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The Product of *HUM1*, a Novel Yeast Gene, Is Required for Vacuolar Ca^{2+}/H^+ Exchange and Is Related to Mammalian Na^+/Ca^{2+} Exchangers

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Calcineurin, or PP2B, plays a critical role in mediating Ca^{2+}-dependent signaling in many cell types. In yeast cells, this highly conserved protein phosphatase regulates aspects of ion homeostasis and cell wall synthesis. We show that calcineurin mutants are sensitive to high concentrations of Mn^{2+} and identify two genes, *CCC1* and *HUM1*, that, at high dosages, increase the Mn^{2+} tolerance of calcineurin mutants. *CCC1* was previously identified by complementation of a Ca^{2+}-sensitive (*csg1*) mutant. HUM1 (for "high copy number undoes manganese") is a novel gene whose predicted protein product shows similarity to mammalian Na^+/Ca^{2+} exchangers. *hum1* mutations confer Mn^{2+} sensitivity in some genetic backgrounds and exacerbate the Mn^{2+} sensitivity of calcineurin mutants. Furthermore, disruption of *HUM1* in a calcineurin mutant strain results in a Ca^{2+}-sensitive phenotype. We investigated the effect of disrupting *HUM1* in other strains with defects in Ca^{2+} homeostasis. The Ca^{2+} sensitivity of *pmc1* mutants, which lack a P-type ATPase presumed to transport Ca^{2+} into the vacuole, is exacerbated in a *hum1* mutant strain background. Also, the Ca^{2+} content of *hum1 pmc1* cells is less than that of *pmc1* cells. In contrast, the Ca^{2+} sensitivity of *vph1* mutants, which are specifically defective in vacuolar acidification, is not significantly altered by disruption of Hum1p function. These genetic interactions suggest that Hum1p may participate in vacuolar Ca^{2+}/H^+ exchange. Therefore, we prepared vacuolar membrane vesicles from wild-type and *hum1* cells and compared their Ca^{2+} transport properties. Vacuolar membrane vesicles from *hum1* mutants lack all Ca^{2+}/H^+ antiport activity, demonstrating that Hum1p catalyzes the exchange of Ca^{2+} for H^+ across the yeast vacuolar membrane.

Ca^{2+}, the intracellular mediator of many extracellular signaling events, exerts much of its effect on cell physiology by modulating the phosphorylation state of cellular proteins. Calcineurin, a protein phosphatase that is stimulated by Ca^{2+} and calmodulin, is an effector of Ca^{2+}-dependent changes in protein phosphorylation (26). Calcineurin, or PP2B, is specifically inhibited by the immunosuppressant drugs FK506 and cyclosporin A (33). In addition to being powerful therapeutic agents, these compounds have served as tools to assess the physiological functions of calcineurin in many cell types. Thus, calcineurin activity has been shown to be critical for many calcium-regulated processes in mammalian cells, including T-cell activation (10, 33, 46) and neutrophil chemotaxis (23, 30). Calcineurin has also been shown to affect the activities of a number of ion transporters in both plant and animal cells (2, 4, 31, 36). Calcineurin is highly conserved and is found in both higher and lower eukaryotes. We have focused on characterizing the physiological role of this enzyme in baker's yeast (*Saccharomyces cerevisiae*), using a combination of biochemical and genetic approaches.

Calcineurin is a heterodimer made up of a catalytic and a regulatory subunit. In *S. cerevisiae* two genes, *CNA1/CMP1* and *CNA2/CMP2*, encode functionally redundant catalytic subunits of calcineurin (12, 34), and the *CNB1* gene encodes the regulatory subunit (13, 27). Thus, yeast calcineurin function can be disrupted in vivo either by mutating both of the two catalytic subunit genes (*cna1 cna2*), by mutating the sole regulatory subunit gene (*cnb1*), or by adding an immunosuppressant (FK506 or cyclosporin A) to the growth medium (20). In all cases, disruption of calcineurin function results in viable cells that display a number of specific phenotypes.

Analyses of calcineurin-deficient yeast cells indicate that this protein phosphatase regulates cell wall synthesis and ion homeostasis. Some yeast strains with defective cell walls require calcineurin for growth (14, 16, 22), and the expression of *FKS2*, a gene involved in synthesis of cell wall β-1,3-glucan, is regulated by calcineurin (39). Several aspects of ion homeostasis are also perturbed in calcineurin mutants. First, cells lacking calcineurin grow poorly in the presence of high concentrations of Na^+ and Li^+ (9, 40, 41). The role of calcineurin in Na^+ and Li^+ tolerance can be partly explained by an effect on gene expression; in calcineurin mutants there is reduced transcriptional induction of *ENA1*, which encodes a P-type ATPase involved in Na^+ efflux (40). However, calcineurin likely has additional effects on Na^+ and Li^+ homeostasis, as cells completely lacking *ENA1* are rendered even more Na^+ sensitive in the absence of calcineurin function (19). Second, calcineurin mutants grow better than wild-type cells on media containing high concentrations of Ca^{2+} (56). Measurements of intracellular Ca^{2+} revealed that Ca^{2+} sequestration is stimulated when calcineurin is inhibited with FK506 (56). These findings are consistent with known genetic interactions between calcineurin and *PMC1*, a gene which encodes a vacuolar P-type ATPase proposed to pump Ca^{2+} from the cytosol into the vacuole (11). Mutations that disrupt calcineurin function suppress the Ca^{2+} sensitivity of *pmc1* mutants (11). Third, calcineurin mutants fail to grow on high-pH media (41). In summary, many experimental observations suggest that calcineurin affects the homeostasis of multiple ions in yeast cells. The protein or proteins that are directly dephosphorylated by calcineurin in vivo in order to bring about these effects have yet to be identified.

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TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Source or reference
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	55
YPH500	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	55
YPH501	*MAT***a**/α YPH499 × YPH500	55
DD09	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3 cnb1Δ1::hisG*	13
MCY3-1D	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cnb1Δ1::LEU2*	13
MCY300	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3*	12
TPY1	*MAT***a**/α YPH501 *HUM1/hum1Δ1::URA3*	This work
TPY2	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 hum1Δ1::URA3*	This work
TPY3	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cnb1Δ1::LEU2 hum1Δ1::URA3*	This work
ASY1	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 pmc1::TRP1*	This work
TPY4	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 hum1Δ1::URA3 pmc1::TRP1*	This work
BJ7289	*MAT*α *ura3-52 lys2-801 ade2-101 his3-Δ200 leu2 vph1::LEU2*	37
BJ7290	*MAT***a** *ura3-52 lys2-801 leu2*	37
TPY5	*MAT***a** *ura3-52 lys2-801 leu2 hum1Δ1::URA3*	This work
TPY6	*MAT*α *ura3-52 lys2-801 ade2-101 his3-Δ200 leu2 vph1::LEU2 hum1Δ1::URA3*	This work
TDY689	*MAT***a** *ura3-52 leu2*	21
TDY689-ccc1Δ	*MAT***a** *ura3-52 leu2 CCC1::LEU2*	21
TPY7	*MAT***a** *ura3-52 lys2-801 ade2-101trp1-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3 cnb1Δ1::hisG CCC1::LEU2*	This work

In this report we show that calcineurin affects Mn^{2+} homeostasis. Little is known about the regulation of intracellular Mn^{2+} levels in yeast cells or about the mechanism by which high levels of Mn^{2+} in the medium inhibit growth. Mn^{2+} is an essential trace element for *S. cerevisiae*, and it may, under some circumstances, substitute for Ca^{2+} (35). The growth of strains lacking functional calcineurin (cnΔ strains) is more severely inhibited by Mn^{2+} than that of wild-type strains. We describe here a screen to identify genes that, when present at high dosages, increase the Mn^{2+} tolerance of calcineurin mutants. Our objectives in undertaking this screen were twofold: to identify genes whose products participate in Mn^{2+} homeostasis and to gain insight into how calcineurin affects Mn^{2+} homeostasis. We report here the characterization of two genes identified in that screen: *CCC1* and *HUM1*. *CCC1* encodes a predicted protein product containing five putative transmembrane domains (21). The *CCC1* gene was previously isolated by its ability, when present on a low-copy-number plasmid, to complement a Ca^{2+}-sensitive (*csg1*) mutant (21). Our findings suggest that Ccc1p also participates in Mn^{2+} homeostasis. *HUM1*, a novel gene, encodes a predicted protein product that contains 11 putative transmembrane domains and has regions of sequence similarity to mammalian Na^+/Ca^{2+} exchangers. Although *HUM1* was identified by its ability to improve Mn^{2+} tolerance at a high dosage, the biochemical and genetic characterizations presented here indicate that a major physiological function of Hum1p is to promote intracellular Ca^{2+} sequestration through vacuolar Ca^{2+}/H^+ exchange.

MATERIALS AND METHODS

Yeast strains and culture conditions. The yeast strains used are listed in Table 1. Strain TPY1 was created by introducing the *hum1Δ1::URA3* allele (see below) into YPH501 by DNA-mediated transformation. Strain TPY2 is a haploid segregant of the TPY1 diploid. TPY3 was isolated as a haploid segregant of the diploid formed by mating TPY2 to MCY3-1D. The *hum1Δ1::URA3* allele was also introduced into strain BJ7290 and BJ7289 to create strains TPY5 and TPY6, respectively. Strain ASY1 was generated by introducing the *pmc1::TRP1* allele (from plasmid pKC59 [11]) into YPH499 by DNA-mediated transformation. The *hum1Δ1::URA3* allele was also introduced into strain ASY1 by DNA-mediated transformation to create strain TPY4. Strain TPY7 was generated by introducing *CCC1::LEU2* (21) into DD09 by DNA-mediated transformation. Yeast cultures were grown in either YAPD (1% yeast extract, 2% Bacto Peptone, 0.4% adenine [40 mg/liter], 2% dextrose) or synthetic medium (54) containing twice the recommended levels of supplements and with 3.5 g of ammonium chloride per liter substituted for ammonium sulfate. Where noted, salts were added to synthetic medium at the specified level.

