ*, *pmr1*::*lacZ*, and *pmr2A*::*lacZ* reporters was also examined. The *vcx1*::*lacZ* reporter was repressed 2.2-fold and *pmr1*::*lacZ* was induced 2.3- to 2.9-fold by calcineurin-dependent mechanisms in response to high-Ca^{2+} conditions (Fig. 8C and Table 2). Lastly, a *pmr2A*::*lacZ* reporter was induced over 25-fold by the actions of Ca^{2+} and calcineurin (Table 2) and up to 500-fold in some conditions (Fig. 8B). As for *pmc1*::*lacZ*, calcineurin-dependent expression of the *pmr2A*::*lacZ* reporter was exacerbated in the *pmc1* and *pmc1 vcx1* mutants; however, the range of added Ca^{2+} necessary to induce *pmr2A*::*lacZ* expression was much lower than that of *pmc1*::*lacZ* (Fig. 8). Other differences between the two reporters in the different yeast strains can be observed in the highest-Ca^{2+} conditions. These results suggest that Pmc1p and to a lesser degree Vcx1p serve to lower $[Ca^{2+}]c$ in high-Ca^{2+} conditions and thereby decrease calcineurin activation. There was no detectable induction of either *pmc1*::*lacZ*, *pmr1*::*lacZ*, or *pmr2A*::*lacZ* in *cmd1-3* mutants (data not shown). Thus, activation of calcineurin by calmodulin and elevated $[Ca^{2+}]c$ can differentially affect the expression of at least four reporter genes. No conserved sequence elements are obvious in the promoter regions of these genes. Calcineurin-dependent repression of *VCX1* and induction of *PMC1* and *PMR1* provide at least partial explanations for all the observed effects of different mutations on Ca^{2+} tolerance (Fig. 6).

Calmodulin and calcineurin mediate responses to Mn^{2+} and Na^+. Because *PMR1* and *PMR2A* have been implicated in tolerance to added Mn^{2+} and Na^+, respectively (28, 37), we tested whether calcineurin may be involved in tolerance to these ions. Inactivation of calcineurin with mutations or FK506 also causes a significant decrease in tolerance to Mn^{2+} and Na^+ (Fig. 9 and references 40 and 45). To test whether the consequences of calcineurin inactivation are dependent on *PMR1* and *PMR2A* function, we compared Mn^{2+} and Na^+ tolerances in *pmr1* and *pmr2* null mutants both with and without a functional calcineurin. Addition of FK506 to the growth medium markedly decreased Mn^{2+} tolerance of wild-type and *vcx1* mutants but had no significant effect on the tolerance of *pmr1* and *pmr1 vcx1* mutants (Fig. 9A). The nonadditive effect of FK506 plus *pmr1* mutations suggests that the role of calcineurin in Mn^{2+} tolerance is dependent on *PMR1* function. A similar experiment to assess Na^+ tolerance of *pmr2* mutants (which lack *PMR2A* and three additional repeats at this locus) with and without the *cnb1* null mutation (Fig. 9B) showed that there was only a slight additive effect of the two mutations. The introduction of *vcx1* mutations had only small quantitative effects on the Mn^{2+} and Na^+ tolerance of these strains, indicating that *VCX1* contributes little to Mn^{2+} and Na^+ tolerance with or without calcineurin function. These results are consistent with a model whereby calcineurin promotes Mn^{2+} and Na^+ tolerance through effects on *PMR1* and *PMR2A* (and not *VCX1*), but other possible targets of calcineurin are not ruled out.

