

The Ca²⁺ Homeostasis Defects in a *pgm2Δ* Strain of *Saccharomyces cerevisiae* Are Caused by Excessive Vacuolar Ca²⁺ Uptake Mediated by the Ca²⁺-ATPase Pmc1p*

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Loss of the major isoform of phosphoglucosyltransferase (PGM) causes an accumulation of glucose 1-phosphate when yeast cells are grown with galactose as the carbon and energy source. Remarkably, the *pgm2Δ* strain also exhibits a severe imbalance in intracellular Ca²⁺ homeostasis when grown under these conditions. In the present study, we examined how the *pgm2Δ* mutation alters yeast Ca²⁺ homeostasis in greater detail. We found that a shift from glucose to galactose as the carbon source resulted in a 2-fold increase in the rate of cellular Ca²⁺ uptake in wild-type cells, whereas Ca²⁺ uptake increased 8-fold in the *pgm2Δ* mutant. Disruption of the *PMC1* gene, which encodes the vacuolar Ca²⁺-ATPase Pmc1p, suppressed the Ca²⁺-related phenotypes observed in the *pgm2Δ* strain. This suggests that excessive vacuolar Ca²⁺ uptake is tightly coupled to these defects in Ca²⁺ homeostasis. An *in vitro* assay designed to measure Ca²⁺ sequestration into intracellular compartments confirmed that the *pgm2Δ* mutant contained a higher level of Pmc1p-dependent Ca²⁺ transport activity than the wild-type strain. We found that this increased rate of vacuolar Ca²⁺ uptake also coincided with a large induction of the unfolded protein response in the *pgm2Δ* mutant, suggesting that Ca²⁺ uptake into the endoplasmic reticulum compartment was reduced. These results indicate that the excessive Ca²⁺ uptake and accumulation previously shown to be associated with the *pgm2Δ* mutation are due to a severe imbalance in the distribution of cellular Ca²⁺ into different intracellular compartments.

The regulation of intracellular Ca²⁺ homeostasis in eukaryotic cells is a remarkably intricate process. Ca²⁺ transport across the plasma membrane and its intracellular sequestration is tightly regulated such that the resting cytosolic Ca²⁺ concentration is maintained in a range of 50–200 nM (1–4). Small variations in cytosolic Ca²⁺ that occur in response to a number of stimuli are sufficient to activate a variety of Ca²⁺-sensing proteins, such as calmodulin and calcineurin. This

then leads to the induction of various downstream signal transduction pathways (5). Equally as important, the Ca²⁺ concentrations within the lumen of the endoplasmic reticulum (ER)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 Ca²⁺ storage compartment and contains >95% of total cellular Ca²⁺ (7). This large store of Ca²⁺ is maintained through the action of two transporters, the Ca²⁺-ATPase Pmc1p and the Ca²⁺/H⁺ exchanger Vcx1p (8, 9). Once thought to be relatively static by virtue of its association with inorganic polyphosphate (7), the vacuolar Ca²⁺ 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 Ca²⁺ efflux out of the vacuole (10). Additional reports have shown that vacuolar Ca²⁺ 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 Ca²⁺ homeostasis in yeast. The transporters responsible for maintaining proper Ca²⁺ levels in the secretory pathway include the Ca²⁺-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 Ca²⁺ 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 Ca²⁺ homeostasis, including an increased rate of cellular Ca²⁺ uptake from the extracellular environment and a greater sensitivity to elevated extracellular Ca²⁺ levels (15). The elevated Ca²⁺ uptake observed in the *pmr1Δ* mutant is mediated by the *MIDI* and *CCH1* gene products and is reminiscent of the mammalian capacitance Ca²⁺ entry (CCE) response (21). The depletion of secretory pathway Ca²⁺ stores caused by the *pmr1Δ* 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 Ca²⁺ 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, capacitance Ca²⁺ entry; PGM, phosphoglucosyltransferase; 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; DTT, dithiothreitol.

sion is mediated by the transcription factor Hac1p/Ern4p (25–27).