General methods. Standard procedures were used for genetic manipulations of *S. cerevisiae* (54). DNA was introduced into yeast cells by lithium acetate transformation (5). Northern (RNA) and Southern analyses were performed according to established protocols (5). Radioactive probes were synthesized by using random primers (Pharmacia) and [α-^{32}P]dCTP (NEN Dupont). Biotinylated probes were generated by using the New England Biolabs NEBlot Phototope kit. *HUM1* and *CCC1* were localized on the physical map of the yeast genome by hybridization to a panel of lambda phages carrying ordered segments of *S. cerevisiae* chromosomal DNA (ATCC 77284) (32).

Phenotypic characterization of ion sensitivities. Solid synthetic medium was made by using the chloride salt of the specified ion. Sensitivity was determined by one or both of two assays.

(i) Sector assay. Saturated overnight cultures were diluted in medium, and equal numbers of cells from each strain tested were spread on plates containing a range of ion concentrations. The plates were then incubated at 30°C for 1 to 3 days, and the growth of each strain was assayed by noting colony size and number.

(ii) Dilution spot assay. For each strain tested, six serial fivefold dilutions were made from a saturated overnight culture diluted to a starting optical density at 600 nm (OD_{600}) of 1. Ten microliters of each dilution was spotted onto synthetic plates containing a range of ion concentrations. The plates were then incubated at 30°C for 1 to 3 days.

For each assay, every strain was always tested on a wide range of ion concentrations. One particular concentration was then chosen for use in the figures presented here.

Isolation of plasmids conferring Mn^{2+} tolerance to calcineurin mutants. Strain DD09 was grown to log phase, transformed with a yeast genomic library constructed in the yeast high-copy-number plasmid YEp351 (17, 25), and grown at 30°C on solid synthetic medium lacking leucine. Colonies were then replica plated to medium containing 10 mM $MnCl_2$. Those colonies which grew after 2 days of incubation at 30°C were purified. Approximately 20,000 colonies (representing more than five genomes of yeast DNA) were screened by this method, resulting in the isolation of 45 colonies that grew on Mn^{2+}-containing medium. The library plasmid present in each colony was then isolated and transformed into DH5α bacteria (5). The entire collection of 45 Mn^{2+}-tolerant colonies yielded 15 plasmids with unique *Hin*dIII-*Eco*RI restriction patterns. When retransformed into yeast strain DD09, seven of these plasmids, some of which contained overlapping inserts of genomic DNA, clearly improved the growth of DD09 on Mn^{2+}-containing medium. Two of these seven plasmids were further characterized: YEpF-101, because it conferred the most dramatic increase in Mn^{2+} tolerance, and YEpC-101, because all or part of its genomic insert was present in 17 of 45 plasmids originally identified in the screen.

Plasmids. (i) F/*CCC1* plasmids. YEpF-101 from the genomic library contained a 12-kb insert of yeast genomic DNA (see Fig. 2A). An 8.3-kb *Sac*I fragment of YEpF-101, containing the *CCC1* open reading frame (ORF) and four other ORFs (see Fig. 2A), was subcloned into pBluescript (Stratagene), creating pF-201, and into the *Sac*I site of YEp351, creating YEpF-104. Digestion of pF-201 with *Spe*I removed a 1.57-kb fragment, truncating *CCC1*; the remaining plasmid was religated, creating pF-202. The truncated insert of pF-202 was subcloned back into YEp351, creating YEpF-105. Digestion of YEpF-201 with *Spe*I liber-

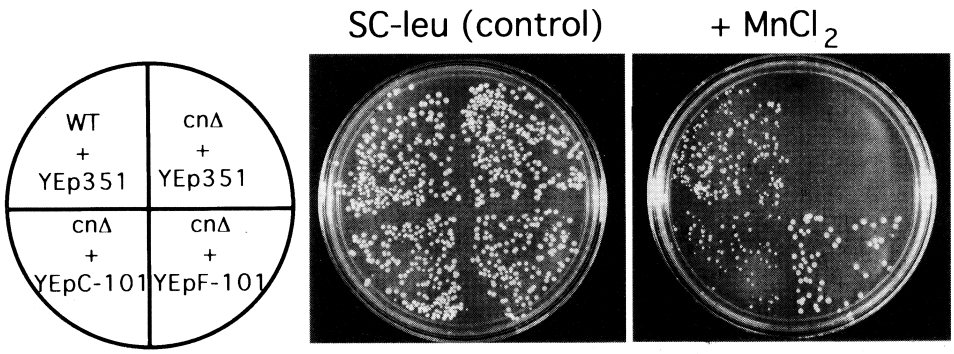

FIG. 1. High-copy-number plasmids YEpC-101 and YEpF-101 increase the Mn^{2+} tolerance of calcineurin mutants. Saturated overnight cultures were diluted and spread on synthetic medium lacking leucine (SC-leu) with or without 10mM $MnCl_2$ and grown for 2 days at 30°C. The strains were YPH499 transformed with YEp351 (WT + YEp351), DD09 transformed with YEp351 (cnΔ + YEp351), DD09 transformed with YEpC-101 (cnΔ + YEpC-101), and DD09 transformed with YEpF-101 (cnΔ + YEpF-101).

ated a 386-bp fragment and a 1,185-bp fragment. The 1,185-bp *Spe*I fragment, containing the L8083.7 ORF, was cloned into the *Xba*I site of YEp351, generating YEpF-106. The 1.3-kb *Kpn*I-*Hin*dIII fragment containing the *CCC1* gene (isolated from a plasmid obtained from Teresa Dunn [21]) was subcloned into YEp351, creating plasmid YEp-CCC1.

(ii) *HUM1* plasmids. YEpC-101 from the genomic library contained a 5.1-kb insert of yeast genomic DNA. A 2.8-kb *Hin*dIII fragment of YEpC-101 was subcloned into YEp351 to create pHUM-108 and into pBluescript to create pHUM-201. (One of the *Hin*dIII sites in the 2.8-kb fragment originates from the polylinker of YEp351, not from genomic DNA [see Fig. 3A].) A 1.5-kb *Hin*cII-*Hin*dIII fragment was subcloned from pHUM-201 into YEp351 to create YEpHUM-109. A 1.3-kb *Hin*cII fragment was subcloned from pHUM-201 into YEp351 to create YEpHUM-110. A 1.4-kb *Pvu*II-*Hin*dIII fragment was subcloned from pHUM-201 to create YEpHUM-111.

(iii) Disruption of *HUM1*. The knockout strategy is outlined in Fig. 3B. pHUM-201 was transformed into *dam* mutant bacteria. Subsequently, pHUM-205 was generated by removing the 979 bp between the two *Bcl*I sites in the *HUM1* ORF and ligating in the 3.8-kb *Bam*HI-*Bgl*III fragment containing the *URA3 hisG* cassette from pNKY51 (1). The genomic copy of *HUM1* was disrupted by transforming yeast strains with the 5.3-kb *Sac*I-*Xho*I fragment from pHUM-205. Disruption of the endogenous *HUM1* gene was confirmed by Southern analysis, using the 2.8-kb *Hin*dIII fragment from pHUM-108 as a probe.

DNA sequence analysis of *HUM1*. An ordered set of exonuclease III deletions from both ends of the 2.8-kb insert in pHUM-201 was made by the method of Henikoff (24). Double-stranded DNA templates were prepared according to the manufacturer's instructions (Promega Magic Miniprep). The nucleotide sequences of both strands were determined by using the deletions and, when necessary, custom-synthesized oligonucleotide primers. The Sequenase version of phage T7 DNA polymerase and nonradioactive nucleotides were obtained from U.S. Biochemical Corporation, and sequencing reactions were conducted according to the manufacturer's instructions. α-^{35}S-dATP was obtained from Amersham.

Determination of whole-cell Ca^{2+}. $^{45}CaCl_2$ (from Amersham or NEN Dupont) was added to YPD to a final specific activity of 33 μCi/μmol. (The Ca^{2+} concentration of YPD is 0.3 mM, as determined by Dunn et al. [15].) This radioactive YPD was inoculated to a final OD_{600} of 0.05 with log-phase cultures, grown at 30°C with agitation, and sampled twice (at 6 and 7 h) to determine the Ca^{2+} content. Results obtained at the two times were equivalent, indicating that labeling had reached a steady-state level. At each time point, duplicate 100-μl aliquots of cells were collected on glass fiber filters (Whatman 934-AH) that had been prewashed twice with 10 ml of wash buffer (100 mM Tris [pH 6.5] with 50 mM $CaCl_2$). The filters were then washed twice more with 10 ml of ice-cold wash buffer. The background radioactivity was determined by addition of the isotope (final concentration, 10 μCi/ml) to an equivalent aliquot of a nonradioactive culture followed by immediate filtration. Filters were dried at 80°C overnight, and the radioactivity in each sample was determined by liquid scintillation counting. The total number of counts was normalized to the cell number at each time point, as determined by OD_{600}. Each datum point was determined by averaging the results for duplicate samples.

