The Ca\textsuperscript{2+} Homeostasis Defects in a pgm2\textDelta Strain of Saccharomyces cerevisiae Are Caused by Excessive Vacuolar Ca\textsuperscript{2+} Uptake Mediated by the Ca\textsuperscript{2+}-ATPase Pmc1p*

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Loss of the major isof orm of phosphoglucomutase (PGM) causes an accumulation of glucose 1-phosphate when yeast cells are grown with galactose as the carbon and energy source. Remarkably, the pgm2\textDelta strain also exhibits a severe imbalance in intracellular Ca\textsuperscript{2+} homeostasis when grown under these conditions. In the present study, we examined how the pgm2\textDelta mutation alters yeast Ca\textsuperscript{2+} homeostasis in greater detail. We found that a shift from glucose to galactose as the carbon source resulted in a 3-fold increase in the rate of cellular Ca\textsuperscript{2+} uptake in wild-type cells, whereas Ca\textsuperscript{2+} uptake increased 5-fold in the pgm2\textDelta mutant. Disruption of the PMC1 gene, which encodes the vacuolar Ca\textsuperscript{2+}-ATPase Pmc1p, suppressed the Ca\textsuperscript{2+}-related phenotypes observed in the pgm2\textDelta strain. This suggests that excessive vacuolar Ca\textsuperscript{2+} uptake is tightly coupled to these defects in Ca\textsuperscript{2+} homeostasis. An in vitro assay designed to measure Ca\textsuperscript{2+} sequestration into intracellular compartments confirmed that the pgm2\textDelta mutant contained a higher level of Pmc1p-dependent Ca\textsuperscript{2+} transport activity than the wild-type strain. We found that this increased rate of vacuolar Ca\textsuperscript{2+} uptake also coincided with a large induction of the unfolded protein response in the pgm2\textDelta mutant, suggesting that Ca\textsuperscript{2+} uptake into the endoplasmic reticulum compartment was reduced. These results indicate that the excessive Ca\textsuperscript{2+} uptake and accumulation previously shown to be associated with the pgm2\textDelta mutation are due to a severe imbalance in the distribution of cellular Ca\textsuperscript{2+} into different intracellular compartments.

The regulation of intracellular Ca\textsuperscript{2+} homeostasis in eukaryotic cells is a remarkably intricate process. Ca\textsuperscript{2+} transport across the plasma membrane and its intracellular sequestration is tightly regulated such that the resting cytosolic Ca\textsuperscript{2+} concentration is maintained in a range of 50–200 nM (1–4). Small variations in cytosolic Ca\textsuperscript{2+} that occur in response to a number of stimuli are sufficient to activate a variety of Ca\textsuperscript{2+}-sensing proteins, such as calmodulin and calcineurin. This then leads to the induction of various downstream signal transduction pathways (5). Equally as important, the Ca\textsuperscript{2+} concentrations within the lumen of the endoplasmic reticulum (ER)\textsuperscript{1} and Golgi apparatus are carefully maintained to ensure the proper folding and processing of proteins transported through the secretory pathway (6).

In the budding yeast Saccharomyces cerevisiae, the vacuole is the major cellular Ca\textsuperscript{2+} storage compartment and contains >95% of total cellular Ca\textsuperscript{2+} (7). This large store of Ca\textsuperscript{2+} is maintained through the action of two transporters, the Ca\textsuperscript{2+}-ATPase Pmc1p and the Ca\textsuperscript{2+/H\textsuperscript{+}} exchanger Vcx1p (8, 9). Once thought to be relatively static by virtue of its association with inorganic polyphosphate (7), the vacuolar Ca\textsuperscript{2+} store has recently been suggested to be more dynamic in nature. The recently identified yeast transient receptor potential channel homologue, Yvc1p, was shown to localize to the vacuolar membrane and mediate Ca\textsuperscript{2+} efflux out of the vacuole (10). Additional reports have shown that vacuolar Ca\textsuperscript{2+} efflux by Yvc1p can be specifically induced by hypotonic shock, which may be mediated by a mechano-sensitive mechanism (11, 12).

In addition to the vacuole, the ER and Golgi apparatus are also important for maintaining proper intracellular Ca\textsuperscript{2+} homeostasis in yeast. The transporters responsible for maintaining proper Ca\textsuperscript{2+} levels in the secretory pathway include the Ca\textsuperscript{2+}-ATPases Pmr1p (3, 13–16) and Cod1p/Spf1p (17–20). Pmr1p is localized primarily to the Golgi apparatus, where it plays an essential role in maintaining the luminal Ca\textsuperscript{2+} concentration required for the proper glycosylation and processing of proteins in this compartment (13, 14). The loss of Pmr1p results in a number of alterations in Ca\textsuperscript{2+} homeostasis, including an increased rate of cellular Ca\textsuperscript{2+} uptake from the extracellular environment and a greater sensitivity to elevated extracellular Ca\textsuperscript{2+} levels (15). The elevated Ca\textsuperscript{2+} uptake observed in the pmr1\textDelta mutant is mediated by the MID1 and CCH1 gene products and is reminiscent of the mammalian capacitative Ca\textsuperscript{2+} entry (CCE) response (21). The depletion of secretory pathway Ca\textsuperscript{2+} stores caused by the pmr1\textDelta mutation also leads to improper folding and processing of proteins that transit through the ER and Golgi (14, 22). It was recently reported that this luminal Ca\textsuperscript{2+} depletion induces the unfolded protein response (UPR) (23). The UPR is activated by the presence of unfolded proteins in the ER and results in the increased expression of molecular chaperones that aid in protein folding in this compartment (24). This increased expres-

