The Golgi Apparatus Plays a Significant Role in the Maintenance of Ca\(^{2+}\) Homeostasis in the vps33\(\Delta\) Vacuolar Biogenesis Mutant of Saccharomyces cerevisiae*

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The vacuole is the major site of intracellular Ca\(^{2+}\) storage in yeast and functions to maintain cytosolic Ca\(^{2+}\) levels within a narrow physiological range. In this study, we examined how cellular Ca\(^{2+}\) homeostasis is maintained in a vps33\(\Delta\) vacuolar biogenesis mutant. We found that growth of the vps33\(\Delta\) strain was sensitive to high or low extracellular Ca\(^{2+}\). This strain could not properly regulate cytosolic Ca\(^{2+}\) levels and was able to retain only a small fraction of its total cellular Ca\(^{2+}\) in a nonexchangeable intracellular pool. Surprisingly, the vps33\(\Delta\) strain contained more total cellular Ca\(^{2+}\) than the wild type strain. Because most cellular Ca\(^{2+}\) is normally found within the vacuole, this suggested that other intracellular compartments compensated for the reduced capacity to store Ca\(^{2+}\) within the vacuole of this strain. To test this hypothesis, we examined the contribution of the Golgi-localized Ca\(^{2+}\) ATPase Pmr1p in the maintenance of cellular Ca\(^{2+}\) homeostasis. We found that a vps33\(\Delta\)/pmr1\(\Delta\) strain was hypersensitive to high extracellular Ca\(^{2+}\). In addition, certain combinations of mutations effecting both vacuolar and Golgi Ca\(^{2+}\) transport resulted in synthetic lethality. These results indicate that the Golgi apparatus plays a significant role in maintaining Ca\(^{2+}\) homeostasis when vacuolar biogenesis is compromised.

Like all eukaryotes, the yeast Saccharomyces cerevisiae normally maintains a resting cytosolic Ca\(^{2+}\) concentration of 50–200 nM (1–3). This tight regulation of intracellular Ca\(^{2+}\) is required to control the complex signaling pathways mediated by cytosolic Ca\(^{2+}\)-sensing proteins such as calmodulin. Remarkably, yeast cells can maintain intracellular Ca\(^{2+}\) homeostasis in the presence of environmental Ca\(^{2+}\) concentrations ranging from <1 μM to >100 mM (4). The vacuole is thought to play a key role in maintaining Ca\(^{2+}\) tolerance over this wide range because it contains >90% of the total cellular Ca\(^{2+}\) (5, 6). Accordingly, many different vacuolar mutations result in an inability to grow in the presence of high concentrations of extracellular Ca\(^{2+}\) (7–13).

Currently, two Ca\(^{2+}\) transporters have been described which act to sequester Ca\(^{2+}\) in the vacuole. The first of these is the vacuolar Ca\(^{2+}\) ATPase encoded by the PMCI gene, a homolog of the mammalian PMCA plasma membrane family of Ca\(^{2+}\) ATPases. The loss of Pmc1p results in an inability to grow in the presence of high environmental Ca\(^{2+}\) (7). The second protein known to be involved in vacuolar Ca\(^{2+}\) transport is the H\(^+/Ca^{2+}\) exchanger encoded by the VCXI (HUM1) gene (14, 15). Although mutants that do not express Vcx1p show little or no decrease in Ca\(^{2+}\) tolerance, the combination of pmci\(\Delta\) and vcx1\(\Delta\) mutations leads to a more severe Ca\(^{2+}\)-sensitive phenotype than the loss of either transporter alone. Both the expression and function of these two vacuolar Ca\(^{2+}\) transporters are regulated by calcineurin, a highly conserved protein phosphatase that is activated by Ca\(^{2+}\)/calmodulin. As in mammalian cells, the activation of yeast calcineurin can be blocked by the immunosuppressant drugs cyclosporin A (CsA) and FK506 (16, 17). Although the functional relationship between these two vacuolar Ca\(^{2+}\) transporters is complex, it has been reported that calcineurin activation stimulates Pmc1p function and inhibits Vcx1p function (14, 15).

Several other genes encoding potential Ca\(^{2+}\) ATPases have been identified within the yeast genome (18); however, the only member of this group demonstrated to play a role in Ca\(^{2+}\) transport is encoded by the PMRI gene. Pmr1p is related to the SERCA family of Ca\(^{2+}\) ATPases and has been shown to reside in the Golgi apparatus of S. cerevisiae (19–22). Although Pmr1p and Pmc1p both act to partition Ca\(^{2+}\) into distinct cellular compartments, their roles in Ca\(^{2+}\) homeostasis do not appear to be equivalent. First, cells lacking Pmc1p are sensitive to high environmental Ca\(^{2+}\), whereas cells lacking Pmr1p cannot grow under low Ca\(^{2+}\) conditions. In addition, the total cellular Ca\(^{2+}\) level in a pmri\(\Delta\) strain is 2–3-fold lower than normal, but the total cellular Ca\(^{2+}\) level in the pmc1\(\Delta\) mutant is 4–5-fold higher than normal. These different phenotypes suggest that the vacuole and the Golgi apparatus normally carry out distinct roles in Ca\(^{2+}\) homeostasis.

Genetic screens have identified at least 60 different genes involved in vacuolar protein localization (23). Among these, the class C vacuolar protein sorting mutants (which include the vpsi1, vpsi6, vpsi8, and vps33 mutants) result in the most severe defects in vacuolar biogenesis. For example, strains carrying the vpsi33\(\Delta\) mutation lack a morphologically distinguishable vacuole but instead accumulate small vesicular and Golgi-like structures (24–26). These anomalous compartments may result from the inability to dock and/or fuse late transport vesicles from the biosynthetic, endocytic, and autophagic pathways with the vacuole (27). A vpsi33\(\Delta\) strain was also found to secrete >90% of soluble vacuolar proteins such as carboxypeptidase Y and to mislocalize nearly 50% of the vacuolar membrane protein α-mannosidase to the cell surface (24).