Isolation of vacuolar membrane vesicles. Vesicles were prepared by a modification of the method of Ohsumi and Anraku (44). Briefly, 6 liters of cells was grown in YPD to an OD_{600} of 2 to 3, washed once in water, and incubated with gentle agitation in 50 mM glycine–2 mM dithiothreitol for 20 min at 30°C. The cells were resuspended to a density of 2×10^8 to 4×10^8 cells per ml in spheroplast buffer (1.2 M sorbitol, 10 mM Tris, 2 mM dithiothreitol) containing 30 μg of Zymolyase 100-T (ICN) per ml and were shaken gently at 30°C for 1 h. All subsequent steps were performed at 0 to 4°C, and protease inhibitors were included in all buffers (0.8 μg [each] of leupeptin, chymostatin and pepstatin per ml; 0.3 mg of benzamidine HCl per ml; and 1 mM phenylmethylsulfonyl fluoride). The spheroplasts were collected and washed twice in 1.2 M sorbitol. The spheroplasts were lysed by resuspension in buffer A (12% Ficoll 400, 10 mM 2-[*N*-morpholino]ethanesulfonic acid [MES] [pH 6.9], 0.1 mM $MgCl_2$) and homogenization in a Dounce homogenizer. Unlysed spheroplasts were pelleted by centrifugation in a Sorvall RC-5B centrifuge (HB-4 rotor; 3,500 rpm for 10 min). The supernatant was collected and centrifuged in a Beckman ultracentrifuge (SW28 rotor; 24,000 rpm for 1 h). The vacuole layer at the top of the tube was collected and homogenized in buffer A. The vacuoles were overlaid with buffer B (7% Ficoll 400, 10 mM MES [pH 6.9], and 0.5 mM $MgCl_2$) and centrifuged (SW41 rotor; 24,000 rpm for 1 h). The top layer was collected and resuspended in 300 μl of 2× buffer C (20 mM MES [pH 6.9], 10 mM $MgCl_2$, and 50 mM KCl) and then in 300 μl of 1× buffer C. The vesicles were pelleted at maximal speed in an Eppendorf microcentrifuge for 15 min at 4°C, resuspended in 1× buffer C containing 10% glycerol, frozen in liquid nitrogen, and stored at −80°C before use. The purity of this preparation was assessed in two ways. First, the bafilomycin-sensitive ATPase activities (i.e., ATPase activity inhibited by 3 μM bafilomycin A) in the initial lysate and in the final vacuolar membrane fraction were assayed; the specific activity was at least 50-fold higher in the final fraction, indicating significant purification of this vacuolar enzyme. Second, we determined that less than 5% of the total ATPase activity in the final vacuolar membrane fraction was inhibited by either vanadate (100 μM) or NaN_3 (5 mM), indicating minimal contamination by the plasma membrane and mitochondrial ATPases, respectively.

ATPase assays. The fraction to be assayed was added (typically in a volume of 1 to 5 μl) to 286 μl of a reaction mixture (10 mM $MgCl_2$, 25 mM KCl, 50 mM MES [pH 7.0], 5 mM Tris, 5 mM phosphoenolpyruvate, 5 mM ATP, and 9 U of pyruvate kinase [Sigma] per ml) that had been preincubated at 30°C for 5 min. After 15 min, 1.25 ml of stop solution (0.34% ammonium molybdate, 0.86 N H_2SO_4, 0.32% sodium dodecyl sulfate, 1.36% ascorbic acid) was added. After 15 min at room temperature, the OD_{820} was read. This value was converted to nanomoles of free phosphate liberated per minute by comparison with phosphate standards used in the same experiment. Protein concentrations were determined with the Bio-Rad Bradford assay kit, with dilutions of bovine serum albumin used to generate the standard curve.

Calcium uptake into vesicles. The ability of the vacuolar vesicles to take up calcium was determined essentially as described by Oshumi and Anraku (45). Two hundred micrograms of thawed vacuolar membrane vesicles was added to reaction buffer (20 mM MES [pH 6.7], 4 mM $MgCl_2$, 20 mM KCl) and incubated at 25°C with gentle agitation. When indicated, ATP was added to a final concentration of 0.3 mM and bafilomycin was added to a final concentration of 3 μM. Uptake was initiated by the addition of $^{45}CaCl_2$ to a final concentration of 0.5 mM (specific activity of 2 mCi/mmol). Duplicate 100-μl aliquots were withdrawn at each time point, added to 3 ml of ice-cold wash buffer (10 mM MES [pH 6.7], 5 mM $MgCl_2$, and 25 mM KCl), filtered on 0.45-μm-pore-size nitrocellulose filters (Millipore), and washed with 5 ml of ice-cold wash buffer. The filters were air dried, and the radioactivity incorporated into each sample was determined by liquid scintillation counting.

Fluorimetric analysis of vacuolar acidification. Forty micrograms of vacuolar vesicles was diluted into 3 ml of reaction buffer (5 mM MES [pH 7.2], 4 mM $MgCl_2$, 25 mM KCl) containing 1 μM ACMA (9-amino-6-chloro-2-methoxyacridine) (Molecular Probes) in a quartz uvonic cuvette (Uvonic Instruments, Inc.). The stock solution of ACMA was 2 mM in ethanol. The reaction was started by the addition of ATP to a final concentration of 1.33 mM. The formation of the pH gradient was measured by monitoring ACMA fluorescence at 25°C in a SPEX DM3000 spectrofluorimeter (SPEX, Inc.) with a photon-counting mode. The excitation and emission wavelengths used were 410 and 482 nm, respectively. The fluorimetric measurements were performed with constant stirring using a

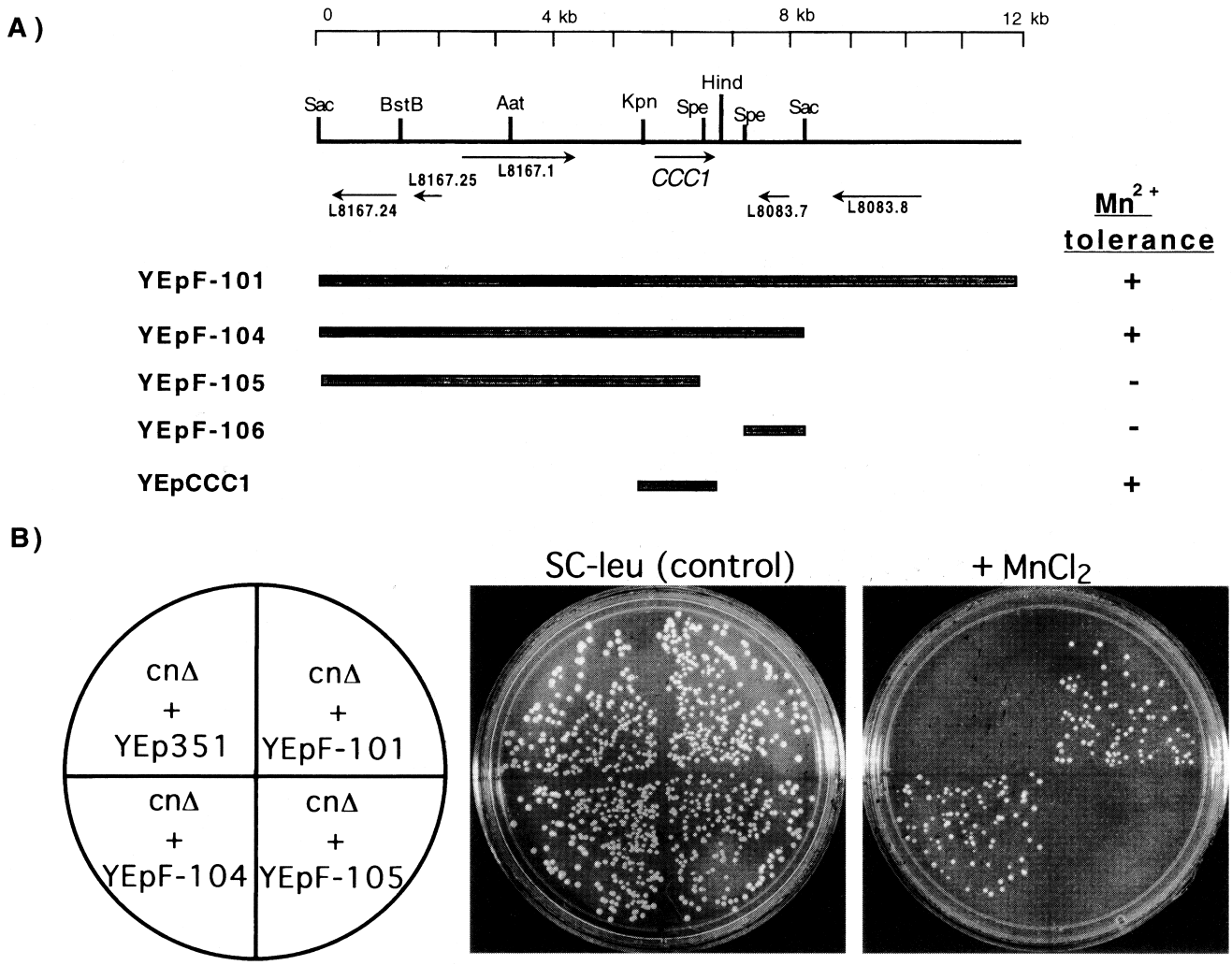

FIG. 2. The increased Mn^{2+} tolerance conferred by YEpF-101 is due to *CCC1*. (A) Restriction map of the genomic insert contained in YEpF-101. All ORFs in the region are indicated by arrows. The dark bars indicate the subcloned fragment of the original YEpF-101 insert present in each plasmid. Abbreviations: Sac, *Sac*I; BstB, *BstB*I; Aat, *Aat*II; Kpn, *Kpn*I; Spe, *Spe*I; Hind, *Hin*dIII. (B) Saturated overnight cultures were diluted and spread on synthetic medium lacking leucine (SC-leu) with or without 10 mM $MnCl_2$ and grown for 2 days at 30°C. The strains were DD09 transformed with YEp351 (cnΔ + YEp351), DD09 transformed with YEpF-101 (cnΔ + YEpF-101); DD09 transformed with YEpF-104 (cnΔ + YEpC-104), and DD09 transformed with YEpF-105 (cnΔ + YEpC-105).

cuvette holder-attached stirrer (model 1069; Instech, Inc.). Addition of 6 μM nigericin (Sigma) was used to dissipate the proton gradient created by the vacuolar H^+-ATPase. When indicated, the vesicles were preincubated for 3 min in 1 μM bafilomycin A (a generous gift from Barry Bowman) before the addition of ATP. The fluorimetric experiments demonstrating Ca^{2+}/H^+ exchange were performed in an identical manner except that the vesicles were incubated in buffer containing 100 μM EGTA [ethylyne glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid] (to reduce residual Ca^{2+} in the reaction buffer) before the addition of ATP. After the acidification, $CaCl_2$ was added to a final concentration of 100 μM. For each subsequent addition of $CaCl_2$, 150 nmol of $CaCl_2$ was added to the reaction mixture.