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1 The abbreviations used are: ER, endoplasmic reticulum; CCE, capacitative Ca\textsuperscript{2+} entry; PGM, phosphoglucomutase; wt, wild-type strain; UPR, unfolded protein response; Glc-6-P, glucose 6-phosphate; Glc-1-P, glucose 1-phosphate; YP, yeast extract/peptone; SM, synthetic medium; MES, 4-morpholineethanesulfonic acid; DTТ, dithiothreitol.
Recent evidence suggests that some products of carbohydrate metabolism also influence intracellular Ca\(^{2+}\) homeostasis in yeast cells. In particular, the sugar phosphates Glc-6-P and Glc-1-P have been proposed to play a role in modulating intracellular Ca\(^{2+}\) homeostasis (28, 29). The enzyme phosphoglucomutase (PGM) interconverts Glc-1-P and Glc-6-P and is required for the metabolism of galactose. Yeast strains lacking the major isomerase of this enzyme (pgm2\(^{+}\)) accumulate a high level of intracellular Glc-1-P when galactose is utilized as the carbon source due to a metabolic bottleneck in the conversion of Glc-1-P to Glc-6-P. This strain also exhibits alterations in the major isoform of this enzyme (pgm2\(^{+}\)) required for the metabolism of galactose. Yeast strains lacking the gene (28) to generate YDB0473. To generate strains harboring deletions of a Whorl Type A (right) Dounce homogenizer. The cell lysates were then cleared of unbroken cells by centrifugation twice at 4°C for 5 min at 2000 rpm (450 \(\times\) g) in a Sorvall SS-34 rotor and gently resuspended in lysis buffer (0.3 M sorbitol, 20 mM triethanolamine acetate, pH 7.2, 1 mM EDTA, and protease inhibitors (0.5 M phenylmethylsulfonyl fluoride, 2 \(\mu\)g/ml chymostatin, 1 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin A, and 1 \(\mu\)g/ml aprotinin; all from Sigma). The spheroplasts were then disrupted mechanically using 20 strokes of a Wheaton Type A (tight) Dounce homogenizer. The cell lysates were then cleared of unbroken cells by centrifugation twice at 4°C for 5 min at 2000 rpm (450 \(\times\) g) in a Sorvall SS-34 rotor. To fractionate membranes, 3 ml of lysate was loaded onto a 10%–54% (in 4% increments) sucrose gradient in 10 mM HEPES, pH 7.5, 1 mM MgCl\(_2\). Gradients were centrifuged at 4°C for 2 h at 27,000 rpm in a Beckman SW-28 rotor. After centrifugation, gradient fractions were collected manually in 3-ml aliquots from top to bottom. Individual fractions from multiple gradients were then pooled and stored at -80°C in 1-ml aliquots. Protein concentrations in each gradient fraction were determined by the method of Bradford using bovine serum albumin to generate a standard curve (37).

Assays in Isolated Membranes—To assay for Ca\(^{2+}\) transport activity in sucrose gradient fractions, 0.7 ml of O-Buffer (10 mM HEPES-NaOH, pH 6.7, 150 mM KCl, 5 mM MgCl\(_2\), 0.5 mM ATP (pre-buffered to pH 6.7), 5 mM NaCN, 0.5 \(\mu\)g/ml \(\beta\)-mercaptoethanol, 1 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin A, and 1 \(\mu\)g/ml aprotinin; all from Sigma). The spheroplasts were then disrupted mechanically using 20 strokes of a Wheaton Type A (tight) Dounce homogenizer. The cell lysates were then cleared of unbroken cells by centrifugation twice at 4°C for 5 min at 2000 rpm (450 \(\times\) g) in a Sorvall SS-34 rotor. To fractionate membranes, 3 ml of lysate was loaded onto a 10%–54% (in 4% increments) sucrose gradient in 10 mM HEPES, pH 7.5, 1 mM MgCl\(_2\). Gradients were centrifuged at 4°C for 2 h at 27,000 rpm in a Beckman SW-28 rotor. After centrifugation, gradient fractions were collected manually in 3-ml aliquots from top to bottom. Individual fractions from multiple gradients were then pooled and stored at -80°C in 1-ml aliquots. Protein concentrations in each gradient fraction were determined by the method of Bradford using bovine serum albumin to generate a standard curve (37).