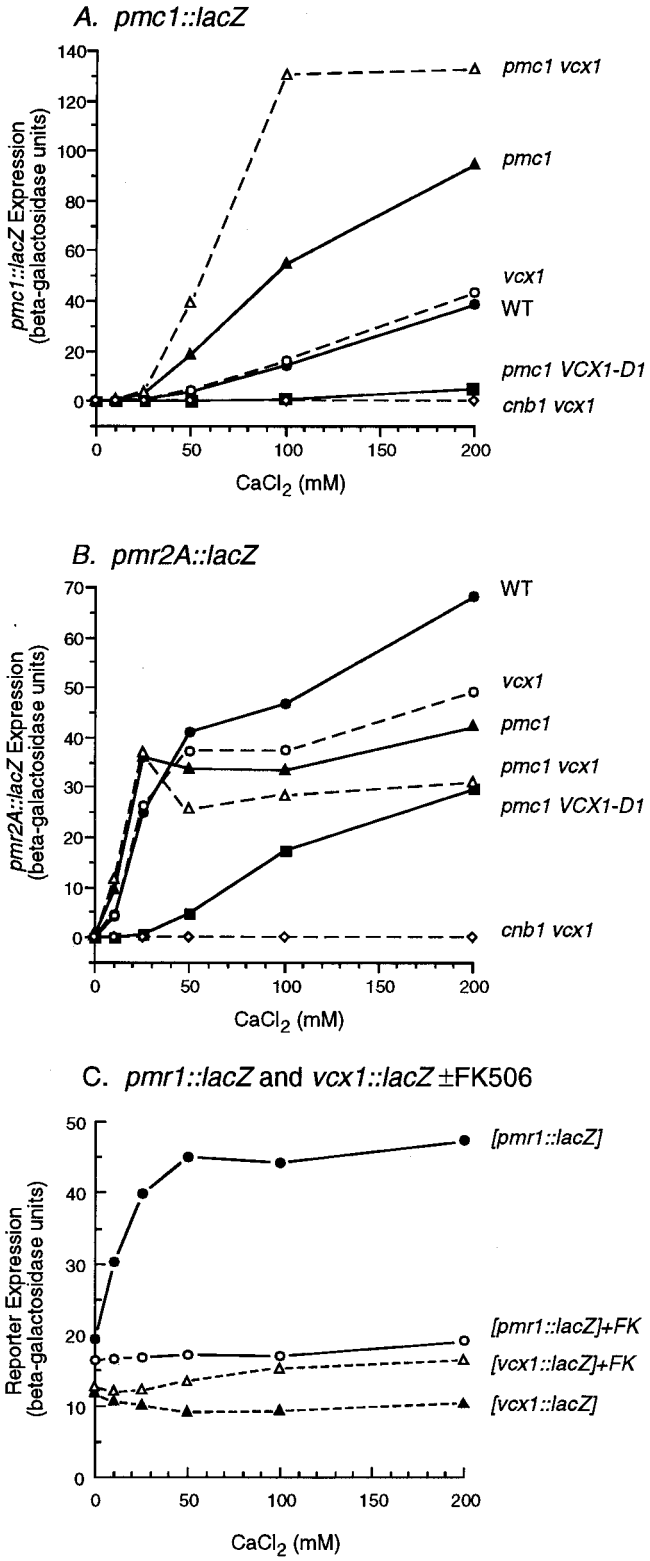

FIG. 8. Expression of *lacZ* reporters for *PMC1*, *PMR2A*, *PMR1*, and *VCX1* in response to added Ca^{2+} and in different mutant strains. Yeast strains were transformed with plasmids pKC190 (A) and pKC201 (B), grown to mid-log phase in SC-Ura medium, collected, and suspended in YPD pH 5.5 medium plus $CaCl_2$ as indicated. After 4.25 h of incubation at 30°C with shaking, total cellular β-galactosidase activity was determined for each culture. Strains: WT (wild type), W303-1A; *pmc1*, K605; *vcx1*, K661; *cnb1 vcx1*, K663; *pmc1 vcx1*, K665; *pmc1 VCX1-D1*, K737. (C) Plasmids pKC199 (*pmr1*::*lacZ*) and pKC200 (*vcx1*::*lacZ*) were transformed into the *vcx1* mutant K661, incubated, and processed as described above except that FK506 (0.5 μg/ml) was included in half of the YPD cultures (+FK). All patterns of expression were reproducible in independent experiments.

TABLE 2. Expression of reporter genes in *vcx1* mutants

Plasmid	Reporter	β-Galactosidase (U)[a]			Ratio[b] (+Ca/ +Ca+FK)
		YPD	+Ca	+Ca+FK	
pKC190	*pmc1*::*lacZ*	<0.2	13.8	<0.2	69 ± 10
pLGΔ312	*cyc1*::*lacZ*	494.2	391.1	445.8	0.88 ± 0.07
pLGΔ178	(Δ*UAS*)*cyc1*::*lacZ*	<0.2	<0.2	<0.2	
pLB178-43	*ho*::*cyc1*::*lacZ*	47.4	41.4	53.0	0.78 ± 0.07
pKC211	(Δ*413*)*pmc1*::*lacZ*	<0.2	<0.2	<0.2	
pKC191	(*413*)*pmc1*::*cyc1*::*lacZ*	0.5	13.5	0.3	52 ± 7
pKC200	*vcx1*::*lacZ*	12.9	9.7	21.9	0.44 ± 0.07
pKC199	*pmr1*::*lacZ*	22.3	41.9	18.3	2.3 ± 0.1
pKC201	*pmr2A*::*lacZ*	0.9	59.2	2.2	27 ± 4