Recent evidence suggests that some products of carbohydrate metabolism also influence intracellular Ca²⁺ 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 Ca²⁺ homeostasis (28, 29). The enzyme phosphoglucosyltransferase (PGM) interconverts Glc-1-P and Glc-6-P and is required for the metabolism of galactose. Yeast strains lacking the major isoform of this enzyme (*pgm2Δ*) 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 cellular Ca²⁺ homeostasis under these conditions, including dramatically increased Ca²⁺ uptake and accumulation, sensitivity to high extracellular Ca²⁺ concentrations, and increased sensitivity to the calcineurin inhibitor cyclosporin A (28). Most recently, it was demonstrated that the simultaneous overproduction of both Glc-1-P and Glc-6-P restored normal Ca²⁺ homeostasis, suggesting that the ratio of these glucose metabolites plays an important role in controlling this process (29). In the present study our results indicate that the *pgm2Δ* strain experiences elevated vacuolar Ca²⁺ uptake and reduced ER Ca²⁺ sequestration when grown with galactose as the carbon source. Furthermore, we show that these phenotypes are suppressed by the deletion of the *MC1* gene. These results demonstrate that the relative levels of Glc-1-P and Glc-6-P play an important role in regulating the distribution of Ca²⁺ into different intracellular compartments.

EXPERIMENTAL PROCEDURES

Strains and Plasmids Used—Yeast strain YDB0316 is a wild-type strain described previously (28). The *PGM2* gene in YDB0316 was disrupted by insertion of a previously described fragment containing the *LEU2* gene (28) to generate YDB0473. To generate strains YDB0484 (*pmc1Δ*) and YDB0483 (*pgm2Δ/pmc1Δ*), the *PMC1* gene was disrupted using standard methods (30) in YDB0316 (wt) and YDB0473 (*pgm2Δ*) using the *S. cerevisiae* *HIS3* open reading frame that was PCR-amplified from pRS313 (31). The primers used to generate this product were DB1158 (5'-TAGAAAAGTG GTTCTAAAAA AAAAAA-ACTG TGTGCGTAAC AAAAAAATA GCTTGGTGAG CGCTAGGAG-T-3') and DB1159 (5'-CAATTTTGAA AATATAACTA TTACACACAT CTTTTCATTT GTTCACCTAC CTGTTTCGTAT ACATACTTAC TGAC-3'). To generate strains YDB0475 (*vcx1Δ*) and YDB0474 (*pgm2Δ/vcx1Δ*), the *VCX1* gene was disrupted using standard methods (30) in YDB0316 (wt) and YDB0473 (*pgm2Δ*) with a previously described knockout construct (3). For each strain, gene disruptions were confirmed by PCR, Southern blotting, and/or PGM enzymatic assays (28). Finally, strain BY4741 (wild type) and the isogenic *ycv1Δ* strain BY4741 (clone 1863) were purchased from Open Biosystems. A *pgm2::LEU2* disruption construct (28) was used to disrupt the *PGM2* gene in BY4741 to generate the isogenic *pgm2Δ* strain YDB0626 and in BY4741 (clone 1863) to generate the isogenic *pgm2Δ/ycv1Δ* strain YDB0627. The induction of UPR signaling was monitored using the reporter plasmid pMCZ-Y (a kind gift from Kazutoshi Mori) that contains *lacZ* under the control of the yeast *CYC1* promoter containing an unfolded protein response element (26).

Culture Media—Bacterial strains used for cloning and plasmid maintenance were grown in standard media as described (32). Similarly, yeast media were prepared as described (30). Yeast extract/peptone (YP) medium and synthetic medium (SM) were supplemented with 2% glucose (dextrose) (YPD or SMD) or 2% galactose (YPGal or SMGal). YPD and YPGal media were routinely buffered to pH 5.5 with 40 mM MES-Tris. To prepare membranes for *in vitro* ⁴⁵Ca²⁺ uptake assays, cells were grown in Yeast Mito medium containing 6.7 g of yeast nitrogen base (Difco) and 0.3% yeast extract (Difco)/liter in the presence of 2% glucose or 2% galactose (YMMG) (33). Liquid cultures were grown for a minimum of five generations to ≤1.0 A₆₀₀ units/ml before harvesting.

Measurement of Whole Cell Ca²⁺ Uptake, Total Cellular Ca²⁺, and Exchangeable Ca²⁺ Pools—Whole cell Ca²⁺ uptake measurements were performed as described previously (15, 29). Measurement of total cell

Ca²⁺ by flame photometry was also carried out as previously described (3, 29).

Cells for Ca²⁺ exchange experiments were grown in YPGal medium to a density of 0.05 A₆₀₀. The medium was then supplemented with ⁴⁵Ca²⁺, and growth was continued to a cell density of 0.5–1 A₆₀₀/ml. The cells were then harvested by centrifugation at 4000 × g for 5 min, washed, and resuspended in fresh YPGal supplemented with 50 mM CaCl₂. At the indicated times, aliquots were removed, filtered, washed, and processed for scintillation counting as previously described (3).