**Measurement of Whole Cell Ca\(^{2+}\) Uptake, Total Cell Ca\(^{2+}\), and Exchangeable Ca\(^{2+}\) Pools—**Whole cell Ca\(^{2+}\) uptake measurements were performed as described previously (15, 29). Measurement of total cell Ca\(^{2+}\) by flame photometry was also carried out as previously described (3, 29).

**Isolation and Subcellular Fractionation of Total Cell Membranes—**Total cell membranes were isolated from yeast using a protocol based on previous publications (14, 16, 36). Yeast strains were grown at 30°C 24. Cells were then harvested by centrifugation and permeabilized by repeated freeze-thawing in liquid nitrogen (34). β-Galactosidase activity was measured using the colorimetric substrate 2-Nicotinamide-2-deoxy-β-D-galactopyranoside according to a previously described protocol (35). To examine the effect of extracellular Ca\(^{2+}\) and dithiothreitol (DTT), cells were treated with 20 mM CaCl\(_2\) or 5 mM DTT, respectively, for 4 h before harvest. Units of β-galactosidase activity are defined as the absorbance at 420 nm x10^{-3} A\(_{500}\) units of cell.
showed). These results suggest that vacuolar Ca$^{2+}$ is substantially higher than that observed for the wild-type and pmc1Δ mutant when the yeast is grown on YPGal plates supplemented with 50 mM CaCl$_2$ (Fig. 2, A). After the indicated incubation period, equal numbers of cells were counted in a hemocytometer to monitor the cell density and dilution of the culture as needed. The YPGal cultures were maintained in mid-log phase by monitoring the cell density and dilution of the culture as needed.

Intracellular Ca$^{2+}$ accumulation in yeast has been shown to exist in two distinct states termed the exchangeable and non-exchangeable pools (7). The exchangeable pool is thought to be largely synonymous with the vacuole pool, whereas the non-exchangeable pool is thought to exist in complex with polyphosphate (7). The exchangeable pool of Ca$^{2+}$ serves as the major Ca$^{2+}$ storage compartment in yeast (7, 39). This model predicts that wild-type cells shifted from the metabolism of glucose to galactose should also experience a significant increase in Ca$^{2+}$ uptake. To test this hypothesis, we measured $^{45}$Ca$^{2+}$ uptake in the wild-type and pgm2Δ strains after a carbon source shift from glucose to galactose (Fig. 1). The rates of $^{45}$Ca$^{2+}$ uptake in the wild-type and pgm2Δ strains were similar before the shift. We found that the rate of $^{45}$Ca$^{2+}$ uptake in the wild-type strain increased 2-fold within 6 h of re-suspending the cells in medium containing galactose as the carbon source and remained constant thereafter. In contrast, the pgm2Δ mutant exhibited a 4-fold increase in $^{45}$Ca$^{2+}$ uptake 6 h after the shift, and uptake increased to 8-fold higher than the pre-shift level after 12 h. These results support the hypothesis that a component of the normal adaptive response of yeast cells to the utilization of galactose as the carbon source is an increase in Ca$^{2+}$ transport across the plasma membrane. Furthermore, these findings suggest that the defect in Ca$^{2+}$ homeostasis observed in the pgm2Δ strain may result from an inability to properly regulate this normal physiological response due to the overproduction of Glc1-P in this strain.

Disruption of the PMC1 Gene Partially Suppresses Phenotypes Associated With the pgm2Δ Mutation—The vacuole serves as the major Ca$^{2+}$ storage compartment in yeast (7, 39). Given the high rate of cellular Ca$^{2+}$ uptake observed when the pgm2Δ strain utilizes galactose as carbon source, we reasoned that efficient vacuolar Ca$^{2+}$ sequestration might be critical for the viability of this strain. To determine the consequences of reducing the level of vacuolar Ca$^{2+}$ sequestration in the pgm2Δ mutant, we disrupted the genes encoding the vacuolar Ca$^{2+}$ ATPase Pmc1p and the vacuolar Ca$^{2+}$/H$^+$ exchanger Vcx1p both independently and together in the pgm2Δ strain. The loss of Vcx1p activity had no effect on the growth of the pgm2Δ mutant (data not shown). Surprisingly, the pmc1Δ mutation partially suppressed the slow growth and Ca$^{2+}$ sensitivity phenotypes of the pgm2Δ mutant on both standard YPGal plates and on YPGal plates supplemented with 50 mM CaCl$_2$ (Fig. 2, A and B). Combining both the pmc1Δ and vcx1Δ mutations together in the pgm2Δ mutant provided no greater suppression than that observed for the pmc1Δ mutation alone (data not shown). These results suggest that vacuolar Ca$^{2+}$ sequestration mediated by Pmc1p may be detrimental to growth of the pgm2Δ strain.