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1 The abbreviations used are: CsA, cyclosporin A; kh, kilobase; MES, 4-morpholineethanesulfonic acid; L, light emission; L\(_{\text{max}}\), maximum light emission.
In this study we asked how the severe defects in vacuolar biogenesis associated with the vps33Δ mutation affect cellular Ca\(^{2+}\) homeostasis. We found that the vps33Δ strain was sensitive to both high and low levels of environmental Ca\(^{2+}\) and was unable to regulate cytosolic Ca\(^{2+}\) levels properly when exposed to a sudden, large increase in environmental Ca\(^{2+}\). Despite its defect in vacuolar biogenesis, we found that the vps33Δ strain contains more total cellular Ca\(^{2+}\) than a wild type strain. To determine whether other intracellular compartments compensate for reduced vacuolar Ca\(^{2+}\) storage, we examined whether the Golgi-localized Ca\(^{2+}\) ATPase Pmr1p plays a significant role in Ca\(^{2+}\) homeostasis in the vps33Δ strain. We found that Pmr1p expression is elevated in the vps33Δ strain. We also found that a vps33Δ/pmr1Δ strain is hypersensitive to high extracellular Ca\(^{2+}\), and the combination of certain mutations effecting both vacuolar and Golgi Ca\(^{2+}\) transport results in synthetic lethality. These results indicate that the Golgi apparatus plays a significant role in maintaining Ca\(^{2+}\) homeostasis when vacuolar biogenesis is compromised.

**MATERIALS AND METHODS**

**Strains Used**—Strains used in this study are listed in Table I. The PMC1 and VCX1 genes were disrupted using the one-step gene replacement method (29). A 1.62-kb fragment of the PMC1 gene was generated by PCR using wild type yeast genomic DNA as template. The forward primer used was 5′-ATGAGTACCA CTCTTGAATG AT-3′, and the reverse primer was 5′-CATGATGGCT GCCATCTCA-3′. These primers contained KpnI and BamHI restriction endonuclease sites respectively (underlined). The PCR product was digested with KpnI and BamHI and cloned into a pBluescript II KS (+) plasmid. The 1.06-kb segment of the PMC1 gene was then removed by digestion with AflIII and EcoRI and replaced by the TRP1 gene taken from pJ280 plasmid (29). A KpnI/BamHI fragment containing the disrupted pmc1Δ::TRP1 fragment was then used to transform yeast. Trp\(^{+}\) colonies were selected, and the correct gene replacement was confirmed by PCR.

Similarly, a 2.04-kb fragment of the VCX1 gene was generated by PCR using genomic DNA as template. The forward primer used was 5′-CGTGGATCC TTGCTCCATC-3′, and the reverse primer was 5′-GCTAGATGTC CCTAACATG G-3′. Again, these primers contained KpnI and BamHI restriction endonuclease sites, respectively (underlined). The fragment was digested with these enzymes and cloned into a pBluescript II KS (+) plasmid. A 1.56-kb fragment was removed from the VCX1 DNA by digestion with HindIII and HindIIII endonucleases and replaced with a fragment containing the URA3 gene obtained from pJ244 (29). A KpnI/BamHI fragment containing the disrupted vcx1Δ::URA3 fragment from this plasmid was used to transform yeast. The replacement of wild type VCX1 was confirmed by PCR analysis. Other genetic manipulations were carried out by standard methods (30).

**Culture Media**—Bacterial strains were grown on standard media (31). Yeast strains were maintained on YP medium containing 2% D-glucose (YPD) or synthetic minimal medium containing 2% D-glucose (SM) and other supplements as required (30). Growth media were routinely buffered with 40 mM MES-Tris, pH 5.5.

**Determination of Ca\(^{2+}\) Concentration in Media**—EGTA was used to reduce the Ca\(^{2+}\) concentration of buffered media. Because YPD and SM media contain divalent cations other than Ca\(^{2+}\), the effective concentrations of Mg\(^{2+}\), Mn\(^{2+}\), Fe\(^{2+}\), K\(^{+}\), and Na\(^{+}\) were considered when calculating free Ca\(^{2+}\) concentrations. Known quantities of CaCl\(_2\) stock solutions were added, and the resulting free Ca\(^{2+}\) concentrations were calculated based on the total concentration of Ca\(^{2+}\) as well as other cations, pH, and temperature of the medium. These calculations were done using the Maxchelator 1.2 program.

**Measurements of Total Cellular Ca\(^{2+}\), Mg\(^{2+}\), Na\(^{+}\), K\(^{+}\), and Phosphate Levels**—50–100 A\(_{600}\) units of yeast growing in YPD supplemented with CaCl\(_2\) or EGTA were harvested by centrifugation at 5,000 × g for 5 min. The cell pellets were washed with fresh YP and transferred to microcentrifuge tubes whose mass had previously been determined gravimetrically to an accuracy of 0.1 mg on an analytical balance. The tubes were centrifuged at 15,000 × g for 5 min, and the supernatants were removed carefully. The tubes were then resuspended, and any remaining supernatant was again removed. The tubes containing the pellets were weighed to determine the net weight of the pellet, and the pellets were then dried to completion in a Savant SpeedVac system. The tubes were then weighed again to determine the dry weight of the pellet. 1 × HCl was added to the dry pellets, and the capped microcentrifuge tubes were vortexed and incubated on a rocker for at least 24 h. Thereafter each sample was centrifuged briefly in a microcentrifuge, and multiple aliquots of each supernatant were taken for ion measurements. Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\) measurements of aliquots were carried out with an Eppendorf FOUO-5070 flame photometer; Mg\(^{2+}\) levels of aliquots were determined using a Varian AA-20 atomic absorption spectrophotometer. Cellular ion concentrations were then calculated based on the dry weight of the samples and dilution factors. Total combined orthophosphate and polyphosphate levels (referred to as total inorganic phosphate) were determined in the 1 M HCl hydrolysate described above using an acid molybdate-based diagnostic kit (Sigma). The phosphorus levels measured represent the sum of the acid-hydrolyzed polyphosphate and the inorganic phosphate present (32).