Nucleotide sequence accession number. The *HUM1* sequence was submitted to GenBank and is listed there under accession number U18944.

RESULTS

Identification of high-copy-number plasmids that increase the Mn^{2+} tolerance of calcineurin mutants. Yeast strains lacking functional calcineurin (cnΔ strains) display an increased sensitivity to Mn^{2+} relative to that of isogenic wild-type strains (Fig. 1). We isolated two high-copy-number plasmids from a yeast genomic library, YEpC-101 and YEpF-101, that significantly improve the growth of the calcineurin mutant strain DD09 on medium containing 10 mM $MnCl_2$ (Fig. 1) (see Materials and Methods). These plasmids also increase the Mn^{2+} tolerance of the isogenic wild-type parent strain, YPH499 (data not shown).

Calcineurin mutants exhibit specific growth defects under a number of other environmental conditions: they are more sensitive to Na^+, Li^+, and high pH than are isogenic wild-type cells (9, 41), and they display a defect in recovery from pheromone arrest (12, 13). In contrast to the Mn^{2+} sensitivity of calcineurin mutants, these additional phenotypes were unchanged by the YEpC-101 and YEpF-101 plasmids (data not shown). Thus, YEpC-101 and YEpF-101 specifically increase tolerance to Mn^{2+} but do not substitute generally for a lack of calcineurin in vivo.

***CCC1* is present on YEpF-101 and increases the Mn^{2+} tolerance of calcineurin mutants when present at high dosage.** Expression of the YEpF-101 plasmid leads to strong rescue of the Mn^{2+} sensitivity of cnΔ strains (Fig. 1). This plasmid contains a 12-kb insert of yeast genomic DNA (Fig. 2A). Hybridization of a fragment from YEpF-101 to a collection of ordered

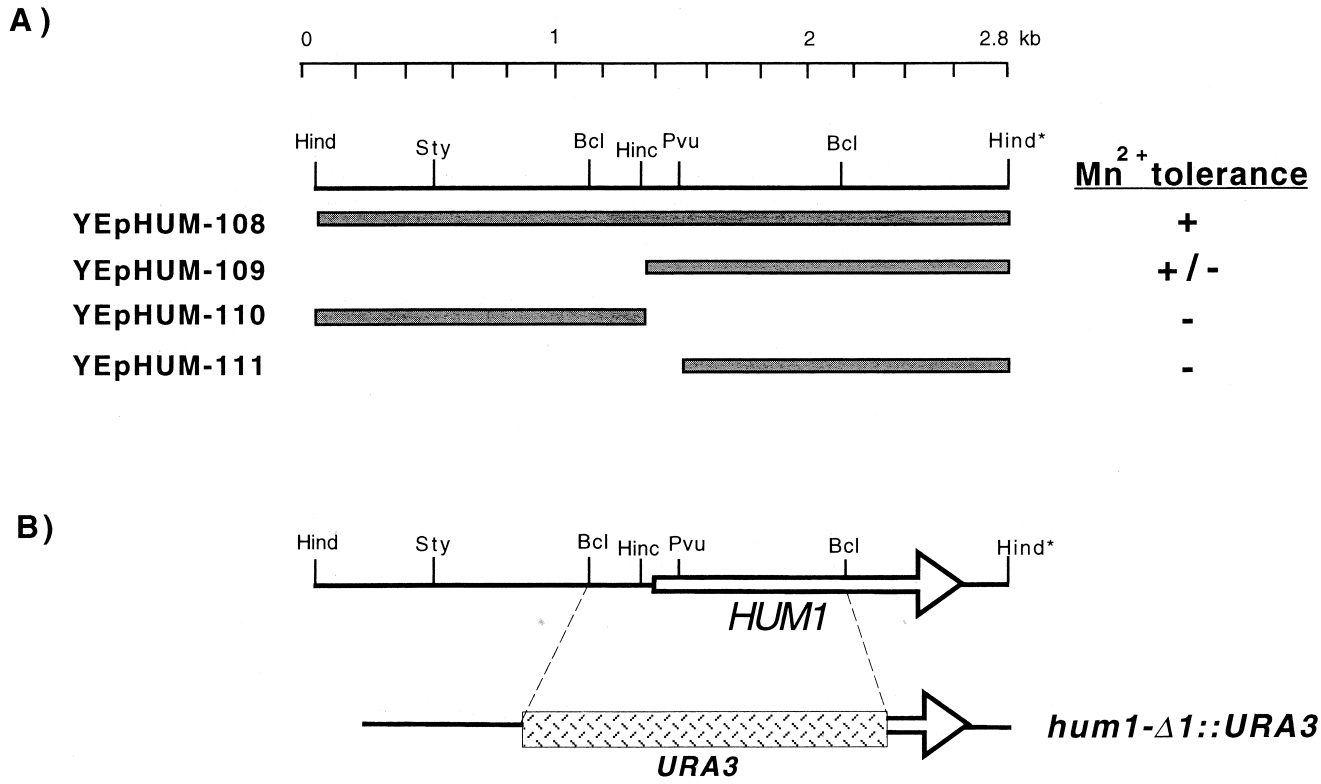

FIG. 3. The increased Mn^{2+} tolerance conferred by YEpC-101 is due to *HUM1*. (A) Restriction map of the 2.8-kb insert from YEpC-101. The dark bars indicate the subcloned fragment of YEpC-101 present in each plasmid. Abbreviations: Sty, *Sty*I; Bcl, *Bcl*I; Hinc, *Hinc*II; Pvu, *Pvu*II; Hind, *Hin*dIII. The *Hin*dIII site marked with an asterisk is a site from YEp351 and not from the genomic insert. (B) Localization of the *HUM1* ORF and construction of the *hum1-Δ1::URA3* allele.

lambda phage representing the yeast genome (32) revealed that the genomic DNA in this plasmid originates from chromosome XII, between *NMT1* and *CDC42* (ATCC clone 70072). The complete DNA sequence of this region of chromosome XII has been determined (GenBank accession numbers U19027 and U14913), and we used this information to establish that a single ORF contained in the YEpF-101 insert was sufficient to confer Mn^{2+} tolerance (Fig. 2) (see Materials and Methods). This ORF was previously identified as *CCC1* (21). We examined the response of cells lacking *CCC1* to Mn^{2+} and Ca^{2+} stress. Cells lacking *CCC1* (strain TDY689-ccc1Δ) are viable, and we observed, as had been previously reported (21), that this strain is not Ca^{2+} sensitive. The TDY689-ccc1Δ strain also demonstrates growth identical to that of its isogenic wild-type strain (strain TDY689) on Mn^{2+}-containing medium (data not shown). Furthermore, the growth of a strain lacking both calcineurin and Ccc1p (TPY7) did not differ significantly from that of an isogenic calcineurin mutant strain on either Mn^{2+}- or Ca^{2+}-containing medium (data not shown).

***HUM1* is present on YEpC-101 and increases the Mn^{2+} tolerance of calcineurin mutants when present at high dosage.** cnΔ cells containing the YEpC-101 plasmid are able to grow on medium containing 10mM $MnCl_2$ (Fig. 1). YEpC-101 contained a 5-kb insert of yeast genomic DNA. A 2.8-kb fragment of this insert was as effective as the entire insert in conferring Mn^{2+} tolerance to cnΔ cells (Fig. 3, YEpHUM-108). Hybridization of this 2.8-kb fragment to an ordered collection of lambda phage representing the entire yeast genome (32) localized this DNA to chromosome IV, between *RPC53* and *PHO2* (ATCC clone 70057). Hybridization of this same fragment to RNA by Northern analysis detected a single transcript, approximately 1.2 kb in size (data not shown).

The region of the 2.8-kb insert able to confer Mn^{2+} tolerance was further localized to a single 1.2-kb ORF, which we named *HUM1* for high copy number undoes Mn^{2+} sensitivity (Fig. 3) (see Materials and Methods).