To determine whether this partial suppression of the pmc1Δ mutation correlated with changes in cellular Ca$^{2+}$ homeostasis, we next measured the level of total cellular Ca$^{2+}$ accumulation in strains grown with galactose as the carbon source (Fig. 3). As reported previously, the pgm2Δ mutant exhibited a 4-fold higher level of total cell Ca$^{2+}$ than the wild-type strain. Consistent with the observed growth phenotypes, the pgm2Δ/vcx1Δ double mutant had a level of total cell Ca$^{2+}$ that was similar to the level measured in the pgm2Δ mutant. In contrast, the pgm2Δ/pmc1Δ strain had a level of total cell Ca$^{2+}$ that was only 1.5-fold higher than that found in the wild-type strain. These results demonstrate that the introduction of the pmc1Δ mutation (and presumably a reduction in vacuolar Ca$^{2+}$ sequestration) coincides with the suppression of the Ca$^{2+}$ homeostasis phenotypes observed in the pgm2Δ strain.

Intracellular Ca$^{2+}$ in yeast has been shown to exist in two distinct states termed the exchangeable and non-exchangeable pools (7). The exchangeable pool was so named because it was rapidly released from yeast cells when they were introduced into medium containing a limiting level of extracellular Ca$^{2+}$. In contrast, the non-exchangeable pool of cellular Ca$^{2+}$ was released from the cell under these conditions at a much slower rate. Until recently, the non-exchangeable pool of cellular Ca$^{2+}$ was thought to be largely synonymous with the vacuole pool, where it is thought to exist in complex with polyphosphate (7).
These differences in Ca\(^{2+}\) exchangeable pool in either the wild-type or
the mean versus the corresponding decrease in its contribution to the total ex-
changeable pool in the

Thus, our finding that the exchangeable Ca\(^{2+}\) in these strains (Fig. 4A), and the presence of the yvc1\(\Delta\) mutation reduced the exchangeable Ca\(^{2+}\) pool from 17.2 to 13.5% in the wild-type background (a decrease of 21.5%), whereas the yvc1\(\Delta\) mutation reduced the fraction of total cellular Ca\(^{2+}\) in the exchangeable pool of the pgm2\(\Delta\) strain from 20.4 to 13.9% (a decrease of 31.9%). These results confirm that the vacuole contains a significant portion of the total exchangeable fraction. Furthermore, the 50% increase in the size of the exchangeable pool within the vacuole (based on the 31.9% decrease in the exchangeable vacuolar Ca\(^{2+}\) pool in the pgm2\(\Delta\) strain versus the corresponding 21.5% decrease observed in the wild-type strain) suggests that the size of the exchangeable Ca\(^{2+}\) pool in the ER or Golgi may undergo a corresponding decrease in its contribution to the total exchangeable pool in the pgm2\(\Delta\) strain.

The Unfolded Protein Response Is Activated in the pgm2\(\Delta\) Strain and Reversed by the pmc1\(\Delta\) Mutation—We previously used two assays to ask whether the Ca\(^{2+}\) level in the ER or Golgi was reduced. First, we found that the rate of ER degrada-
tion of a mutant form of carboxypeptidase Y (CPY\(^{\ast}\)) was normal, suggesting that the level of divalent cations in the ER is normal. Similarly, we found that the glycosylation of invertase (more specifically, outer chain addition) was normal, suggesting that the level of divalent cations in the Golgi was also normal. Both of these processes are dependent upon the presence of Ca\(^{2+}\) or Mn\(^{2+}\). Complicating the use of these assays to estimate compartmental Ca\(^{2+}\) levels is the fact that Mn\(^{2+}\) can effectively replace the requirement for Ca\(^{2+}\) to promote the growth of yeast cells (40), and Mn\(^{2+}\) was found to suppress the defects in invertase glycosylation caused by the pmr1 mutation more effectively than Ca\(^{2+}\) (22). Thus, these results did not provide conclusive evidence that the Ca\(^{2+}\) level was normal in either compartment. In addition, our previous finding that a yeast strain carrying both the pgm2\(\Delta\) and pmr1\(\Delta\) mutations is unable to grow on media containing galactose as the carbon source (28) suggested that the pgm2\(\Delta\) mutation may further reduce the depleted level of divalent cations in the secretory pathway caused by the pmr1\(\Delta\) mutation. This led us to re-examine the level of divalent cations in the ER of the pgm2\(\Delta\) strain.