[a] Total cellular β-galactosidase activity was assayed as described in Materials and Methods after 4 h of growth in YPD pH 5.5 medium supplemented as indicated with 100 mM $CaCl_2$ (Ca) and 0.2 μg of FK506 (FK) per ml. Data are averages of two independent experiments; 0.2 U was the limit of detection.

[b] Average of two independent experiments (± standard deviation).

Is calcineurin required for induction of the *PMR1* and *PMR2A* reporter genes in response to Mn^{2+} and Na^+? Expression of the *pmr1*::*lacZ* reporter was induced significantly in *vcx1* mutants, but not *cmd1-3 vcx1* mutants, after a shift to media supplemented with 1 mM $MnCl_2$ (Fig. 10A). Addition of FK506 to the $MnCl_2$-supplemented cultures decreased expression by approximately 2.5-fold in *vcx1* mutants but had no significant effect in *vcx1 cmd1-3* mutants. There was also no significant FK506-sensitive induction of *ho*::*lacZ* in *vcx1* mutants, indicating that calmodulin and calcineurin are required for the specific induction of *pmr1*::*lacZ* in response to Mn^{2+}. Similarly, *pmr2A*::*lacZ* expression was induced by 500 mM NaCl in *pmc1* mutants, and induction was markedly decreased either by FK506 addition (Fig. 10B) or by introduction of *cmd1-3* or *cnb1* mutations (data not shown). Qualitatively similar results were obtained when wild-type strains, *vcx1* mutants, and *pmc1 vcx1* mutants were each analyzed, although the relative levels of calcineurin-dependent induction appeared to increase as Ca^{2+} transporters were inactivated (data not shown). Significant calcineurin-dependent induction of Na^+ stress has been shown previously to increase accumulation of *PMR2A* mRNAs in wild-type yeast strains and to a lesser degree in *cnb1* mutants (20). Thus, functions of calmodulin and calcineurin may be involved in the induction of *PMR1* and *PMR2A* gene expression in response to elevated extracellular Mn^{2+} and Na^+. These findings provide at least one plausible explanation for the decreased Mn^{2+} and Na^+ tolerance of calcineurin mutants.

Activated variants of Vcx1p decrease Ca^{2+} signaling. Our results suggest that $[Ca^{2+}]c$ becomes elevated in response to added Ca^{2+}, Mn^{2+}, or Na^+ in order to promote the calcineurin-dependent induction of specific tolerance factors. The latter two responses may require inactivation of Vcx1p for efficient production of Ca^{2+} signals. To test this idea, we isolated *VCX1-D* mutants which are active despite the function of calcineurin and tested whether such mutants would diminish the calcineurin-dependent induction of *pmr2A*::*lacZ* in response to Na^+. In our original screen for spontaneous Ca^{2+}-tolerant revertants of *pmc1* strains, recessive mutations comprising three complementation groups were isolated (14). Six additional revertants were dominant over wild-type *VCX1* for Ca^{2+} tolerance, segregated 2:2 in backcrosses, and segregated