***HUM1* encodes a novel yeast protein with limited homology to mammalian Na^+/Ca^{2+} exchangers.** The predicted protein product of the *HUM1* gene is 411 amino acids in length, with an approximate molecular mass of 45 kDa (Fig. 4). Kyte-Doolittle hydropathy analysis of the predicted protein sequence revealed 11 highly hydrophobic regions predicted to encode membrane-spanning domains (Fig. 4, underlined regions; Fig. 5A). Comparison of the *HUM1* ORF with the current database of known protein sequences revealed two regions with significant similarity to the bovine retinal Na^+/Ca^{2+}-K^+ exchanger (Fig. 5B). These regions of similarity include predicted transmembrane domains 3, 4, and 8 of Hum1p and predicted transmembrane domains 2, 3, and 8 of the mammalian retinal Na^+/Ca^{2+}-K^+ exchanger (50). The retinal Na^+/Ca^{2+}-K^+ exchanger is also distantly related to the mammalian cardiac Na^+/Ca^{2+} exchanger (43, 50). As shown in Fig. 5B, the two areas of amino acid similarity between the retinal and cardiac Na^+/Ca^{2+} exchangers overlap exactly with the regions of similarity between the retinal Na^+/Ca^{2+} exchanger and Hum1p (50). Thus, although limited, the similarity between Hum1p and the Na^+/Ca^{2+} exchangers is likely to be functionally significant.

Hum1p shows a significant degree of homology to the predicted protein products of two additional sequences. The C-terminal half of a hypothetical yeast protein of unknown func-

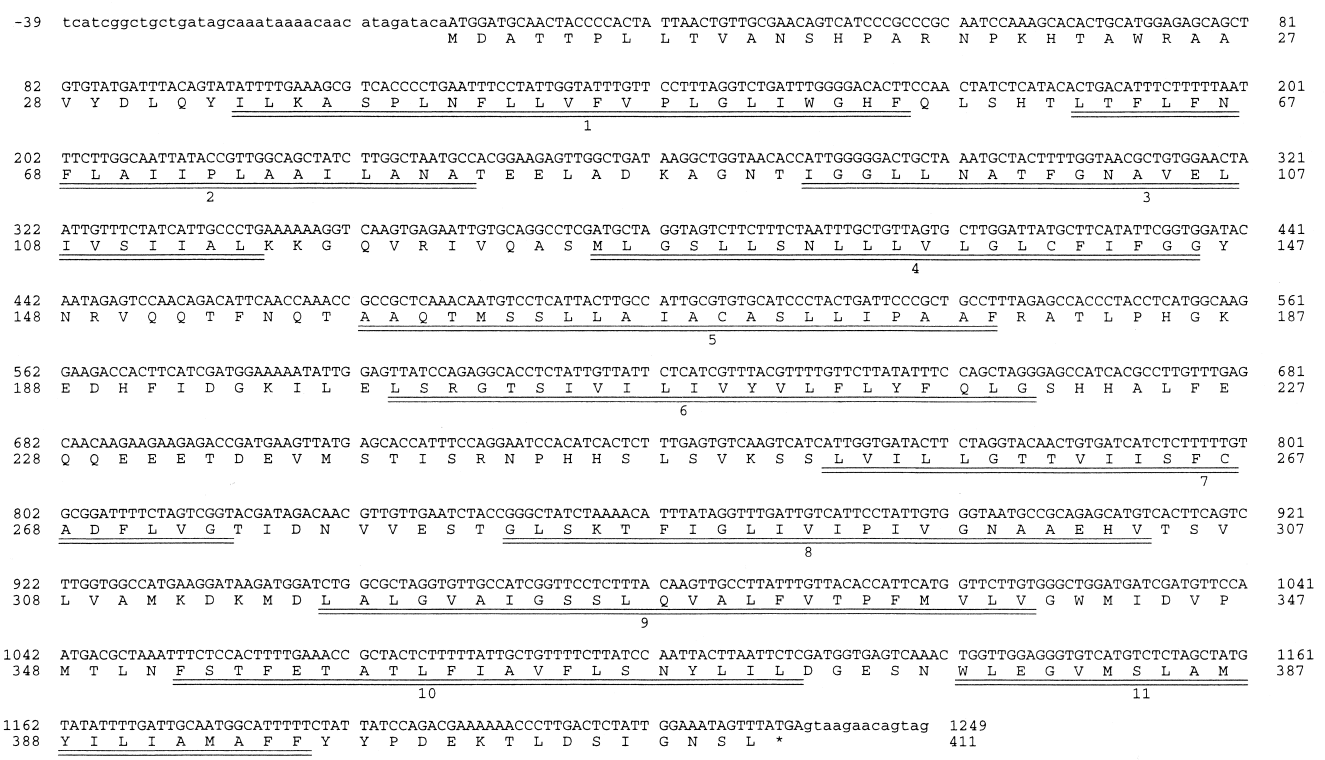

FIG. 4. Molecular analysis of *HUM1*. The nucleotide sequence of *HUM1* and the predicted amino acid sequence are shown. Predicted transmembrane regions are double underlined and numbered.

tion, NO339 (Swiss-Prot accession number P42839), is 22% identical to Hum1p. This homology extends throughout the whole HUM1 protein sequence and includes the regions similar to Na^+/Ca^{2+} exchangers. Also, the C-terminal third of Hum1p is 35% identical to the entire amino acid sequence predicted by a partial rice cDNA sequence (GenBank accession number D15647).

***HUM1* is not essential for growth.** TPY1, a diploid strain that was heterozygous for a disruption allele of *HUM1* (*hum1Δ1*::*URA3*/*HUM1*) (see Materials and Methods) (Fig. 3B) was induced to undergo sporulation, and its haploid meiotic products were analyzed. All tetrads dissected produced four viable spores and displayed 2:2 segregation of Ura^+. Haploid cells carrying the *hum1Δ1*::*URA3* allele (TPY2) are viable and show no obvious defects in growth or morphology; thus, the *HUM1* gene is not required for vegetative growth. Furthermore, the tolerance of TPY2 to Mn^{2+} and Ca^{2+} does not differ significantly from that of the wild type (Fig. 6), although a slight resistance of this strain to Na^+ has been observed (data not shown). Similar results were obtained when the *hum1Δ1*::*URA3* allele was introduced into a different genetic background (i.e., TPY5); however, this strain did display Mn^{2+} sensitivity relative to the wild-type level (see Fig. 8).

***HUM1* and calcineurin display a genetic interaction.** *HUM1* was initially identified by its ability to modify a calcineurin mutant phenotype; therefore, we further investigated the genetic relationship between *HUM1* and calcineurin by constructing strains that lack both proteins (see Materials and Methods). The *cnb1Δ1*::*LEU2 hum1Δ1*::*URA3* double mutants (e.g., TPY3) are viable and display a normal growth rate on standard growth media (i.e., synthetic medium and YPD). Like cnΔ strains, TPY3 fails to grow on media containing high concentrations of NaCl (data not shown). However, the *cnb1Δ1*::*LEU2 hum1Δ1*::*URA3* cells differ from the parental strains in two respects. First, they are more sensitive to Mn^{2+} than cnΔ cells. Second, their growth is inhibited in medium containing high concentrations of Ca^{2+} (Fig. 6). In contrast, the growth of *hum1* cells is indistinguishable from that of wild-type cells on Ca^{2+}-supplemented medium, and cnΔ strains grow even better than wild-type strains under these conditions (Fig. 6).

***HUM1* exacerbates the Ca^{2+} sensitivity of strains lacking *PMC1*.** The similarity of the *HUM1* gene product to Na^+/Ca^{2+} exchangers and the Ca^{2+} sensitivity of *cnb1 hum 1* double mutants suggested that *HUM1* might participate in cellular Ca^{2+} homeostasis. We therefore investigated the effect of disrupting the *HUM1* gene in mutant strains with known defects in Ca^{2+} sequestration.

PMC1 encodes a vacuolar protein with homology to mammalian Ca^{2+}-ATPases and has been proposed to transport Ca^{2+} from the cytosol into the vacuole (11). We compared the growth of isogenic wild-type, *hum1*, *pmc1*, and *hum1 pmc1* mutant strains on medium containing Ca^{2+}. As previously described, the growth of *pmc1* mutants is compromised on media containing Ca^{2+} (11) (Fig. 6). We observed that a *hum1 pmc1* double mutant strain (strain TPY4) shows significantly more growth inhibition than a *pmc1* mutant (ASY1) on medium containing 600 mM $CaCl_2$ (Fig. 6). In contrast, the *pmc1* mutation did not affect the ability of yeast cells to tolerate Mn^{2+}, as the growth of both the *pmc1* and the *pmc1 hum1* strains was indistinguishable from that of wild-type cells on medium containing Mn^{2+} (Fig. 6).

Mutations in *HUM1* decrease the cellular Ca^{2+} concentration. To further examine the effect of Hum1p on Ca^{2+} homeostasis, the total concentration of Ca^{2+} in mutant and wild-type strains was determined by $^{45}Ca^{2+}$ labeling. We