**Ca\(^{2+}\) Concentration in a Vacuolar Biogenesis Mutant**—A pEVP11-based plasmid containing a functional apoaequorin gene (pAEQ) was transformed into yeast using the LEU2 gene as selectable marker (1). This plasmid was a gift from Patrick Masson. Cells containing the pAEQ plasmid were grown in SMD medium containing other necessary supplements and were harvested in the logarithmic growth phase. 1 A\(_{600}\) units of cells were resuspended in 0.2 ml of aquorin test medium, which consists of 1 mM Ca\(^{2+}\) supple-

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**Table I**

**Yeast strains used in this study**

| Strain       | Relevant genotype | Complete genotype | Source  |
|-------------|------------------|-------------------|---------|
| SEY2601     | Wild type        | MATa, ura3–2, leu2–3,112, his3–Δ200, trp1–Δ901, lys2–801, suc2–49 | S. Emr  |
| YDB224      | pmc1Δ            | MATa, ura3–2, leu2–3,112, his3–Δ200, trp1–Δ901, lys2–801, suc2–49, pmc1Δ::TRP1 | This study |
| YDB225      | vcx1Δ            | MATa, ura3–2, leu2–3,112, his3–Δ200, trp1–Δ901, lys2–801, suc2–49, vcx1Δ::URA3 | This study |
| YDB224      | pmc1Δ/vcx1Δ      | MATa, ura3–2, leu2–3,112, his3–Δ200, trp1–Δ901, lys2–801, suc2–49, pmc1Δ::TRP1, vcx1Δ::URA3 | This study |
| YDB279      | pmr1Δ            | MATa, ura3–2, leu2–3,112, his3–Δ200, trp1–Δ901, lys2–801, suc2–49, pmr1Δ::LEU12 | This study |
| LBY317      | vps33Δ           | MATa, ura3–2, leu2–3,112, his3–Δ200, trp1–Δ901, lys2–801, suc2–49, vps33Δ::HIS3 | S. Emr  |
| YDB282      | vps33Δ/pmr1Δ     | MATa, ura3–2, leu2–3,112, his3–Δ200, trp1–Δ901, lys2–801, ade2, suc2–49, vps33Δ::HIS3, pmr1Δ::LEU12 | This study |
| YDB255      | vps33Δ/pmc1Δ/vcx1Δ | MATa, ura3–2, leu2–3,112, his3–Δ200, trp1–Δ901, lys2–801, suc2–49, vps33Δ::HIS3, pmc1Δ::TRP1, vcx1Δ::URA3 | This study |
Calcium Homeostasis in a Vacuolar Biogenesis Mutant

RESULTS

Sensitivity of Yeast Vacuolar Mutants to Different Environmental Ca\(^{2+}\) Concentrations—We initially compared the Ca\(^{2+}\) tolerance of yeast strains containing knockout of genes involved in vacuolar Ca\(^{2+}\) transport (pmc1\(\Delta\), vcx1\(\Delta\), or pmc1\(\Delta\)/vcx1\(\Delta\)) vascular biogenesis (ups33\(\Delta\)) or a combination of both classes (ups33\(\Delta\)/pmc1\(\Delta\)/vcx1\(\Delta\)). Each strain was streaked onto YPD plates supplemented with increasing concentrations of CaCl\(_2\) or with 10 mM EGTA and incubated at 30 °C for 48 h. The wild type, pmc1\(\Delta\), vcx1\(\Delta\), and pmc1\(\Delta\)/vcx1\(\Delta\) strains grew similarly on standard YPD plates (buffered to pH 5.5) containing 0.3 mM Ca\(^{2+}\) (Fig. 1A), whereas the colony size of the ups33\(\Delta\) and ups33\(\Delta\)/pmc1\(\Delta\)/vcx1\(\Delta\) strains was slightly smaller. The wild type, pmc1\(\Delta\), and vcx1\(\Delta\) strains also grew similarly on YPD medium supplemented with 100 mM CaCl\(_2\), whereas the growth rate of the pmc1\(\Delta\)/vcx1\(\Delta\) double mutant was reduced significantly on this medium (Fig. 1B). In contrast, neither the ups33\(\Delta\) strain nor the ups33\(\Delta\)/pmc1\(\Delta\)/vcx1\(\Delta\) strain was able to form visible colonies under these growth conditions during the 48-h incubation period.

When the YPD plates were supplemented with 200 mM CaCl\(_2\), both the wild type and vcx1\(\Delta\) strains grew somewhat more slowly than on YPD plates supplemented with 100 mM CaCl\(_2\). The pmc1\(\Delta\)/vcx1\(\Delta\) double mutant was unable to grow under these conditions, whereas the pmc1\(\Delta\) strain grew much more slowly than the wild type strain (Fig. 1C). A further doubling of the Ca\(^{2+}\) concentration in the YPD plate to 400 mM completely inhibited growth of the pmc1\(\Delta\) strain but not the growth of the wild type and vcx1\(\Delta\) strains (not shown). None of these strains was inhibited by the addition of either 400 mM NaCl or 400 mM KCl to the YPD plates, indicating that the increased osmolarity associated with 200 mM CaCl\(_2\) did not cause the growth sensitivity described above. We conclude that strains harboring the ups33\(\Delta\) mutation show greater sensitivity to high extracellular Ca\(^{2+}\) than strains carrying the pmc1\(\Delta\) mutation, the vcx1\(\Delta\) mutation, or both mutations together. Overall, the rank order of Ca\(^{2+}\) sensitivity observed for these strains was: ups33\(\Delta\)/pmc1\(\Delta\)/vcx1\(\Delta\) and ups33\(\Delta\) strains > pmc1\(\Delta\)/vcx1\(\Delta\) strain > pmc1\(\Delta\) strain > vcx1\(\Delta\) and wild type strains.

We also examined whether the growth of these strains was sensitive to inhibition by the chelating agent EGTA. We found that pmc1\(\Delta\), vcx1\(\Delta\), and pmc1\(\Delta\)/vcx1\(\Delta\) strains grew similarly to the wild type strain on YPD plates buffered to pH 5.5 and supplemented with 10 mM EGTA (Fig. 1D). In contrast, the growth of the ups33\(\Delta\) and ups33\(\Delta\)/pmc1\(\Delta\)/vcx1\(\Delta\) strains was severely inhibited under these conditions, suggesting that they require a higher minimal level of environmental Ca\(^{2+}\) for efficient growth than the other strains. However, not only Ca\(^{2+}\) but other cations such as Zn\(^{2+}\), Fe\(^{2+}\), and Mn\(^{2+}\) are also com-
plicated effectively by EGTA. To confirm that low environmental Ca$^{2+}$ was responsible for EGTA sensitivity, we supplemented EGTA-prepared media with different divalent cations to determine the component(s) required for growth of the $ups33\Delta$ strain. We found that the addition of Ca$^{2+}$ could restore a significant amount of growth in YPD medium treated with EGTA, whereas several other cations (Mg$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, Zn$^{2+}$, and Cu$^{2+}$) could not (data not shown). These results lead us to conclude that the $ups33\Delta$ and $ups33\Delta/pmc1\Delta/vcx1\Delta$ strains are more sensitive to either high or low levels of environmental Ca$^{2+}$ than the wild type, $pmc1\Delta$, $vcx1\Delta$, and $pmc1\Delta/vcx1\Delta$ strains.