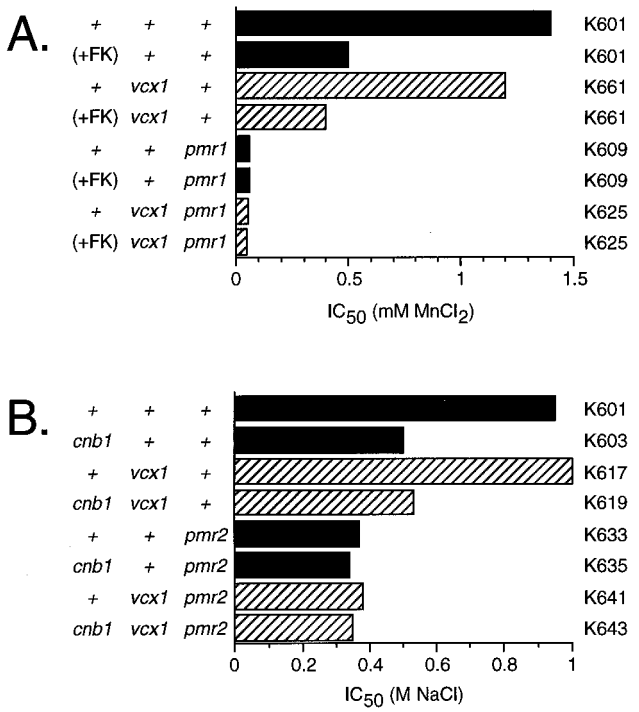

FIG. 9. Roles of calcineurin in Mn^{2+} and Na^+ tolerance. The indicated yeast strains were inoculated into YPD pH 5.5 medium supplemented with 0.5 μg of FK506 (FK) per ml, $MnCl_2$, or NaCl as indicated. After 16 h of growth at 30°C, culture density and IC_{50} were determined by interpolation as described in Materials and Methods. +, wild-type allele. Similar results were obtained in three independent experiments.

4:0 in crosses with each other. Crosses between these strains and *vcx1*::*URA3* demonstrated tight linkage of the dominant mutations to the *VCX1* locus. We recovered all of the dominant mutations on plasmids by using an allele rescue strategy (26) with *Stu*I-digested plasmid pKC106. Subcloning and sequencing demonstrated that point mutations within the *VCX1* open reading frame, all of which resulted in amino acid substitutions, were responsible for the Ca^{2+} tolerance phenotype. A *pmc1 VCX1-D1* double mutant was also found to sequester high levels of Ca^{2+} in the nonexchangeable pool (approximately 10 times that of *pmc1* mutants; data not shown) and to block partially the induction of *pmc1*::*lacZ* and *pmr2A*::*lacZ* in response to added Ca^{2+} (Fig. 8). Complete sequencing of *VCX1-D1* revealed a single nucleotide change, G-1344→A, which changes codon 383 from Met to Ile in the 11th putative transmembrane domain. These results suggest that *VCX1-D1* produces a variant vacuolar H^+/Ca^{2+} exchanger that is very active in strains in which calcineurin is nominally functional.

Calcineurin-dependent induction of *pmr2A*::*lacZ* by Na^+ was also found to be much less in *pmc1 VCX1-D1* mutants than in *pmc1* mutants (Fig. 10B). There was no significant change, however, in the calcineurin-independent (FK506-resistant) induction of *pmr2A*::*lacZ* by Na^+. Thus, inappropriately high activity of the vacuolar H^+/Ca^{2+} exchanger decreases a number of calcineurin-dependent responses. *PMC1* function also appears to modulate calcineurin-dependent gene expression (Fig. 8) presumably through effects on $[Ca^{2+}]c$. We conclude that calcineurin activation by Ca^{2+}/calmodulin is a physiological response to high Ca^{2+}, Mn^{2+}, and Na^+ concentrations which regulates the appropriate detoxification mechanisms. Feedback interactions between calcineurin and vacuolar Ca^{2+} transporters may ensure that such processes occur efficiently over a wide range of conditions.

DISCUSSION

Ca^{2+} homeostasis in *S. cerevisiae*. Our previous work suggested that calcineurin might inhibit a factor required for Ca^{2+} tolerance of *pmc1* mutants (14). Here we have identified *VCX1*, encoding the major vacuolar H^+/Ca^{2+} exchanger, as a direct or indirect target of calcineurin required for this effect. The simplest model consistent with the new results is that Ca^{2+} homeostasis in yeast cells is achieved by a sophisticated feedback mechanism involving at least calmodulin, calcineurin, and three intracellular Ca^{2+} transporters encoded by *VCX1*, *PMC1*, and *PMR1* (Fig. 11). Together these Ca^{2+} transporters control $[Ca^{2+}]c$ and appear to prevent the toxic accumulation of Ca^{2+} in the cytosol, which is especially important in conditions of high extracellular Ca^{2+} concentrations. We propose that calcineurin activation by calmodulin and elevated $[Ca^{2+}]c$ leads to increased expression of *PMC1* and possibly *PMR1* and to slightly decreased expression of *VCX1*. It is more likely that calcineurin inactivates Vcx1p by a posttranslational mechanism because the repression of Vcx1p expression seems insufficient