The efficient sequestration of divalent cations is required for the proper folding and processing of proteins in the secretory pathway. Consequently, mutations that prevent the uptake of divalent cations into the ER, such as the pmr1\(\Delta\) and cod1\(\Delta\) mutations, result in an elevated UPR (23). To further examine whether the level of divalent cations in the ER is affected in the pgm2\(\Delta\) mutant, we assayed the level of UPR induction in different strains metabolizing galactose as the carbon source. This analysis was carried out using a reporter plasmid that contained UPR elements upstream of the \(\beta\)-galactosidase gene (26). Remarkably, we found that expression of the UPR reporter protein was 40-fold higher in the pgm2\(\Delta\) mutant than in the wild-type strain (Fig. 5A). This finding provides strong evidence that the level of divalent cations in the ER is reduced. Significantly, the pgm2\(\Delta\)/PMC1\(\Delta\) double mutant exhibited a level of UPR induction that was only 3-fold higher than the level observed in the wild type strain when grown in a medium with galactose as carbon source. This indicates that the loss of
We found that the addition of DTT to the growth medium led to a 7-fold increase in β-galactosidase activity in the wild-type strain and a 4.5-fold increase in the mutant. These results are consistent with the hypothesis that

**Fig. 6. Ca\(^{2+}\) sequestration into membrane vesicles isolated from wild-type cells correlates with Pmc1p activity.** 

![Graph showing Ca\(^{2+}\) sequestration into membrane vesicles isolated from wild-type (squares) or pmc1Δ (diamonds) cells grown in SMGal medium. ATP-dependent Ca\(^{2+}\) uptake assays were carried out in the presence of 25 μM carbonyl cyanide m-chlorophenylhydrazone. The data are presented as mean ± S.D. See “Experimental Procedures” for further details.](https://example.com/graph6)

pmc1Δ mutant (Fig. 5C). Both of these values are much lower than the 40-fold induction that was observed when the pgm2Δ strain was grown with galactose as the carbon source. Interestingly, the pgm2Δ mutant showed no further UPR induction upon exposure to DTT, suggesting that a maximal level of induction had already been reached. Overall, the massive induction of UPR in the pgm2Δ mutant suggests that Ca\(^{2+}\) stores in the ER are significantly reduced when this strain is grown with galactose as the carbon source. The suppression of this UPR response by the introduction of the pmc1Δ mutation indicates that this depletion of ER Ca\(^{2+}\) becomes much less severe when vacuolar Ca\(^{2+}\) uptake is reduced.

**Ca\(^{2+}\) Transport Activity in Cellular Membranes Is Increased in the pgm2Δ Strain—**Our results indicate that yeast cells normally increase Ca\(^{2+}\) uptake and accumulation when shifted to a growth medium containing galactose as the carbon source. Furthermore, Ca\(^{2+}\) uptake is increased much more in the pgm2Δ strain under these conditions. To gain further insights into how this additional Ca\(^{2+}\) is sequestered inside the cell, we next assayed Ca\(^{2+}\) transport into isolated intracellular membrane vesicles after a sucrose step gradient as previously described (9, 14, 16, 36). We first examined ATP-dependent Ca\(^{2+}\) transport into membranes isolated from wild-type cells grown with galactose as the carbon source. As reported previously, we found 45Ca\(^{2+}\) transport capacity to be greatest in the higher density membrane fractions, with a peak in activity occurring in Fraction 7 (Fig. 6). Furthermore, we found that the majority of the 45Ca\(^{2+}\) transport observed was dependent on the presence of Pmc1p. A pmc1Δ strain exhibited a large decrease in total ATP-dependent 45Ca\(^{2+}\) transport activity (and a 6.8-fold reduction in the activity centered on Fraction 7) as compared with the wild-type strain. These results indicate that the vacuolar transporter Pmc1p is primarily responsible for the ATP-dependent Ca\(^{2+}\) transport activity in yeast membranes.

We next compared 45Ca\(^{2+}\) transport into membrane fractions harvested from the wild-type and pgm2Δ strains grown with galactose as the carbon source. We previously found that the pgm2Δ strain grows very slowly in synthetic media containing galactose as the carbon source. To normalize the growth rates between the wild-type and pgm2Δ strains as much as possible, the synthetic medium used to grow both strains was supplemented with 0.3% yeast extract. As shown above, membranes from both the wild-type and pgm2Δ strains again showed peak 45Ca\(^{2+}\) transport activity in Fraction 7 (Fig. 7). However, peak 45Ca\(^{2+}\) transport activity was 3.2-fold higher in this peak fraction of membranes harvested from the pgm2Δ mutant. These results are consistent with the hypothesis that
grown in YMMG medium. The data are presented as the mean ± S.D. See "Experimental Procedures" for further details.