Measurement of Rapid Changes in the Cytosolic Ca$^{2+}$ Concentration upon External Ca$^{2+}$ Challenge—Yeast cells, like mammalian cells, have been reported to maintain cytosolic free Ca$^{2+}$ levels in the range of 50–200 nM (1–3). To determine how the above mutations affect the ability of yeast to maintain cytosolic Ca$^{2+}$ homeostasis, we introduced a plasmid encoding a cytosolic form of apoaequorin into each strain (1). Aposeaquin can be converted to aequorin by incubating the strains with the membrane-permeant cofactor coelenterazine. Once active aequorin is generated, it is capable of emitting light as a function of the free Ca$^{2+}$ concentration present in the cytosol (33). In the experiments described here, the aequorin-dependent light emission of each strain was sampled throughout the experiment at 200-ms intervals. To determine the cytosolic Ca$^{2+}$ concentration as a function of light emission, a standard curve was prepared using crude extracts from the wild type strain where the light emission at each Ca$^{2+}$ concentration was correlated to the $I_{\text{max}}$ for each sample (Fig. 2A). Using this method, the relative light units/s emitted from the wild type strain corresponds to a resting free cytosolic Ca$^{2+}$ concentration of $\sim 75$ nM when cells were incubated in a medium containing low (6 μM) free Ca$^{2+}$ (for further details, see “Materials and Methods”).

To determine how various mutations affect the ability of these strains to respond to a sudden increase in extracellular Ca$^{2+}$, 50 μM CaCl$_2$ was injected rapidly into the cell suspension while the cytosolic aequorin-dependent light emission was continuously monitored. We found that the light emission of the wild type strain increased rapidly and reached a peak level corresponding to $\sim 300$ nM cytosolic Ca$^{2+}$ within 5 s (Fig. 2B). The Ca$^{2+}$ concentration decreased rapidly thereafter and returned to a new steady-state free cytosolic Ca$^{2+}$ concentration of $\sim 80–85$ nM within 90 s.

The light emission measured in the $pmc1\Delta/vcx1\Delta$ strain corresponded to a basal cytosolic Ca$^{2+}$ concentration of 75–80 nM. When 50 μM CaCl$_2$ was injected, the light emission reached a peak value corresponding to $\sim 385$ nM cytosolic free Ca$^{2+}$, which was somewhat higher than the peak observed with the wild type strain. The recovery phase of the $pmc1\Delta/vcx1\Delta$ strain was also much weaker than the wild type control. The post-shock steady-state cytosolic Ca$^{2+}$ concentration was $\sim 310$ nM, which was 4-fold higher than the steady-state cytosolic Ca$^{2+}$ concentration observed in the wild-type strain after the same Ca$^{2+}$ shock. This suggests that the loss of the Pmc1p and Vcx1p vacuolar Ca$^{2+}$ transporters severely compromises the ability of this strain to return its cytosolic Ca$^{2+}$ concentration to a low resting level after exposure to elevated extracellular Ca$^{2+}$.

We next examined the response of strains carrying the $ups33\Delta$ mutation to Ca$^{2+}$ shock. We found that the initial resting cytosolic Ca$^{2+}$ concentration was $\sim 165$ nM, which was 2-fold higher than the wild type strain. The baseline cytosolic Ca$^{2+}$ level measured in the $ups33\Delta/pmc1\Delta/vcx1\Delta$ strain was $\sim 210$ nM, which was almost 3-fold higher than the wild type strain. When the $ups33\Delta$ strain was exposed to Ca$^{2+}$ shock, the maximum light emission was nearly 100-fold higher than observed with the wild type strain and corresponded to a peak cytosolic Ca$^{2+}$ concentration of $\sim 1.75$ μM (Fig. 2B). This level was 5-fold higher than the peak observed with the wild type strain. Like the $pmc1\Delta/vcx1\Delta$ strain, the recovery of the $ups33\Delta$ strain from the peak cytosolic Ca$^{2+}$ level was much weaker than the wild type control and reached a new steady-state level at $\sim 470$ nM (6-fold higher than the wild type strain). The $ups33\Delta/pmc1\Delta/vcx1\Delta$ strain exhibited a high peak of cytosolic Ca$^{2+}$, which corresponded to $\sim 1.5$ μM, which was somewhat lower than was observed with the $ups33\Delta$ strain. However, the recovery of this strain from the peak level was even weaker than the $ups33\Delta$ strain and reached a new steady-state level of $\sim 660$ nM (more than 8-fold higher than the wild type strain). The weaker recovery of this strain may indicate that a low level of residual function of the Pmc1p and/or the Vcx1p transporters remains within the vesicles that accumulate in the $ups33\Delta$ strain. When taken together, these results indicate that strains carrying the $ups33\Delta$ mutation are severely compromised in their ability to regulate basal cytosolic Ca$^{2+}$ levels and are unable to sequester efficiently the cytosolic Ca$^{2+}$ that enters the cell after an acute Ca$^{2+}$ shock.
The vps33Δ mutation causes a large decrease in cellular Mg2+ and phosphate but does not reduce the amount of cellular Ca2+. Cultures of the indicated strains were grown in standard YPD medium, and the relative amounts of Mg2+ (panel A), inorganic phosphate (panel B), and Ca2+ (panel C) were determined as described under “Materials and Methods.” WT, wild type.

Measurement of Total Cellular Ca2+, Mg2+, and Phosphate Levels in Yeast Vacuolar Mutants—A large fraction of total cellular Ca2+, Mg2+, and polyphosphate normally resides within the vacuole (6, 8, 35, 36). To determine how these various vacuolar mutations affect the capacity to store these compounds within the vacuole, we measured their total cellular levels (Fig. 3). We did not detect a significant change in the level of Mg2+ in the pmc1Δ/vcx1Δ strain, although a small (22%) decrease in total cellular inorganic phosphate (orthophosphate and polyphosphate) was observed. In contrast, the total cellular Ca2+ level was reduced nearly 2-fold in the pmc1Δ/vcx1Δ strain.

The cellular levels of these three compounds were significantly different in strains carrying the vps33Δ mutation. We found that the total amount of cellular Mg2+ was 3-fold lower in both the vps33Δ and vps33Δ/pmc1Δ/vcx1Δ strains. Similarly, the total inorganic phosphate level was reduced more than 4-fold in the vps33Δ strain and 6-fold in the vps33Δ/pmc1Δ/vcx1Δ strain. Thus, strains carrying the vps33Δ mutation exhibited a severe reduction in the cellular content of Mg2+ and inorganic phosphate, consistent with a reduced capacity to store these ions within the vacuole of strains carrying the vps33Δ mutation.