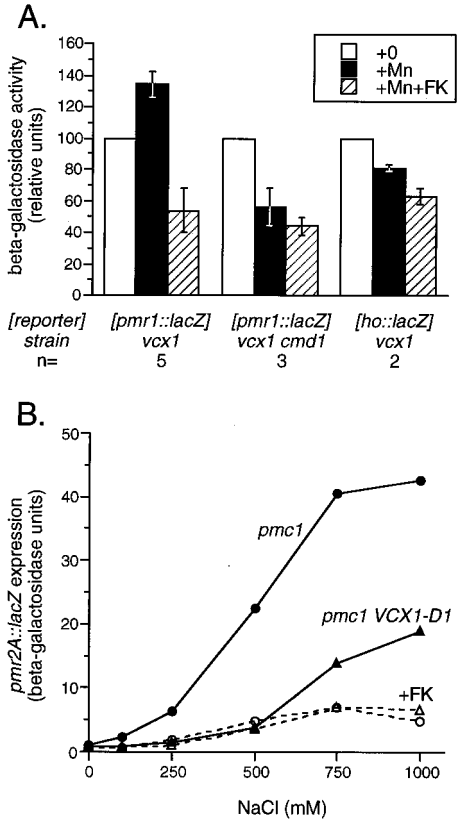

FIG. 10. Calcineurin mediates responses to Mn^{2+} and Na^+. (A) Expression of *pmr1*::*lacZ* or *ho*::*lacZ* reporters in yeast strains K661 (*vcx1*) and K669 (*vcx1 cmd1-3*) after 4 h of growth in YPD pH 5.5 medium (+0) or in the same medium supplemented with 1 mM $MnCl_2$ (+Mn) and 0.2 μg of FK506 per ml (+Mn +FK). β-Galactosidase activity from independent cultures was determined, and all values were normalized to that of the unsupplemented cultures (arbitrarily set at 100 relative units), averaged, and plotted as bars. Error markers indicate standard deviations from *n* independent experiments. (B) Expression of *pmr2A*::*lacZ* in *pmc1* (K605) and *pmc1 VCX1-D1* (K737) strains was determined after 4 h of growth in YPD pH 5.5 medium supplemented with the indicated amounts of NaCl (filled symbols, solid lines) and with 0.4 μg of FK506 (FK) per ml (open symbols, dashed lines).

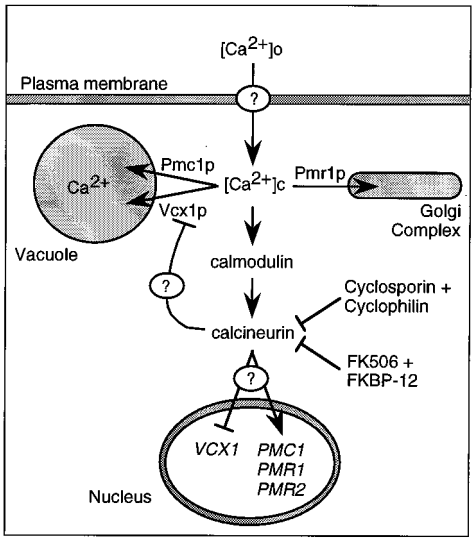

FIG. 11. Working model of Ca^{2+} homeostasis in *S. cerevisiae*. Lines with arrowheads indicate positive functions or activating interactions, and lines with bars indicate inhibitory interactions. Question marks denote unknown factors or interactions that may be indirect. $[Ca^{2+}]o$, extracellular free Ca^{2+} concentration.

to account for the dramatic effects of calcineurin on *VCX1*-dependent Ca^{2+} tolerance and sequestration. Although the exact mechanisms by which calcineurin accomplishes all these functions are not yet clear, this model provides a plausible explanation for why calcineurin function decreases Ca^{2+} tolerance of *pmc1* mutants (14) and increases Ca^{2+} tolerance of *vcx1* mutants and *pmc1 vcx1* double mutants (Fig. 6).