FIG. 7. Increased Ca\(^{2+}\) sequestration occurs in membrane vesicles harvested from the pgm2Δ strain metabolizing galactose as the carbon source. In vitro 45Ca\(^{2+}\) uptake assay on membrane fractions isolated from wild-type (squares) or pgm2Δ (diamonds) cells grown in YMMG medium. The data are presented as the mean ± S.D. for further details.

vacuolar Ca\(^{2+}\) sequestration is significantly increased in the pgm2Δ mutant. Ca\(^{2+}\) Transport into Membranes Isolated from Wild-type and pgm2Δ Strains Is Not Stimulated Directly by Glc-6-P or Glc-1-P—A previous report demonstrated that the altered Ca\(^{2+}\) homeostasis in the pgm2Δ mutant correlates with an increase in the intracellular ratio of Glc-1-P relative to Glc-6-P (29). To determine whether this effect is due to a direct stimulation of Ca\(^{2+}\) transport, we used the in vitro 45Ca\(^{2+}\) transport assay to test whether Glc-1-P or Glc-6-P can directly stimulate Ca\(^{2+}\) transport into membranes harvested from the wild-type or pgm2Δ strains. For both strains membranes were again prepared from cells grown with galactose as the carbon source, and the fractions showing the peak 45Ca\(^{2+}\) transport activity in preliminary experiments were examined further. For the wild-type strain, we found that neither Glc-6-P (Fig. 8A) nor Glc-1-P (Fig. 8B) stimulated 45Ca\(^{2+}\) transport into these membranes. Instead they decreased 45Ca\(^{2+}\) transport in a dose-dependent manner. In the presence of 10 mM Glc-6-P, 45Ca\(^{2+}\) accumulation decreased 33%. Similarly, the presence of 10 mM Glc-1-P decreased 45Ca\(^{2+}\) accumulation 45%. To determine whether these decreases were specific for Glc-6-P and Glc-1-P, we measured 45Ca\(^{2+}\) transport activity in the presence of 5 mM fructose 6-phosphate or mannose 6-phosphate. Interestingly, although no significant change was observed on 45Ca\(^{2+}\) transport in the presence of 5 mM fructose 6-phosphate, 5 mM mannose 6-phosphate led to a ∼20% increase in 45Ca\(^{2+}\) uptake that was reproducible in multiple experiments (Fig. 8C). Because previous reports predicted that Ca\(^{2+}\) is retained in the yeast vacuole by an interaction with polyphosphate (7), we also assayed 45Ca\(^{2+}\) transport in the presence of 5 mM sodium phosphate. Under these conditions, membranes from the wild-type strain reproducibly exhibited an increase in 45Ca\(^{2+}\) accumulation of greater than 50% (Fig. 8C).

We also examined the effects of sugar phosphates on 45Ca\(^{2+}\) transport using membranes harvested from the pgm2Δ mutant grown with galactose as carbon source. As described above for membranes harvested from the wild-type strain, we found that both Glc-6-P and Glc-1-P decreased 45Ca\(^{2+}\) accumulation into membranes from the pgm2Δ mutant in a dose-dependent manner. The addition of 10 mM Glc-6-P decreased 45Ca\(^{2+}\) transport 37% (Fig. 8D), whereas the addition of 10 mM Glc-1-P decreased 45Ca\(^{2+}\) transport nearly 50% (Fig. 8E). As observed with the wild-type strain, the addition of 5 mM fructose 6-phosphate had no significant effect on 45Ca\(^{2+}\) transport, whereas the addition of 5 mM mannose 6-phosphate led to an increase of ∼20% (Fig. 8F). The addition of 5 mM sodium phosphate increased 45Ca\(^{2+}\) transport in vitro in membranes harvested from the pgm2Δ mutant by 75% (Fig. 8F). When taken together, these results indicate that the sugar phosphates Glc-6-P and Glc-1-P are unable to directly stimulate Ca\(^{2+}\) transport into the vacuolar compartment.

DISCUSSION

In the current study we found that cellular Ca\(^{2+}\) uptake in the pgm2Δ strain increased 8-fold after a shift from glucose to galactose as the carbon source. In addition, several observations indicate that increased vacuolar Ca\(^{2+}\) uptake by Pmc1p plays a key role in the manifestation of the Ca\(^{2+}\) homeostasis defects observed in the pgm2Δ mutant. First, we found that the introduction of the pmc1Δ mutation reduced total cellular Ca\(^{2+}\) in the pgm2Δ strain to a level similar to that observed in the wild-type strain. The observation that the massive 40-fold induction of UPR observed in the pgm2Δ strain is greatly reduced in the pgm2Δ/pmc1Δ strain closely parallels the levels of Ca\(^{2+}\) uptake and accumulation observed in these strains. Further evidence that these Ca\(^{2+}\) homeostasis defects are largely attributable to Pmc1p function is provided by experiments in which the genes encoding other intracellular Ca\(^{2+}\) transporters are disrupted. Disruption of the vacuolar Ca\(^{2+}\)/H\(^{+}\) exchanger Vcx1p did not significantly alter the Ca\(^{2+}\) homeostasis phenotype observed in the pgm2Δ mutant. Similarly, disruption of the gene encoding the ER Ca\(^{2+}\)-ATPase Cod1p was unable to rescue the pgm2Δ-related Ca\(^{2+}\) defects (data not shown), whereas the pmr1Δ mutation was found to exacerbate those defects (28, 29). When taken together, our data suggest that the pgm2Δ mutation simultaneously leads to elevated vacuolar Ca\(^{2+}\) uptake and reduced ER/Golgi Ca\(^{2+}\) accumulation in yeast cells grown with galactose as the carbon source. Given our previous finding that a Glc-1-P/Glc-6-P ratio is responsible for the imbalance in Ca\(^{2+}\) homeostasis in the pgm2Δ strain (29), this suggests that the relative levels of these key glucose metabolites play a key role in determining the distribution of intracellular Ca\(^{2+}\) into different intracellular compartments.