Because >90% of total cellular Ca2+ is normally stored within the vacuole (5, 6), we expected the vps33Δ strain to contain a much lower level of total Ca2+. However, we found that both the vps33Δ and vps33Δ/pmc1Δ/vcx1Δ strains contained 15–20% more cellular Ca2+ than the wild-type strain. Thus, the vacuolar biogenesis defect associated with the vps33Δ mutation resulted in a net increase in total cellular Ca2+, and this phenotype was epistatic to the decrease in total cellular Mg2+ observed in the pmeIΔ/vcx1Δ mutant. When taken in conjunction with the observation that the vacuolar storage of Mg2+ and inorganic phosphate is compromised in strains carrying the vps33Δ mutation, these results suggest that another intracellular compartment is capable of compensating for the defects in Ca2+ storage and homeostasis in strains carrying the vps33Δ mutation.

Membrane Permeability of the Vacuolar Mutants—The results described above indicate that the vps33Δ mutation has effects on Ca2+ homeostasis which differ significantly from the combined loss of the Pmc1p and Vcx1p vacuolar Ca2+ transporters. One possible explanation for the higher level of Ca2+ observed is that the rate of Ca2+ uptake in the vps33Δ strain is increased. To test this possibility, we measured the rate of 45Ca2+ uptake in each strain (Fig. 4). A CaCl2 solution containing the radionuclide was added to cells at a final concentration of 1 mM. Aliquots were then collected at intervals over a period of 90 s to determine the rate of Ca2+ uptake. All four strains (wild type, pme1Δ/vcx1Δ, vps33Δ, and vps33Δ/pme1Δ/vcx1Δ) showed a similar rate of Ca2+ uptake under the conditions examined (1 mM extracellular Ca2+). If the vps33Δ mutation altered the plasma membrane permeability in a more general, nonspecific way, it is likely that the concentration of other intracellular ions would also be altered. To examine this possibility, we measured the steady-state concentrations of the monovalent K+ and Na+ ions in each strain when grown in YPD medium (Fig. 5). We found that none of the strains had any significant differences in the total cellular concentrations of either cation. Taken together, these results indicate that the membrane permeabilities of Ca2+, K+, and Na+ are not altered significantly in strains carrying the vps33Δ mutation under the conditions examined.

45Ca2+ Exchange in the Vacuolar Mutants—The total cellular...
lar Ca\(^{2+}\) found in yeast cells exists in two distinct forms, termed the exchangeable and nonexchangeable pools (6, 8). The exchangeable pool represents Ca\(^{2+}\) that can readily leave the cell, whereas the nonexchangeable pool is thought to represent a more stable pool of Ca\(^{2+}\) located primarily within the vacuole.

In a complex with polyphosphate. To determine the partitioning of cellular Ca\(^{2+}\) between the exchangeable and nonexchangeable pools in the \textit{vps33} \textit{Δ} strains, we measured \(^{45}\text{Ca}^{2+}\) efflux. Strains were grown in YPD medium containing \(^{45}\text{Ca}^{2+}\) for four generations. After washing and resuspending the cells in fresh YPD medium containing 50 mM CaCl\(_2\), the amount of \(^{45}\text{Ca}^{2+}\) that remained associated with cells from each strain was determined at various times (Fig. 6A). We found that the wild type and \textit{pmc1} \textit{Δ}/\textit{vcx1} \textit{Δ} strains quickly exchanged a small portion of the total cellular Ca\(^{2+}\) during the first 15 min and subsequently exchanged Ca\(^{2+}\) at a much slower rate. In contrast, both strains carrying the \textit{vps33} \textit{Δ} mutation exhibited a much longer period of Ca\(^{2+}\) exchange which extended for 90 min for the \textit{vps33} \textit{Δ}/\textit{pmc1} \textit{Δ}/\textit{vcx1} \textit{Δ} strain and 210 min for the \textit{vps33} \textit{Δ} strain. This indicates that most of the Ca\(^{2+}\) within strains carrying the \textit{vps33} \textit{Δ} mutation does not reside within a nonexchangeable pool. Although the larger size of the exchangeable Ca\(^{2+}\) pool may partially account for the increased period of time required to release the exchangeable Ca\(^{2+}\) pool in these strains, other factors may also be involved.

Because the strains carrying the \textit{vps33} \textit{Δ} mutation exhibited a prolonged time of release of their exchangeable pools, we compared the nonexchangeable and exchangeable Ca\(^{2+}\) pools in each strain after Ca\(^{2+}\) efflux was allowed to proceed for 210 min (Fig. 6, B and C). Under these conditions, we found that the wild type strain contained 4.9 mmol of Ca\(^{2+}\)/kg dry mass in its nonexchangeable pool. In contrast, the \textit{pmc1} \textit{Δ}/\textit{vcx1} \textit{Δ} strain retained only 0.5 mmol of Ca\(^{2+}\)/kg of dry mass after 210 min of efflux. Similarly, the \textit{vps33} \textit{Δ} strain held 1.0 mmol of Ca\(^{2+}\)/kg dry mass, and the \textit{vps33} \textit{Δ}/\textit{pmc1} \textit{Δ}/\textit{vcx1} \textit{Δ} strain held 0.6 mmol of Ca\(^{2+}\)/kg dry mass in their nonexchangeable pools. Thus, the nonexchangeable pool in each of these mutant strains is 5–10-fold smaller than in the wild type strain, indicating that all three mutant strains are severely compromised in their ability to store Ca\(^{2+}\) within the vacuolar nonexchangeable pool.

When we calculated the amount of Ca\(^{2+}\) that was readily mobilized during 210 min of efflux, we found that the exchangeable pool in the wild type strain contained 1.2 mmol of Ca\(^{2+}\)/kg dry mass. This pool held 6.9 mmol of Ca\(^{2+}\)/kg dry mass in the \textit{vps33} \textit{Δ} strain and 8.1 mmol of Ca\(^{2+}\)/kg dry mass in the \textit{vps33} \textit{Δ}/\textit{pmc1} \textit{Δ}/\textit{vcx1} \textit{Δ} strain. Thus, the absolute amount of Ca\(^{2+}\) in the exchangeable pool in these strains was 6–7-fold larger than in the wild type strain. In contrast, the amount of Ca\(^{2+}\) in the exchangeable pool in the \textit{pmc1} \textit{Δ}/\textit{vcx1} \textit{Δ} strain was 1.8 mmol of Ca\(^{2+}\)/kg dry mass, which was only 1.5-fold higher.