Mutant strains lacking all three Ca^{2+} transporters (*VCX1*, *PMC1*, and *PMR1*) are inviable in all growth media tested to date. However, mutants containing only a single functional Ca^{2+} transporter gene are viable and provide insight into the relative roles of each Ca^{2+} transporter and its regulators. For example, *PMR1* provides functions essential for the viability of *cnb1 pmc1 vcx1* triple mutants, which implies that *PMR1* retains significant activity in the absence of calcineurin activation. This finding is consistent with the relatively high basal expression of *pmr1::lacZ* (Fig. 8) and with the known secretory roles of *PMR1* in low-Ca^{2+} conditions (4, 57). The viability of *cnb1 pmr1 vcx1* triple mutants suggests that basal expression of *PMC1* is also sufficient to carry out essential functions. Furthermore, the inviability of *pmc1 pmr1* double mutants and viability and high Ca^{2+} tolerance of *cnb1 pmc1 pmr1* triple mutants (14) and of *pmc1 pmr1 VCX1-D1* triple mutants (data not shown) can be interpreted to indicate that *VCX1* can provide the essential functions when the inhibitory effects of calcineurin are removed. Inhibition of *VCX1* function by calcineurin is somewhat incomplete because residual activity of *VCX1* was detected in *pmc1* mutants (Fig. 6). Thus, all three transporters appear to function to some degree with or without calcineurin activation. In Ca^{2+} tolerance assays, *PMC1* provides the largest contribution whereas *VCX1* and *PMR1* ordinarily contribute much less.

In *S. cerevisiae*, Ca^{2+}/calmodulin regulates the intracellular Ca^{2+} transporters indirectly through effects on calcineurin. Animal cells, in contrast, regulate the plasma membrane Ca^{2+} ATPases and both the retinal and cardiac Na^+/Ca^{2+} exchangers by direct binding of Ca^{2+}/calmodulin and also by phosphorylation (for reviews, see references 12 and 52); regulation by calcineurin has not been reported. Sequence alignments indicate that the calmodulin-binding regulatory domains of mammalian plasma membrane Ca^{2+} ATPases and the retinal $Na^+/Ca^{2+},K^+$ exchangers are not conserved in Pmc1p and Vcx1p (Fig. 2 and reference 14). We observed that calcineurin-dependent inhibition of *VCX1* function and promotion of *PMC1* function are both abolished by *cmd1-3* mutations except when the calmodulin-independent *CNA1ΔC* derivative of calcineurin is expressed (14). Thus, yeast cells differ from mammalian cells in that the vacuolar Ca^{2+} transporters are regulated predominantly by calcineurin activation rather than direct binding of Ca^{2+}/calmodulin. Although our model fits the extant data, it does not rule out other possible interactions among these factors. For example, all *cmd1-3* strains and *cmd1-3 cnb1* double mutants are less Ca^{2+} tolerant than corresponding *cnb1* mutants (Fig. 6 and data not shown), suggesting that Ca^{2+}/calmodulin may have some other roles in Ca^{2+} tolerance that are independent of calcineurin, *PMC1* and *VCX1*.

Kinetic analyses of the vacuolar H^+/Ca^{2+} exchanger from *S. cerevisiae* suggested that despite a relatively high K_m for Ca^{2+}, this enzyme would be sufficient to account for the levels of Ca^{2+} sequestration observed over a wide range of environmental conditions (17). Similar conclusions have been reached for vacuolar H^+/Ca^{2+} exchangers of filamentous fungi and higher plants (8, 41). These considerations, taken together with the finding that *vma1* mutants (and other mutants deficient in the vacuolar H^+ ATPase necessary for H^+/Ca^{2+} exchange activity) are extremely sensitive to added Ca^{2+}, have led to the prediction that mutants lacking the H^+/Ca^{2+} exchanger would similarly be unable to grow in high-Ca^{2+} conditions (3). Our results show that *vcx1* mutants lack the major vacuolar H^+/Ca^{2+} exchanger but are only slightly less tolerant of Ca^{2+} than wild-type cells and much more Ca^{2+} tolerant than *pmc1* mutants (Fig. 6) and *vma1* mutants (49). Furthermore, *VCX1* contributes little to Ca^{2+} sequestration in the range of 0.2 to 20 mM extracellular Ca^{2+} (Fig. 5 and data not shown). We presume that the optimized assay for H^+/Ca^{2+} exchange activity (Fig. 4A) does not effectively reconstitute calcineurin-dependent inhibition of *VCX1* function because wild-type and *cnb1* extracts were found to be indistinguishable (data not shown). The model described above does not explain why *vma1* mutants are sensitive to Ca^{2+}, but it predicts that *PMC1* and *PMR1* would be important for the residual Ca^{2+} tolerance of *vma1* mutants. Consistent with this prediction, *pmr1 vma1* double mutants are not viable (62) and *cnb1 vma1* double mutants are either extremely Ca^{2+} sensitive or inviable (21, 30, 62) as a result of decreased expression of *PMC1* and *PMR1* or other effects.