The results of in vitro Ca\(^{2+}\) transport assays carried out with membranes harvested from wild-type and pgm2Δ strains also suggest that the bulk of Ca\(^{2+}\) transport activity in intracellular membranes is attributable to the vacuolar Ca\(^{2+}\)-ATPase Pmc1p. This finding is consistent with the previous conclusion that the vacuole contains >95% cellular Ca\(^{2+}\) in yeast (7). Other studies have also shown that pmc1Δ strains exhibit a reduced tolerance to high levels of extracellular Ca\(^{2+}\) and contain reduced amounts of total cellular Ca\(^{2+}\) (Refs. 3 and 39 and this study). However, a recent study using a similar Ca\(^{2+}\) uptake assay with isolated yeast membranes concluded that the Golgi Ca\(^{2+}\)-ATPase Pmr1p rather than the vacuolar Pmc1p is the major intracellular Ca\(^{2+}\) transporter under normal growth conditions (16). The reason for this discrepancy remains to be determined.

The UPR can be induced by a defect in protein glycosylation or a reduction in the level of ER Ca\(^{2+}\) (23). Because the pgm2Δ mutation alters the relative cellular levels of Glc-1-P and Glc-6-P, it is possible that this imbalance in glucose metabolites inhibits the core glycosylation of proteins after their translocation into the ER. However, our finding that both the Ca\(^{2+}\) homeostasis defects and UPR induction can be suppressed by the pmc1Δ mutation strongly suggests that the primary defect associated with the pgm2Δ mutation is a defect in Ca\(^{2+}\) homeostasis rather than a defect in protein glycosylation. Based on this reasoning we propose the following model to explain how the pgm2Δ mutation alters Ca\(^{2+}\) homeostasis in yeast (Fig. 9). First, growth of the pgm2Δ strain in media containing galactose as the carbon source causes Glc-1-P to accumulate due to the metabolic bottleneck in the conversion of Glc-1-P to Glc-6-P (28). This results in an altered cellular ratio of Glc-1-P to
Glc-6-P (29) that leads to an increase in Pmc1p activity (this study). The increased rate of vacuolar Ca\(^{2+}\) uptake reduces the level of cytosolic Ca\(^{2+}\), which in turn causes a depletion of free Ca\(^{2+}\) in the ER. This decrease in the level of free Ca\(^{2+}\) in the lumen of the ER has two consequences. First, it has an adverse effect on protein folding in the ER, which leads to an induction of the UPR. In addition, it leads to an increase in Ca\(^{2+}\) uptake across the plasma membrane by a CCE-like mechanism. Evidence that excessive vacuolar Ca\(^{2+}\) uptake can deplete ER Ca\(^{2+}\) stores was previously provided by Cunningham and co-workers (21), who found that increased vacuolar Ca\(^{2+}\) sequestration could effectively out-compete the secretory pathway Ca\(^{2+}\) transporters for free Ca\(^{2+}\) in the cytosol. Notably, this led to an induction of a CCE-like response due to the depletion of ER Ca\(^{2+}\) stores (21). According to our model, the suppression of these Ca\(^{2+}\) homeostasis defects by the pmc1\(\Delta\) mutation occurs by moderating the excessive vacuolar Ca\(^{2+}\) sequestration, thus allowing the Ca\(^{2+}\) levels in the cytoplasm and ER lumen to rise to concentrations that are closer to normal levels.

It was previously shown that yeast possess both high affinity and low affinity Ca\(^{2+}\) uptake mechanisms (21). The high affinity pathway is mediated by the MID1 and CCH1 gene products, which are thought to oligomerize to form a single high affinity Ca\(^{2+}\) channel in the plasma membrane. In contrast, the low affinity pathway has not been characterized in detail, and the gene products that encode components of this channel have not been identified. The high affinity pathway was considered to be a good candidate to mediate the CCE-like response proposed for the pgm2\(\Delta\) strain, since the MID1/CCH1 gene products were previously shown to be required for a CCE-like mechanism in a pmr1\(\Delta\) strain (21). However, we found that the introduction of a mid1\(\Delta\) mutation into the pgm2\(\Delta\) strain was unable to suppress the Ca\(^{2+}\) homeostasis defects associated with the pgm2\(\Delta\) mutation. Because both the rate of cellular Ca\(^{2+}\) uptake and UPR induction are much larger in the pgm2\(\Delta\) mutant than was previously shown to be associated with the pmr1\(\Delta\) strain (21), it is possible that the more robust CCE response observed in this strain is mediated by the low affinity Ca\(^{2+}\) uptake pathway or a combination of both.