![Fig. 5](image5.png)

**Fig. 5. Measurement of total cellular Na\(^{+}\) and K\(^{+}\) levels.** Cultures of the indicated strains were grown in standard YPD medium, and the relative amounts of K\(^{+}\) (panel A) and Na\(^{+}\) (panel B) were determined as described under “Materials and Methods.” WT, wild type.

![Fig. 6](image6.png)

**Fig. 6. Measurement of \(^{45}\text{Ca}^{2+}\) efflux.** The indicated strains were grown for four generations in YPD medium supplemented with \(^{45}\text{Ca}^{2+}\). To initiate Ca\(^{2+}\) release, the strains were harvested, washed, and resuspended in YPD supplemented with 50 mM CaCl\(_2\). The amount of \(^{45}\text{Ca}^{2+}\) that remained cell-associated was determined at the indicated times and converted to total cellular Ca\(^{2+}\) as described under “Materials and Methods.” **Panel A,** absolute amounts of cell-associated Ca\(^{2+}\): Squares, wild type; diamonds, \textit{pmc1} \textit{Δ}/\textit{vcx1} \textit{Δ}; circles, \textit{vps33} \textit{Δ}; and triangles, \textit{vps33} \textit{Δ}/\textit{pmc1} \textit{Δ}/\textit{vcx1} \textit{Δ}. **Panel B,** nonexchangeable Ca\(^{2+}\) pools. **Panel C,** exchangeable Ca\(^{2+}\) pools. WT, wild type.
than the wild type strain. These results indicate that although the nonexchangeable Ca\(^{2+}\) pools within the three vacuolar mutant strains are similar, the exchangeable pools found in the \(vps33\Delta\) and \(vps33\Delta/pmc1\Delta/vcx1\Delta\) strains are roughly 4-fold larger than those found in the \(pmc1\Delta/vcx1\Delta\) strain.

**Sensitivity of Vacular Mutants to Cyclosporin A**—Several studies have found that the loss of calcineurin function leads to a significant increase in the steady-state level of cellular Ca\(^{2+}\) (7, 14, 37–39). To determine how the \(vps33\Delta\) strain responds to such an increase in intracellular Ca\(^{2+}\), we compared the growth of these strains on YPD plates (pH 5.5) with and without 20 \(\mu\)g/ml CsA (Fig. 7). In the absence of CsA, the \(vps33\Delta\) and the \(vps33\Delta/pmc1\Delta/vcx1\Delta\) strains again had a slightly slower growth rate than the other strains. However, when CsA was added to the plates the growth of the \(vps33\Delta/pmc1\Delta/vcx1\Delta\) strain was completely blocked, whereas the \(vps33\Delta\) strain showed a severe growth defect compared with plates lacking CsA. In contrast, growth of the wild type and \(pmc1\Delta/vcx1\Delta\) strains was unaffected by the presence of CsA. These results are consistent with the possibility that the \(vps33\Delta\) mutation reduces the ability of these strains to sequester adequately the increased intracellular Ca\(^{2+}\) that accumulates upon the inhibition of calcineurin function.

The Golgi Ca\(^{2+}\) ATPase Pmr1p Participates in the Maintenance of Cellular Ca\(^{2+}\) Homeostasis during Ca\(^{2+}\) Stress—The results presented above clearly demonstrate that the \(vps33\Delta\) mutation severely disrupts intracellular Ca\(^{2+}\) homeostasis. Despite the severe defects in vacuolar structure and Ca\(^{2+}\) sequestration which result from this mutation, they remain viable and accumulate a normal amount of total cellular Ca\(^{2+}\). This raised the possibility that other intracellular organelles may compensate for the loss of vacuolar Ca\(^{2+}\) storage in these strains. Besides the vacuole, two compartments within the secretory pathway have also been implicated in Ca\(^{2+}\) storage in yeast. The PMR1 gene encodes a Ca\(^{2+}\) ATPase that has been localized to the Golgi apparatus (19, 21, 22) and was also recently reported to influence the rate of degradation of proteins within the endoplasmic reticulum (40). Given this well defined role of Pmr1p as a Ca\(^{2+}\) ATPase within a non-vacuolar compartment, we next tested whether Pmr1p may be involved in maintaining Ca\(^{2+}\) homeostasis in strains defective in vacuolar biogenesis.

First, we examined PMR1 mRNA levels to determine whether its expression changes in response to either the concentration of environmental Ca\(^{2+}\) or mutations that effect Ca\(^{2+}\) homeostasis. To provide the broadest range of environmental Ca\(^{2+}\) concentrations during this experiment, strains were grown in YPD containing 1 mM EGTA (calculated to reduce the free Ca\(^{2+}\) concentration to approximately 0.01 mM) or in YPD supplemented with 50 mM CaCl\(_2\). RNA was extracted from each strain, and the level of PMR1 mRNA was determined (relative to an ACT1 control). In the wild type strain, we found that the relative level of PMR1 mRNA increased 1.4-fold as extracellular Ca\(^{2+}\) increased (Fig. 8). In the \(pmr1\Delta/vcx1\Delta\) strain, we found that the PMR1 mRNA level was slightly elevated in the low Ca\(^{2+}\) medium and was increased to 1.6-fold above the wild type control when the environmental Ca\(^{2+}\) was increased. Finally, the PMR1 mRNA level in the \(vps33\Delta\) strain was 1.6-fold higher than the wild type strain when grown in the presence of low Ca\(^{2+}\) and was increased to 2.2-fold higher than the wild type control when grown in the presence of 50 mM Ca\(^{2+}\). These results indicate that PMR1 gene expression increases moderately as a function of the Ca\(^{2+}\) stress on a wild type strain or as a consequence of mutations that effect the maintenance of intracellular Ca\(^{2+}\) homeostasis.