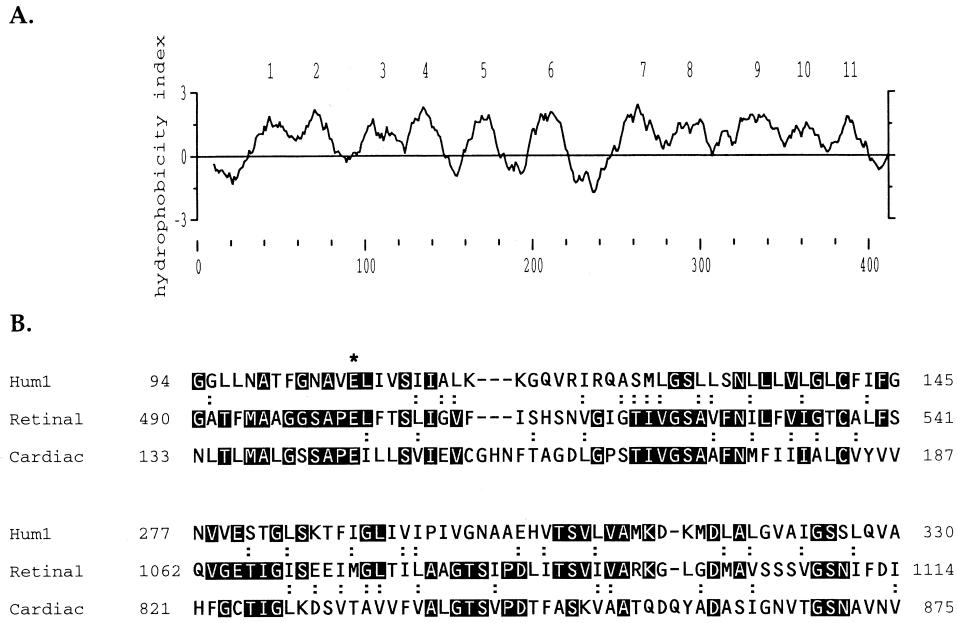

FIG. 5. (A) Kyte-Doolittle hydropathy analysis of the predicted HUM1 amino acid sequence, using a window size of 19 amino acids. (B) Alignment of regions of HUM1, the bovine retinal Na^+/Ca^{2+}-K^+ exchanger (50), and the canine cardiac Na^+/Ca^{2+} exchanger (43). Homology was identified by using the BLAST program. Residues in HUM1 or the cardiac exchanger which are identical to those in the retinal exchanger are highlighted. Similar amino acids are indicated by dots. Amino acids considered similar are as follows: E and D; V, L, I, and M; A and G; F, Y, and W; S and T; Q and N; and R and K. The conserved glutamate denoted by the asterisk has been shown to play a critical role in the function of the cardiac exchanger (42).

determined, as shown in Fig. 7, that the Ca^{2+} content of *pmc1* cells was reduced relative to that of the wild type. This finding is similar to those previously published for this mutant (11). We also observed that the Ca^{2+} content of *hum1* cells was slightly reduced relative to that of the wild type. Finally, *hum1 pmc1* cells showed a more dramatic reduction in cellular Ca^{2+} than that observed for either single mutant strain. Thus, both *pmc1* and *hum1* disrupt intracellular Ca^{2+} homeostasis, and the effects of the two mutations are additive.

***HUM1* does not exacerbate the Ca^{2+} sensitivity of strains lacking *VPH1*.** The major store for intracellular Ca^{2+} in a yeast cell is the vacuole. In vitro experiments established that the major mechanism of Ca^{2+} transport into the yeast vacuole is Ca^{2+}/H^+ antiport, which is dependent on the proton gradient generated by the vacuolar H^+-ATPase (45). We tested the possibility that *HUM1* contributes to this Ca^{2+}/H^+ exchange by examining genetic interactions between *VPH1* and *HUM1*. *VPH1* encodes an integral membrane protein required to target the V-ATPase protein complex to the vacuolar membrane (37). Thus, *vph1* mutants are defective in vacuole acidification, and they are Ca^{2+} sensitive as a result of impaired Ca^{2+} uptake into the vacuole via Ca^{2+}/H^+ exchange. We compared the phenotypes of wild-type, *vph1*, *hum1*, and *vph1 hum1* strains (Fig. 8). We observed, as reported previously, that

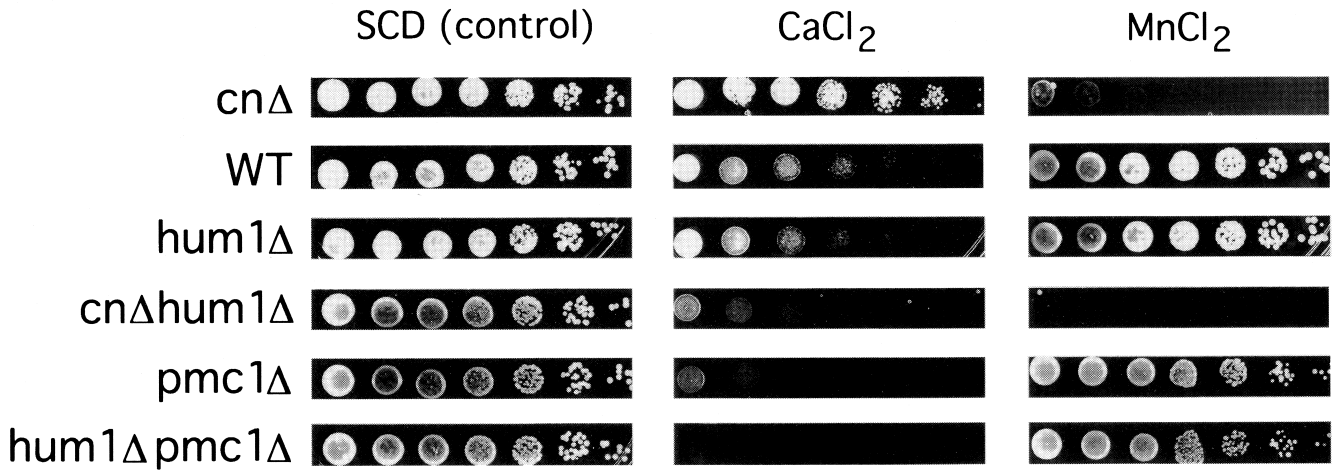

FIG. 6. Genetic interactions of *HUM1* with calcineurin and *PMC1*. Saturated overnight cultures were diluted to an OD_{600} of 1, and 10-μl portions of serial fivefold dilutions were spotted onto synthetic medium containing no added ion [SCD (control)], 600 mM $CaCl_2$, or 8 mM $MnCl_2$. Plates were incubated at 30°C for 2 days. The strains were MCY300 (cnΔ), YPH499 (WT), TPY2 (hum1Δ), TPY3 (cnΔhum1Δ), ASY1 (pmc1Δ), and TPY4 (hum1Δpmc1Δ).

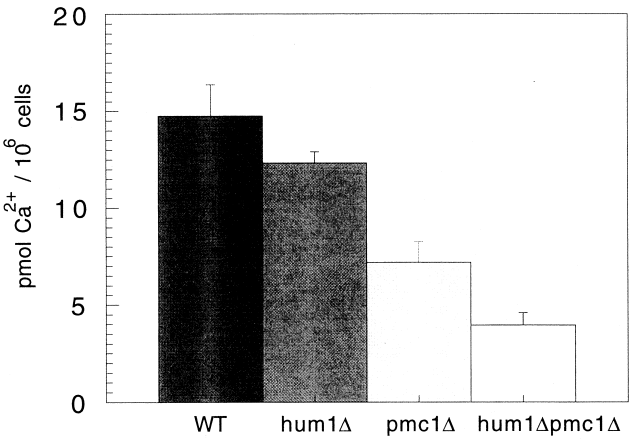

FIG. 7. Effect of *HUM1* and *PMC1* on cellular Ca^{2+} concentration. See Materials and Methods for experimental details. The strains were YPH499 (WT), TPY2 (hum1Δ), ASY1 (pmc1Δ), and TPY4 (hum1Δpmc1Δ). Error bars indicate standard deviations.

*vph1*Δ strains are more sensitive than wild-type strains to Ca^{2+} (38). *vph1*Δ *hum1*Δ double mutants (TPY6) displayed a degree of Ca^{2+} sensitivity equivalent to that of *vph1*Δ strains, indicating that disruption of *HUM1* function in a *vph1* strain does not exacerbate the Ca^{2+} sequestration defect of this strain (Fig. 8). We also observed that *vph1* strains are sensitive to Mn^{2+} and that in this strain background, *hum1* cells display Mn^{2+} sensitivity. In contrast, *vph1 hum1* double mutants are dramatically Mn^{2+} tolerant, growing even better than the wild-type strain on medium containing 12 mM $MnCl_2$ (Fig. 8).

Hum1p is required for Ca^{2+} uptake into vacuolar vesicles. The finding that the Ca^{2+} sensitivity of *vph1* mutants is unchanged by disruption of *hum1* is consistent with the idea that these two gene products function in the same Ca^{2+}-sequestering mechanism, i.e., vacuolar Ca^{2+}/H^+ exchange. Therefore, we prepared vacuolar membrane vesicles from wild-type and *hum1* strains and compared the Ca^{2+} transport activities of these two preparations (see Materials and Methods for experimental details). We observed Ca^{2+} transport activity in the vacuolar vesicles from wild-type cells (Fig. 9A). As shown previously (45), this Ca^{2+} uptake was ATP dependent, and it was sensitive to bafilomycin A, a specific inhibitor of the vacuolar ATPase (8). In contrast, vacuolar vesicles isolated from a *hum1* mutant strain displayed no significant Ca^{2+} uptake activity. Earlier studies indicated that vacuolar Ca^{2+} transport is dependent on the formation of a pH gradient and that Ca^{2+} influx is accompanied by proton efflux, demonstrating that Ca^{2+} accumulates via Ca^{2+}/H^+ antiport (45). We observed proton uptake, as monitored by fluorescence quenching of the pH-sensitive dye ACMA, in vacuolar vesicles prepared from both wild-type and *hum1* mutant strains (Fig. 10). The overall rates and extents of acidification were identical in the two preparations. The fluorescence signal was restored to the original level by addition of nigericin, confirming the formation of a transmembrane pH gradient, with interior acid, in these vesicles. ΔpH formation was dependent on ATP and was inhibited by preincubation with bafilomycin A, indicating that the pH gradient was formed as a result of the activity of the vacuolar H^+-ATPase (Fig. 10). The small reduction in fluorescence observed in the bafilomycin A-treated vesicles is due to a quenching artifact caused by ATP and is not reversed by nigericin (53) (Fig. 10). As previously observed (45) up to 44% of the ΔpH of wild-type vacuolar vesicles was dissipated by addition of $CaCl_2$ (300 μM), indicating the presence of a Ca^{2+}/H^+ exchange activity in these vesicles (Fig. 10). However, in vacuolar vesicles derived from a strain lacking *hum1*, this Ca^{2+}/H^+ exchange activity was completely absent, and the addition of $CaCl_2$ had no effect on the ΔpH in these vesicles. Thus, we conclude that Hum1p is required for Ca^{2+}/H^+ exchange in yeast vacuolar membrane vesicles.