Tanida et al. propose that calcineurin may inhibit an intracellular Ca^{2+} transporter distinct from the vacuolar H^+/Ca^{2+} exchanger and that its increased function after FK506 addition may cause decreased Ca^{2+} tolerance in *vma* mutants (62). They observed that FK506 addition increased Ca^{2+} sequestration into the nonexchangeable pool and rapidly decreased $[Ca^{2+}]c$. These results do not rule out *VCX1* as the target of calcineurin inhibition because a small pH gradient persists in the vacuoles of *vma* mutants (43), which may be sufficient to promote vacuolar H^+/Ca^{2+} exchange activity. That FK506 or *cnb1* mutations cause decreased Ca^{2+} tolerance of *vma* mutants (21, 30, 62) can also be explained by a variety of secondary defects in cell physiology and not necessarily changes in Ca^{2+} transport. For example, a drastic reduction of vacuolar polyphosphate levels in *vma* mutants (67) could lead to decreased Ca^{2+} tolerance simply by lowering the Ca^{2+} buffering in this organelle (17) irrespective of effects on Ca^{2+} transport. Furthermore, *vma* mutants display pleiotropic defects in ion

homeostasis, sorting of vacuolar proteins, general metabolism, and other processes (reviewed in references 3 and 34) which might affect Ca^{2+} tolerance indirectly. Additional studies are necessary to determine how *vma* mutants and other Ca^{2+}-sensitive mutants (6, 48) affect the vacuolar Ca^{2+} transporters encoded by *VCX1* and *PMC1*.

Increased expression of Ca^{2+} ATPases would benefit the cell in high-Ca^{2+} conditions to offset increased Ca^{2+} influx. At present, it is more difficult to appreciate how the cell benefits from *VCX1* when its function seems to be strongly inhibited by calcineurin activation. Because calcineurin and *VCX1* appear to regulate each other in a positive feedback loop, it is not possible to conclude whether *VCX1-D1* produces a H^+/Ca^{2+} exchanger that is specifically resistant to inhibition by calcineurin or whether some other aspect of its function or expression is augmented. The decreased function of calcineurin in *VCX1-D1* strains is consistent with the hypothesis that inhibition of *VCX1* is important to promote the formation of Ca^{2+} signals in *S. cerevisiae*. Presumably *VCX1* functions more significantly during conditions which have yet to be identified.

Ca^{2+} signaling in *S. cerevisiae*. We report here the calcineurin-dependent induction of *pmr1*::*lacZ* and *pmr2A*::*lacZ* in response to added Mn^{2+} and Na^+ as well as to added Ca^{2+} (Fig. 8 and 10). The *cnb1* and *cmd1-3* mutations each block these regulatory effects, suggesting that calcineurin activation by calmodulin may be occurring in response to an increase in $[Ca^{2+}]c$. Because *PMR1* and *PMR2A* encode Mn^{2+} and Na^+ tolerance factors (28, 37), their calmodulin- and calcineurin-dependent induction in response to increased environmental levels of these ions may indicate more physiological means of generating Ca^{2+} signals in yeast cells. Their induction by moderate-Ca^{2+} conditions, as opposed to the high-Ca^{2+} conditions needed for maximal *PMC1* induction, does not necessarily imply roles in Ca^{2+} tolerance but may reflect responsiveness to relatively small increases in $[Ca^{2+}]c$. Introduction of *pmr2* null mutations into *pmc1*, *cnb1*, *vcx1*, and *cnb1 pmc1 vcx1* mutants does not significantly affect Ca^{2+} tolerance (data not shown). Possible means of generating Ca^{2+} signals in response to Na^+ include the gated opening of putative Ca^{2+}-selective channels in the vacuole membrane (7) or plasma membrane (25, 31).

Relevance to other cell types. There is an impressive degree of conservation among *S. cerevisiae* and animals in the mechanisms of calcineurin activation by Ca^{2+} and calmodulin and inhibition by immunosuppressive drugs and their immunophilin receptors (36). Our finding that yeast calcineurin can regulate expression of specific genes has parallels with the major role of calcineurin in the regulation of gene expression in lymphocytes and other mammalian cells. It will be interesting to determine whether the similarities between *S. cerevisiae* and animals in Ca^{2+} signaling mechanisms extend to additional components, such as calcineurin-dependent transcription factors and Ca^{2+} channels. This work provides a number of useful tools for these future studies.

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