Although we found that cellular Ca\(^{2+}\) uptake in the pgm2\(\Delta\) strain increased 8-fold after a shift from glucose to galactose as the carbon source, we also observed that wild-type yeast cells undergo a 2-fold increase in Ca\(^{2+}\) uptake. This finding is consistent with our hypothesis that an increased Glc-1-P/Glc-6-P ratio stimulates cellular Ca\(^{2+}\) uptake via an activation of Pmc1p, since the steady-state Glc-1-P level increases in wild-type cells when galactose is utilized as carbon source. Thus, the increased level of Ca\(^{2+}\) uptake appears to be part of a normal adaptive response to this carbon source shift. In other experiments, we found that this 2-fold elevation in Ca\(^{2+}\) uptake correlated with a -1.5-fold increase in Pmc1p protein levels in both wild-type and pgm2\(\Delta\) strains (data not shown). The much larger increase in Ca\(^{2+}\) uptake observed in the pgm2\(\Delta\) strain suggests that a post-transcriptional mechanism may complement this modest increase in Pmc1p transcription and is again consistent with the hypothesis that Glc-6-P and/or Glc-1-P acts as a signaling molecule whose level provides a sensitive metabolic readout of carbon source that is subsequently amplified via Ca\(^{2+}\) signaling mechanisms. Our finding that neither Glc-

\(a\) D. Aiello and D. Bedwell, unpublished results.
6-P nor Glc-1-P can enhance Ca\(^{2+}\) transport into intracellular membranes in vitro suggests that these metabolites do not directly stimulate the activity of a Ca\(^{2+}\) transporter such as Pmc1p in vitro. However, it remains a formal possibility that a metabolite derived from one of these compounds in vitro could function as the true physiological activator of this process. It is unlikely that a derivative of UDP-glucose (which is derived from Glc-1-P and UDP via the enzyme UDP-Glc pyrophosphorylase) plays this role, since it was previously shown that the overproduction of UDP-glucose in vivo did not result in changes in Ca\(^{2+}\) homeostasis like those observed in the pgm2\(^{11}\) strain. Unexpectedly providing strains and reagents.

If Glc-1-P and Glc-6-P do not stimulate intracellular Ca\(^{2+}\) sequestration by a direct mechanism, how does an imbalance in these sugar phosphates mediate this affect? A large body of evidence suggests that Glc-6-P normally functions as an intracellular signaling molecule. Recent studies have suggested that Snf3p and Rgt2p activate glucose signaling pathways by binding Glc-6-P on the cytosolic side of the cell membrane. This conclusion stems from the observation that the expression of only the C-terminal cytosolic domain of Snf3p can restore relatively normal glucose signaling in snf3Δ cells (43, 44). Consistent with a hypothesized signaling role for Glc-6-P, another study found that the transient elevation of cytosolic Ca\(^{2+}\) response shown to occur upon the re-addition of glucose to cells starved for carbon source was dependent on the ability of the cell to phosphorylate glucose to Glc-6-P (38). Our previous reports that the altered Ca\(^{2+}\) homeostasis phenotypes in the pgm2Δ mutant are due to an altered cellular ratio of Glc-6-P to Glc-1-P are also consistent with a signaling role for these sugar metabolites (28, 29).

Several proteins, including Hxxk2p (45), Snf3p (44), Rgt2p (46), and Gpr1p/Gpa2p (47) have also been proposed to respond to the level of cytosolic Glc-6-P. As such, we sought to determine whether the disruption of the genes encoding any of these putative sugar phosphate sensors could suppress the Ca\(^{2+}\) homeostasis abnormalities observed in the pgm2Δ strain. Unfortunately, none of the mutations tested (or various combinations thereof) could suppress phenotypes associated with the pgm2Δ mutation (data not shown). These results indicate that signaling changes mediated by an altered ratio of Glc-6-P to Glc-1-P in the pgm2Δ strain either occur via a complex interplay of these signaling proteins or is mediated by another mechanism. Although a substantial body of experimental evidence indicates that increased cellular concentrations of Glc-6-P and/or Glc-1-P affect cellular Ca\(^{2+}\) homeostasis, further studies will be required to characterize the nature by which this metabolic signal is recognized and transduced to downstream components of the signaling pathway.

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The Ca$^{2+}$ Homeostasis Defects in a pgm2Δ Strain of *Saccharomyces cerevisiae* Are Caused by Excessive Vacuolar Ca$^{2+}$ Uptake Mediated by the Ca$^{2+}$-ATPase Pmc1p

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