β-Galactosidase Assays—Yeast strains transformed with plasmid pMCZ-Y were grown to mid-log phase in SMGal medium as indicated. Cells were then harvested by centrifugation and permeabilized by repeated freeze-thawing in liquid nitrogen (34). β-Galactosidase activity was assayed using the colorimetric substrate 2-nitrophenyl-β-D-galactopyranoside according to a previously described protocol (35). To examine the effect of extracellular Ca²⁺ and dithiothreitol (DTT), cells were treated with 20 mM CaCl₂ or 5 mM DTT, respectively, for 4 h before harvest. Units of β-galactosidase activity are defined as the absorbance at 420 nm × 10³/min/A₆₀₀ unit of cells.

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 in the indicated media to 0.6–0.8 A₆₀₀ units/ml. Before harvest, NaN₃ was added to a final concentration of 10 mM, and the cells were rapidly chilled in ice water for 10 min. Cells were then harvested by centrifugation at 4 °C for 5 min at 6000 rpm (6000 × g) in a Sorvall SLA-3000 rotor. Cell pellets were resuspended in spheroplasting buffer (1.4 M sorbitol, 50 mM Tris-HCl, pH 7.5, 10 mM NaN₃, 40 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), and 0.3 mg/ml yeast lytic enzyme (ICN). Cells were converted to spheroplasts by incubation for 30–45 min at 37 °C. The spheroplasts were then harvested by centrifugation at 4 °C for 5 min at 2500 rpm (750 × 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 mM phenylmethylsulfonyl fluoride, 2 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/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 × g) in a Sorvall SS-34 rotor. To fractionate membranes, 3 ml of lysate was loaded onto a 10-step (18–54% in 4% increments) sucrose gradient in 10 mM HEPES, pH 7.5, 1 mM MgCl₂. 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).

Ca²⁺ Uptake Assays in Isolated Membranes—To assay for Ca²⁺ transport activity in sucrose gradient fractions, 0.7 ml of O-Buffer (10 mM HEPES-NaOH, pH 6.7, 150 mM KCl, 5 mM MgCl₂, 0.5 mM ATP (pre-buffered to pH 6.7), 5 mM NaN₃, 0.5 μCi/ml ⁴⁵CaCl₂ (9.6 mCi/mg)) was added to 0.3 ml of gradient fraction and incubated for 12 min at 25 °C (36). The entire 1-ml sample was then collected by filtration through a pre-washed nitrocellulose filter (Millipore, HAWP02500) and washed twice with 5 ml of ice-cold wash buffer (10 mM HEPES-NaOH, pH 7.5, 150 mM KCl) (36). Membrane-sequestered ⁴⁵Ca²⁺ was then determined by liquid scintillation counting. Where indicated, Ca²⁺ transport by Vcx1p was inhibited by the addition of 25 μM carbonyl cyanide *m*-chlorophenylhydrazone (Sigma). To test for the effect of sugar phosphates on Ca²⁺ uptake, pre-buffered (pH 6.7) glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, or mannose 6-phosphate was added to the O-Buffer mix at the indicated concentrations as indicated. To test for the effect on Ca²⁺ uptake by free phosphate, 5 mM sodium phosphate was added to the O-Buffer mix as indicated.

RESULTS

A Sustained Increase in Cellular Ca²⁺ Uptake Occurs upon Carbon Source Shift—Previous studies show that the *pgm2Δ* mutation causes increased cellular Ca²⁺ uptake and accumulation when galactose is utilized as the carbon source (28, 29, 38). This increase in Ca²⁺ uptake coincides with a large increase in the level of intracellular Glc-1-P and a resulting alteration in the Glc-1-P/Glc-6-P ratio. It has been proposed that the altered ratio of these glucose metabolites induces a