DISCUSSION

In this report, we showed that yeast cells lacking the serine/threonine phosphatase calcineurin grow poorly in the presence of Mn^{2+} compared with wild-type cells. We also identified two genes (*CCC1* and *HUM1*) that, when present at high dosages, increase the tolerance to Mn^{2+} of both calcineurin mutants and wild-type cells. Calcineurin mutants display a variety of phenotypes, including sensitivities to Na^+, Li^+, and high pH (9, 40, 41), that are not affected by overexpression of Ccc1p or Hum1p. Thus, Ccc1p and Hum1p likely act by altering Mn^{2+} homeostasis directly, rather than by substituting for the loss of calcineurin activity. Furthermore, additional observations indicate that both Ccc1p and Hum1p participate not only in Mn^{2+} homeostasis but also in Ca^{2+} homeostasis.

CCC1 was originally identified by its ability to complement a Ca^{2+}-sensitive (*csg1*) mutant when present on a low-copy-number (centromere-based) plasmid (21). The predicted *CCC1* gene product contains five putative transmembrane regions and possesses no homology to known proteins. The deletion of *CCC1* does not confer sensitivity to high concentrations of Ca^{2+}, nor does deletion of *CCC1* in a *csg1* background exacerbate the Ca^{2+} sensitivity of *csg1* mutants (21). Consistent with our findings, overexpression of *CCC1* was shown to reduce the Mn^{2+} sensitivity of *pmr1* mutant cells (28) (the role of *PMR1* in Mn^{2+} homeostasis is discussed below). However, the growth of *ccc1* mutants on Mn^{2+}-containing medium is

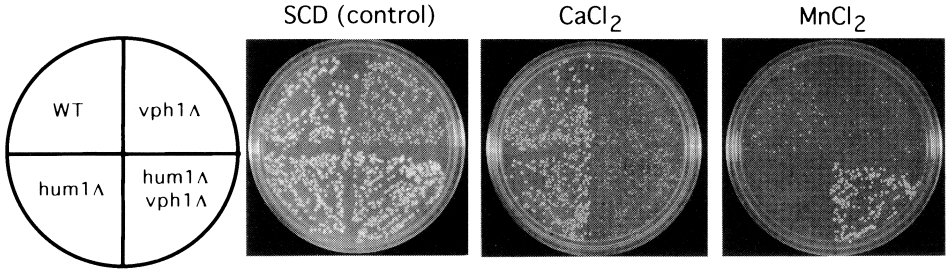

FIG. 8. Genetic interactions of *HUM1* with *VPH1*. Saturated overnight cultures were diluted and spread on synthetic medium containing no added ion [SCD (control)], 400 mM $CaCl_2$, or 12 mM $MnCl_2$. Cells on the control and $MnCl_2$ plates were grown for 2 days at 30°C; the plates containing $CaCl_2$ were incubated for 3 days. The strains were BJ7290 (WT), BJ7289 (vph1Δ), TPY6 (hum1Δ), and TPY7 (hum1Δvph1Δ).

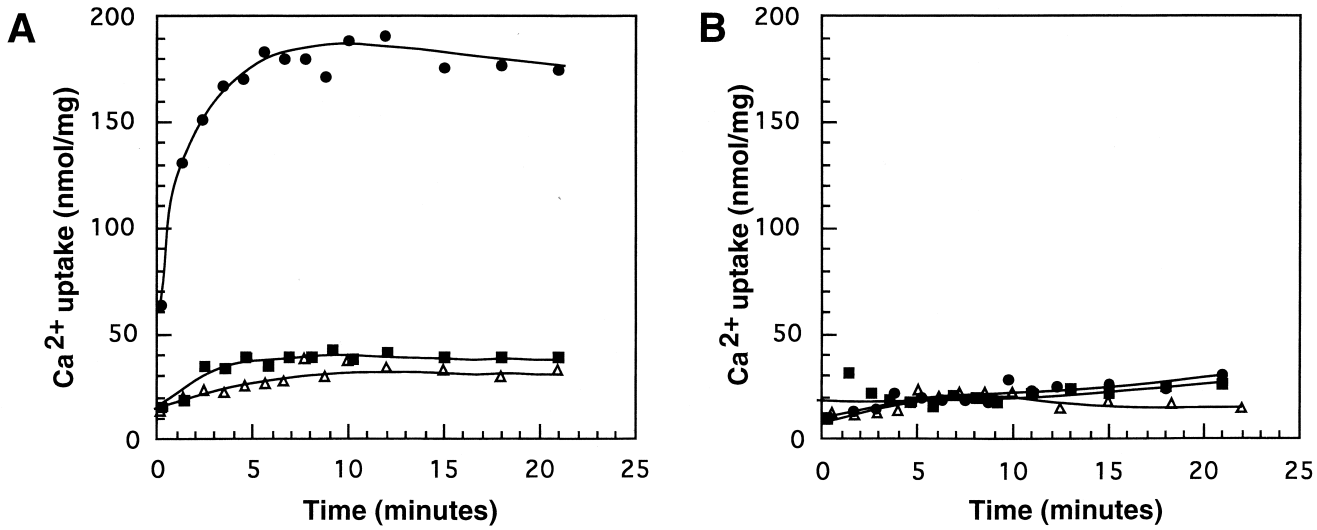

FIG. 9. Vacuolar membrane vesicles from *hum1* cells are defective in ATP-dependent, bafilomycin-sensitive Ca^{2+} transport. (A) Ca^{2+} transport in vacuolar vesicles from a wild-type strain (YPH499). Assays were carried out as described in Materials and Methods with 0.3 mM ATP (●), with 0.3 mM ATP and 3 μM bafilomycin (■), or without ATP (△). (B) Ca^{2+} transport in vacuolar vesicles from a *hum1* strain (TPY2). Conditions and symbols are as described for panel A.

indistinguishable from that of wild-type cells. Despite the apparent failure of *ccc1* mutations to perturb Ca^{2+} or Mn^{2+} homeostasis, the effects of *CCC1* overexpression suggest that this gene product facilitates Ca^{2+} and Mn^{2+} sequestration or efflux. *CCC1* could affect ion homeostasis indirectly. However, the fact that *CCC1* encodes a predicted integral membrane protein suggests that this gene product may represent a novel type of Ca^{2+} and/or Mn^{2+} transporter. Further insights into the exact function of *CCC1* may result from a biochemical characterization of this gene product.

HUM1 is a novel yeast gene predicted to encode a highly hydrophobic protein containing 11 predicted transmembrane domains. This gene product displays significant similarity to mammalian Na^+/Ca^{2+} exchangers in two limited regions. Two types of mammalian Na^+/Ca^{2+} exchangers have been characterized (48). The retinal Na^+/Ca^{2+}-K^+ exchanger is expressed in the outer segment of rod photoreceptor cells, while cardiac myocytes express a distinct Na^+/Ca^{2+} exchanger. The two mammalian proteins are similar in size and topology, containing 11 hydrophobic segments and a large intracellular loop (which serves a regulatory function) between the fifth and sixth hydrophobic segment (43, 50). The retinal and cardiac exchangers have only two short segments of sequence similarity: two hydrophobic regions of about 50 amino acids each that are 30 to 40% identical in the two proteins (50). These same regions of the retinal Na^+/Ca^{2+}-K^+ exchanger are 30% identical and 55% similar in amino acid sequence to regions of the *HUM1* product. Furthermore, mutagenesis has identified several amino acids within these regions that are crucial for ion conduction. One of these residues is a glutamate (marked with an asterisk in Fig. 5B) that is conserved in the *HUM1* product and both mammalian exchangers. Even the most conservative amino acid substitutions (to aspartate or glutamine) when introduced at this position of the cardiac exchanger destroys its activity (42). Thus, the similarities in sequence and predicted topology between the *HUM1* gene product and mammalian Na^+/Ca^{2+} exchangers strongly suggest that Hum1p promotes ion exchange.

Several experimental findings indicate that Hum1p participates in both Ca^{2+} and Mn^{2+} homeostasis in yeast cells. First, some *hum1* mutant strains (i.e., TPY5 but not TPY2) display increased sensitivity to Mn^{2+} (Fig. 8), suggesting a defect in Mn^{2+} homeostasis. Second, although the growth of *hum1* mutants is not impaired by Ca^{2+}, the Ca^{2+} content of these cells is slightly reduced, suggesting that they do have a modest defect in Ca^{2+} sequestration. Further observations indicate that *hum1* mutants fail to display Ca^{2+} sensitivity because of the presence of other cellular ion transporters, especially Pmc1p. *PMC1* encodes a P-type ATPase that is thought to pump Ca^{2+} from the cytosol into the vacuole (11). The growth of *pmc1* mutants is inhibited by high levels of Ca^{2+} in the growth medium, and we found that this inhibition is exacerbated in *pmc1 hum1* double mutants. Also, the Ca^{2+} content of *hum1 pmc1* mutants is dramatically lower than that of *pmc1* mutants. The additive effects of *hum1* and *pmc1* suggest that both Hum1p and Pmc1p remove excess Ca^{2+} from the cytosol and that these two gene products function independently.