To address further the role of Pmr1p in maintaining Ca\(^{2+}\) homeostasis in strains carrying the \(vps33\Delta\) mutation, we examined the progeny of a cross between a \(pmr1\Delta\) strain and a \(vps33\Delta/pmc1\Delta/vcx1\Delta\) strain. A total of 36 tetrads was dissected, and the genotype of the 107 viable spores was determined. We found that all but three possible combinations of mutations were obtained. The nonviable combinations, which all contained the \(pmr1\Delta\) mutation, were: \(pmr1\Delta/pmc1\Delta/vcx1\Delta\), \(pmr1\Delta/vps33\Delta/pmc1\Delta\), and \(pmr1\Delta/vps33\Delta/vcx1\Delta\). This indicates that the loss of both Ca\(^{2+}\) transporters located in the vacuole (\(pmc1\Delta\) and \(vcx1\Delta\)) in conjunction with the Golgi apparatus Ca\(^{2+}\) transporter (\(pmr1\Delta\)) is lethal. Although strains lacking both the vacuolar Ca\(^{2+}\) ATPase (Pmc1p) and Golgi apparatus Ca\(^{2+}\) ATPase (Pmr1p) were viable, the introduction of mutations that further compromised Ca\(^{2+}\) homeostasis (either the \(vps33\Delta\) or the \(vcx1\Delta\) mutation) apparently resulted in a lethal imbalance in Ca\(^{2+}\) homeostasis. These results indicate that specific combinations of both vacuolar and Golgi mutations lead to insurmountable defects in Ca\(^{2+}\) homeostasis.

Previous studies reported that disruption of the PMRI gene does not confer sensitivity to elevated levels of environmental Ca\(^{2+}\) (19–21). This led to the conclusion that the Golgi apparatus does not play a significant role in maintaining cellular Ca\(^{2+}\) homeostasis under conditions of Ca\(^{2+}\) stress. To determine whether the Golgi apparatus plays a more significant role in this process when vacuolar Ca\(^{2+}\) storage is compromised, we next examined the ability of the \(pmr1\Delta/vps33\Delta\) strain to grow in the presence of elevated environmental Ca\(^{2+}\) (Fig. 9). This strain grew somewhat slower than the \(vps33\Delta\) strain on stand-
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and YPD medium. Although the \( \text{vps}33\Delta \) and \( \text{vps}33\Delta/\text{pmc}1\Delta/\text{vcx}1\Delta \) strains were capable of growth on YPD plates containing 50 mM CaCl\(_2\), growth of the \( \text{pmr}1\Delta/\text{vps}33\Delta \) strain was completely inhibited. These results indicate that the Golgi apparatus acts to compensate for the defective Ca\(^{2+}\) homeostasis associated with the \( \text{vps}33\Delta \) strain.

Finally, we examined whether strains carrying the \( \text{pmr}1\Delta \) mutation alone also exhibited a growth defect in the presence of high environmental Ca\(^{2+}\) (Fig. 10). We found that each of the four strains examined (wild type, \( \text{pmr}1\Delta, \text{pmc}1\Delta, \) and \( \text{vcx}1\Delta \)) grew with similar rates on plates containing 100 mM CaCl\(_2\). However, we found that the \( \text{pmr}1\Delta \) and \( \text{pmc}1\Delta \) strains were unable to grow on plates containing 500 mM CaCl\(_2\), whereas the wild type and \( \text{vcx}1\Delta \) strains did grow under these conditions. These results indicate that in cells with intact vacuolar function, Pmr1p plays a more important role in maintaining Ca\(^{2+}\) homeostasis upon exposure to extreme Ca\(^{2+}\) stress than the vacuolar Vcx1p transporter.

**DISCUSSION**

Wild type strains of \( S. \text{cerevisiae} \) are capable of maintaining intracellular Ca\(^{2+}\) levels within a narrow range when faced with extracellular Ca\(^{2+}\) concentrations ranging from \( <1 \mu M \) to \( >100 \mu M \). Consistent with the fact that the yeast vacuole normally contains \( >90\% \) of the total cellular Ca\(^{2+}\), mutations in Ca\(^{2+}\) transporters which limit vacuolar Ca\(^{2+}\) uptake have been shown to cause a 2–3-fold reduction in the total cellular Ca\(^{2+}\) levels (7, 14, 15). Similarly, we observed a 2-fold decrease in total cellular Ca\(^{2+}\) in the \( \text{pmc}1\Delta/\text{vcx}1\Delta \) strain. In contrast, we found that strains carrying the \( \text{vps}33\Delta \) vacuolar biogenesis mutation have total cellular Ca\(^{2+}\) levels that are slightly higher than the wild type strain. This result was surprising based upon the severe defects in vacuolar biogenesis caused by mutations in this gene (24–26) in conjunction with our finding that the steady-state levels of two other substances normally stored primarily within the vacuole (Mg\(^{2+}\) and polyphosphate) were greatly reduced. Because the \( \text{vps}33\Delta/\text{pmc}1\Delta/\text{vcx}1\Delta \) strain (which lacks both known vacuolar Ca\(^{2+}\) transporters) also had this high level of total cellular Ca\(^{2+}\), the increased accumulation of Ca\(^{2+}\) cannot be attributed to the residual function of these transporters in a prevacuolar compartment. Instead, our results suggest that the loss of most (or all) vacuolar Ca\(^{2+}\) storage in strains carrying the \( \text{vps}33\Delta \) mutation leads to the redistribution of a significant portion of intracellular Ca\(^{2+}\) into the Golgi apparatus and possibly other intracellular compartments as well.

The Golgi apparatus contains the only non-vacuolar Ca\(^{2+}\) ATPase (Pmr1p) that has been characterized in yeast (19–22). Because the \( \text{pmr}1\Delta \) strain was not previously found to be sensitive to elevated extracellular Ca\(^{2+}\), it was not thought to play a significant role in maintaining cellular Ca\(^{2+}\) homeostasis. However, we found that a \( \text{pmr}1\Delta/\text{vps}33\Delta \) strain is more sensitive to elevated extracellular Ca\(^{2+}\) than the \( \text{vps}33\Delta \) strain alone, and \( \text{PMR}1 \) gene expression is elevated in the \( \text{vps}33\Delta \) strain. In addition, we found that certain combinations of mutations affecting both vacuolar and Golgi Ca\(^{2+}\) transport (\( \text{pmr}1\Delta/\text{pmc}1\Delta/\text{vcx}1\Delta, \text{pmr}1\Delta/\text{vps}33\Delta/\text{pmc}1\Delta, \) and \( \text{pmr}1\Delta/\text{vps}33\Delta/\text{vcx}1\Delta \)) resulted in synthetic lethality. Taken together, these results indicate that the Golgi apparatus of yeast plays a significant role in cellular Ca\(^{2+}\) homeostasis through a Pmr1p-dependent mechanism when vacuolar Ca\(^{2+}\) storage is compromised. We also found that a \( \text{pmr}1\Delta \) strain with normal vacuolar function is sensitive to high levels of Ca\(^{2+}\) in the growth medium. Given the fact that the Golgi has not previously been observed to play a role in Ca\(^{2+}\) homeostasis under other growth conditions, Golgi Ca\(^{2+}\) sequestration may only play a significant role in cellular Ca\(^{2+}\) homeostasis when the cytosolic Ca\(^{2+}\) load exceeds the capacity of the vacuolar Ca\(^{2+}\) storage system.