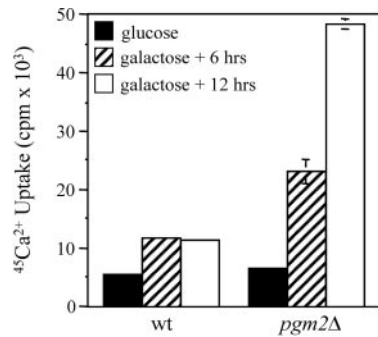


FIG. 1. Yeast strains exhibit an increase in Ca²⁺ uptake and accumulation when shifted to growth media containing galactose as the carbon source. ⁴⁵Ca²⁺ uptake was measured in wild-type and *pgm2Δ* yeast strains after a carbon source shift from YPD to YPGal medium. Data represent the ⁴⁵Ca²⁺ taken up over 10 min as described under “Experimental Procedures.” Cells grown in YPD medium were harvested in mid-log phase and resuspended to the same cell density in YPGal medium. The YPGal cultures were maintained in mid-log phase by monitoring the cell density and dilution of the culture as needed. After the indicated incubation period, equal numbers of cells were harvested for ⁴⁵Ca²⁺ uptake assays. Data are represented as the mean ± S.D. Similar data were obtained in four independent experiments, each performed in triplicate.

mechanism that normally couples their relative abundance to intracellular Ca²⁺ homeostasis (28, 29, 38). This model predicts that wild-type cells shifted from the metabolism of glucose to galactose should also experience a significant increase in Ca²⁺ uptake. To test this hypothesis, we measured ⁴⁵Ca²⁺ uptake in the wild-type and *pgm2Δ* strains after a carbon source shift from glucose to galactose (Fig. 1). The rates of ⁴⁵Ca²⁺ uptake in the wild-type and *pgm2Δ* strains were similar before the shift. We found that the rate of ⁴⁵Ca²⁺ 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 ⁴⁵Ca²⁺ 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 Ca²⁺ transport across the plasma membrane. Furthermore, these findings suggest that the defect in Ca²⁺ homeostasis observed in the *pgm2Δ* strain may result from an inability to properly regulate this normal physiological response due to the overproduction of Glc-1-P in this strain.

Disruption of the *PMC1* Gene Partially Suppresses Phenotypes Associated With the *pgm2Δ* Mutation—The vacuole serves as the major Ca²⁺ storage compartment in yeast (7, 39). Given the high rate of cellular Ca²⁺ uptake observed when the *pgm2Δ* strain utilizes galactose as carbon source, we reasoned that efficient vacuolar Ca²⁺ sequestration might be critical for the viability of this strain. To determine the consequences of reducing the level of vacuolar Ca²⁺ sequestration in the *pgm2Δ* mutant, we disrupted the genes encoding the vacuolar Ca²⁺ ATPase Pmc1p and the vacuolar Ca²⁺/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 Ca²⁺ sensitivity phenotypes of the *pgm2Δ* mutant on both standard YPGal plates and on YPGal plates supplemented with 50 mM CaCl₂ (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 Ca²⁺ sequestra-

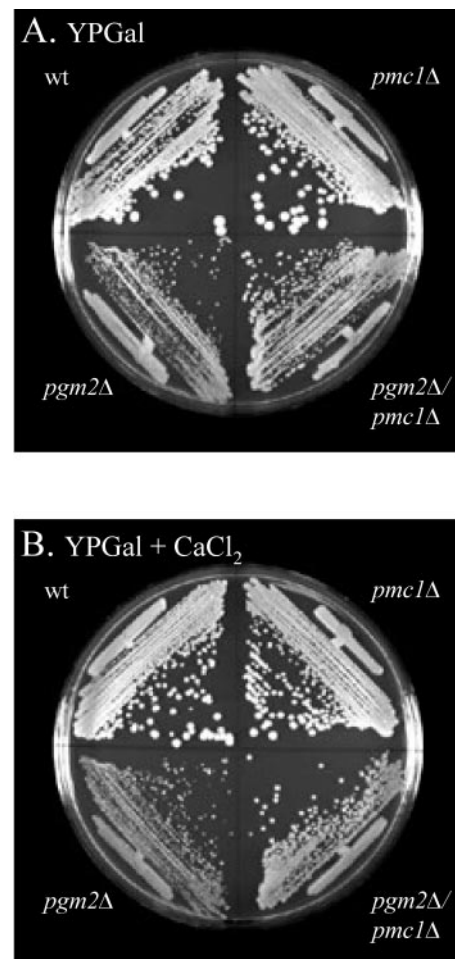


FIG. 2. The *pmc1Δ* mutation partially suppresses the slow growth phenotype associated with the *pgm2Δ* strain on YPGal plates. 50 mM CaCl₂ was added to YPGal medium as indicated. Plates were incubated for 6 days at 30 °C.

tion mediated by Pmc1p may be detrimental to growth of the *pgm2Δ* strain.