In contrast, we find that *vph1* mutants and *vph1 hum1* double mutants are equally Ca^{2+} sensitive. *vph1* mutants are specifically defective in vacuole acidification, which disrupts vacuolar Ca^{2+} transport via Ca^{2+}/H^+ antiport (37, 38, 49). The vacuolar polyphosphate content is also drastically reduced in *vph1* mutants (52), and the Ca^{2+} content of yeast vacuoles is known to vary proportionally with polyphosphate content (15). Therefore, the Ca^{2+} sensitivity of *vph1* mutants probably results from a combination of the vacuolar Ca^{2+} transport and polyphosphate defects. The failure of *hum1* to exacerbate the Ca^{2+} sensitivity of *vph1* mutants and the amino acid similarity of Hum1p to ion-exchange proteins suggest that Hum1p might function in vacuolar Ca^{2+}/H^+ exchange.

We tested the role of Hum1p in vacuolar Ca^{2+} sequestration directly by comparing the Ca^{2+} transport activities of vacuolar membrane vesicles isolated from wild-type and *hum1* mutant cells. Ohsumi and Anraku showed that wild-type vacuolar vesicles exhibit ATP-dependent Ca^{2+} transport that is dependent on a proton gradient generated by the vacuolar H^+-ATPase (45). They also established that this Ca^{2+} transport proceeds via Ca^{2+}/H^+ antiport. We found that vacuolar vesicles derived from a *hum1* strain are defective for Ca^{2+} uptake and completely lack Ca^{2+}/H^+ exchange activity. We conclude, there-

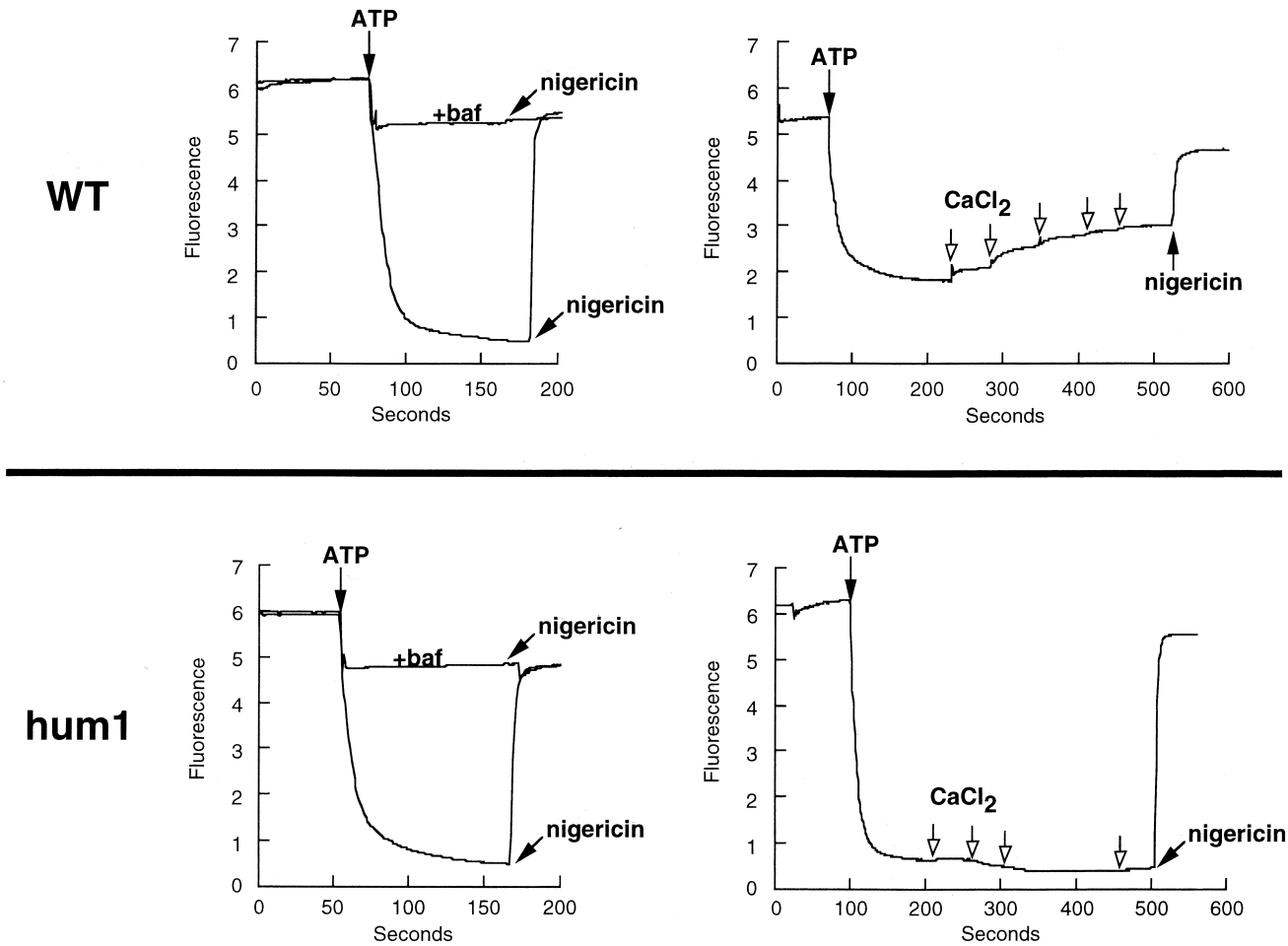

FIG. 10. Vacuolar membrane vesicles from *hum1* cells are defective in Ca^{2+}/H^+ exchange. Vesicle acidification was assayed by the quenching of ACMA fluorescence (graphed as counts per second [10^6]) over time and measured as described in Materials and Methods. The experiments for which results are shown in the upper panel were performed with vacuolar membrane vesicles from a wild-type (WT) strain (YPH499), and those for which results are shown in the lower panel were performed with vesicles from a *hum1* strain (TPY2). Solid arrows mark the addition of ATP or nigericin as indicated. +baf indicates the fluorescence quench that resulted from incubation of vesicles with 1 μM bafilomycin A before ATP addition. Open arrows mark the addition of $CaCl_2$ as follows: the first arrow denotes an initial addition of 300 nmol of $CaCl_2$ to the reaction mixture, which contained 300 nmol of EGTA, and all other arrows denote further additions of 150 nmol of $CaCl_2$ each.

fore, that Hum1p effects Ca^{2+}/H^+ exchange across the yeast vacuolar membrane.

These studies mark the first characterization, at the molecular level, of a Ca^{2+}/H^+ exchange protein. Ca^{2+}/H^+ exchange activities are associated with many plant and fungal vacuoles, and the molecular characterization of *HUM1* provides a critical step towards the identification of this important class of ion exchangers in other organisms. For example, the partial rice cDNA sequence (GenBank accession number D15647) whose predicted protein product displays extensive homology to the *HUM1* product, may prove to encode a Ca^{2+}/H^+ exchanger.

While the genetic and biochemical evidence presented here clarifies the role of Hum1p in Ca^{2+} homeostasis, the role of this protein in Mn^{2+} homeostasis is less clear. As mentioned, some *hum1* strains are Mn^{2+} sensitive, and *hum1* exacerbates the Mn^{2+} sensitivity of calcineurin mutants. Together with the observation that Hum1p overexpression increases the Mn^{2+} tolerance of yeast cells, these findings suggest that Hum1p might transport Mn^{2+} as well as Ca^{2+} into the vacuole. $MnCl_2$, while not as effective as $CaCl_2$, does promote proton efflux from vacuolar membrane vesicles (6). We also report a surprising observation: the growth of *vph1 hum1* double mutants is significantly improved in the presence of Mn^{2+} relative to that of wild-type cells. Presumably this resistance results from alterations in the activity or expression of other ion transporters in the cell. There are two intracellular compartments in *S. cerevisiae* that are known to contain Mn^{2+}: the vacuole and the Golgi. As mentioned above, Mn^{2+} is thought to enter the vacuole via proton exchange (6, 7, 47). Mn^{2+} transport into the yeast Golgi is thought to be mediated by the *PMR1* gene product. *PMR1* encodes a Golgi-localized P-type ATPase, and *pmr1* mutants are sensitive to Mn^{2+} (3, 29, 51). Thus, the increased Mn^{2+} tolerance of *vph1 hum1* mutants could result from an activation of *PMR1*-dependent transport of Mn^{2+} into the Golgi. Alternatively, *CCC1*, or some other, yet-unidentified activity, might be responsible for the increased Mn^{2+} tolerance of these cells.

Finally, these studies provide further evidence of the participation of calcineurin in regulating Ca^{2+} homeostasis. Previous studies have established that calcineurin inhibits intracellular Ca^{2+} sequestration. Cells lacking functional calcineurin grow better than wild-type cells on Ca^{2+}-supplemented media and have a higher cellular Ca^{2+} content than wild-type cells (56). Furthermore, the observations that calcineurin mutations sup-

press the Ca^{2+} sensitivity of *pmc1* mutants and increase the Ca^{2+} content of these cells (11) indicate that the calcineurin-mediated inhibition of Ca^{2+} sequestration is independent of *PMC1*. In contrast, disruption of calcineurin function exacerbates the Ca^{2+} sensitivity of other mutants, including *vma3*, *vph1*, and *csg2* mutants (6, 56, 57) and, as shown here, *hum1* mutants. This effect may be due, as suggested, to calcineurin-mediated inhibition of Ca^{2+} uptake into one or more intracellular compartments (57). Ultimately, the direct in vivo targets of calcineurin must be identified to understand fully the physiological role of this protein phosphatase. As demonstrated by this report, however, studies employing calcineurin mutants have already provided valuable insight into the mechanisms of intracellular ion homeostasis.

ACKNOWLEDGMENTS

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ADDENDUM

An independent report of the sensitivity of calcineurin mutants to Mn^{2+} was recently published (18).

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