Although our study clearly implicates Pmr1p in the maintenance of Ca\(^{2+}\) homeostasis in \( \text{vps}33\Delta \) strains, we observed only a 2-fold increase in \( \text{PMR}1 \) transcription. Although a larger increase might have been expected, it is possible that \( \text{PMR}1 \) expression is regulated primarily at a post-transcriptional level. In this way a significant increase in Pmr1p activity could occur without a concomitant increase in mRNA abundance (or protein abundance if the regulation is exerted at a post-translational level). Alternatively, Pmr1p may be present and active under all conditions, but the vacuolar Ca\(^{2+}\) uptake system may sequester cytosolic Ca\(^{2+}\) more efficiently than the Golgi apparatus under all but the most severe conditions. This could occur, for example, if the vacuolar transporters were activated at a lower cytosolic Ca\(^{2+}\) concentration than Pmr1p. By either mechanism, a high level of Golgi Ca\(^{2+}\) storage would not be observed under most growth conditions that did not subject the cells to high Ca\(^{2+}\) stress. Such an overlapping hierarchy of transporter activation to control Ca\(^{2+}\) homeostasis (either at the level of synthesis or function) would be consistent with the observations obtained in the current study. Such a mechanism would also explain why Pmr1p was not attributed a role in the maintenance of cellular Ca\(^{2+}\) homeostasis in previous studies.

Other results obtained in this study are also consistent with a hierarchical control of Ca\(^{2+}\) homeostasis. First, we found that strains carrying the \( \text{vps}33\Delta \) mutation exhibit a 2–3-fold higher basal level of cytosolic Ca\(^{2+}\) when incubated in a medium containing only 10 \( \mu M \) Ca\(^{2+}\) (Fig. 2). This finding provides
evidence that Pmr1p function may be activated at a higher cytosolic Ca\(^{2+}\) concentration than the vacuolar Ca\(^{2+}\) transporters. Because our results suggest that the secondary system utilizing Pmr1p plays a larger role in Ca\(^{2+}\) homeostasis in strains carrying the \(vps33\) mutation, it would be expected that the basal cytosolic Ca\(^{2+}\) would be maintained near the concentration that activates this transporter. We also found that strains carrying the \(vps33\Delta\) mutation exhibited a severe defect in the maintenance of cytosolic Ca\(^{2+}\) homeostasis when exposed to 50 mM extracellular CaCl\(_2\). Under these conditions, we found that the cytosolic Ca\(^{2+}\) concentration of the \(vps33\Delta\) strain quickly rose to 1.75 \(\mu\)M, a level that was 6-fold higher than the wild type strain. Furthermore, the rate of recovery was slower, and the new steady-state level that was reached was also much higher than the control strain. Again, these results suggest that this secondary system of Ca\(^{2+}\) sequestration cannot remove excess Ca\(^{2+}\) from the cytosol as quickly as the vacuolar system. Despite these limitations, this system remains capable of maintaining intracellular Ca\(^{2+}\) homeostasis (at least to the extent required to maintain growth) in strains carrying the \(vps33\Delta\) mutation when challenged by environmental concentrations as high as 50 mM Ca\(^{2+}\) (see Fig. 9).

Previous studies have shown that strains carrying \(vps33\) mutations mislocalize the vacuolar membrane protein alkaline phosphatase to the cell surface (24–26). Unfortunately, neither the extent of the mislocalization of other vacuolar membrane proteins nor the composition of vesicles that accumulate in the \(vps33\) strain has been characterized further. Nevertheless, it is possible that vacuolar Ca\(^{2+}\) transporters may also be mislocalized to the cell surface and thus could potentially contribute to the increased cytosolic Ca\(^{2+}\) levels observed upon exposure to high extracellular Ca\(^{2+}\). However, we found that the peak cytosolic Ca\(^{2+}\) level was still 5-fold higher than the wild type strain in the \(vps33\)/\(pmc1\) or \(vps33\)/\(vcx1\) strain. This indicates that the mislocalization of the Pmc1p and Vcx1p transporters to the plasma membrane is not responsible for most of the elevated cytosolic Ca\(^{2+}\) observed in strains carrying the \(vps33\) mutation. Two additional lines of evidence suggest that the \(vps33\) mutation does not significantly alter the general permeability of the plasma membrane. First, the steady-state cellular concentrations of two other cations, K\(^{+}\) and Na\(^{+}\), were unaffected by the \(vps33\) mutation. In addition, the rate of \(45\)Ca\(^{2+}\) uptake measured in strains carrying the \(vps33\) mutation was identical to that of the wild type strain. Taken together, these results suggest that the \(vps33\) mutation does not significantly alter the permeability of the plasma membrane in the \(vps33\) strain. As discussed above, it is more likely that the higher peak in cytosolic Ca\(^{2+}\) is caused by a reduced capacity to sequester the Ca\(^{2+}\) into other cellular compartments rapidly.

Several studies have reported that the loss of calcineurin function increases the total cellular Ca\(^{2+}\) level (7, 14, 37–39). Our finding that the \(vps33\) strain shows an increased sensitivity to CsA on standard YPD medium is also consistent with the model that this secondary system of Ca\(^{2+}\) sequestration is not capable of transporting Ca\(^{2+}\) from the cytosol into intracellular compartments as efficiently as the wild type strain. In addition, it has been shown that the induction of \(PMR1\) expression (which functions to inhibit calcineurin activation in a manner analogous to CsA) (14). Thus, the combined effects of increased cellular Ca\(^{2+}\) uptake and lack of \(PMR1\) induction could account for the increased CsA sensitivity that was observed in the \(vps33\) strains.

The results of this study provide evidence that the Golgi apparatus plays a significant role in the maintenance of cellular Ca\(^{2+}\) homeostasis under conditions where the accumulation of cytosolic Ca\(^{2+}\) exceeds the capacity of the vacuole. This suggests that the vacuolar storage system that normally mediates the bulk of Ca\(^{2+}\) homeostasis in yeast may have been superimposed upon another system that is functionally related to the Ca\(^{2+}\) storage and signaling system found within the secretory pathway of mammalian cells. Further studies are required to determine whether other intracellular organelles of yeast (such as mitochondria) also participate in the maintenance of cellular Ca\(^{2+}\) homeostasis under conditions of extreme Ca\(^{2+}\) stress.

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