To determine whether this partial suppression of the *pgm2Δ* growth defect by the *pmc1Δ* mutation correlated with changes in cellular Ca²⁺ homeostasis, we next measured the level of total cellular Ca²⁺ 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 Ca²⁺ than the wild-type strain. Consistent with the observed growth phenotypes, the *pgm2Δ/vcx1Δ* double mutant had a level of total cell Ca²⁺ that was similar to the level measured in the *pgm2Δ* mutant. In contrast, the *pgm2Δ/pmc1Δ* strain had a level of total cell Ca²⁺ 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 Ca²⁺ sequestration) coincides with the suppression of the Ca²⁺ homeostasis phenotypes observed in the *pgm2Δ* strain.

Intracellular Ca²⁺ 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 Ca²⁺. In contrast, the non-exchangeable pool of cellular Ca²⁺ was released from the cell under these conditions at a much slower rate. Until recently, the non-exchangeable pool of cellular Ca²⁺ was thought to be largely synonymous with the vacuole pool, where it is thought to exist in complex with polyphosphate (7).

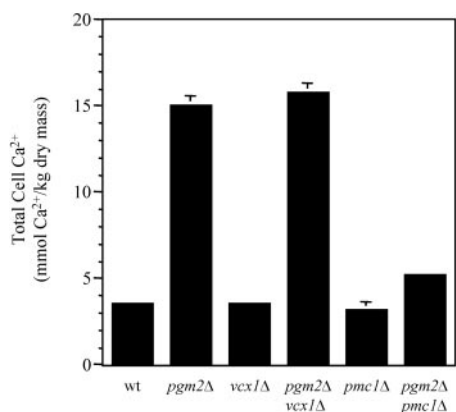


FIG. 3. The *pmc1Δ* mutation suppresses the high Ca²⁺ accumulation phenotype of the *pgm2Δ* strain grown in YPGal medium. The indicated strains were grown in YPGal medium to mid-log phase and harvested for total cell Ca²⁺ determination by flame photometry as described under "Experimental Procedures." Data are represented as the mean ± S.D.

In contrast, the exchangeable pool was thought to represent Ca²⁺ located primarily within the ER and Golgi compartments. Thus, our finding that the exchangeable Ca²⁺ pool in the *pgm2Δ* strain was not reduced when grown with galactose as the carbon source suggested that the Ca²⁺ level was not depleted in the compartments of the secretory pathway. However, Cyert and Denis (11) recently found that a fraction of the vacuolar Ca²⁺ store is more dynamic than previously thought and can be rapidly released from the vacuole into the cytoplasm under certain conditions. They also showed that Yvc1p, a Ca²⁺ channel in the vacuolar membrane, was responsible for mediating this release of vacuolar Ca²⁺. Significantly, these findings indicated that a significant portion of the exchangeable Ca²⁺ pool is localized to the vacuole.

To test whether the exchangeable Ca²⁺ pool in the vacuole is altered by the *pgm2Δ* mutation, we next compared the effect of the *yvc1Δ* mutation on Ca²⁺ partitioning in the wild-type and *pgm2Δ* strains. To do this we measured the relative size of the exchangeable and non-exchangeable pools in wild-type, *yvc1Δ*, *pgm2Δ*, and *pgm2Δ/yvc1Δ* strains. We found that the size of the non-exchangeable pools ranged from 79.6 to 86.5% of total cellular Ca²⁺ in these strains (Fig. 4A), and the presence of the *yvc1Δ* mutation led to a small increase in the size of the non-exchangeable pool in either the wild-type or *pgm2Δ* strains. These differences in Ca²⁺ partitioning were more striking when the exchangeable pools were examined (Fig. 4B). The presence of the *yvc1Δ* mutation reduced the exchangeable Ca²⁺ pool from 17.2 to 13.5% in the wild-type background (a decrease of 21.5%), whereas the *yvc1Δ* mutation reduced the fraction of total cellular Ca²⁺ in the exchangeable pool of the *pgm2Δ* 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 Ca²⁺ pool in the *pgm2Δ* strain versus the corresponding 21.5% decrease observed in the wild-type strain) suggests that the size of the exchangeable Ca²⁺ pool in the ER or Golgi may undergo a corresponding decrease in its contribution to the total exchangeable pool in the *pgm2Δ* strain.

The Unfolded Protein Response Is Activated in the *pgm2Δ* Strain and Reversed by the *pmc1Δ* Mutation—We previously used two assays to ask whether the Ca²⁺ level in the ER or Golgi was reduced. First, we found that the rate of ER degradation of a mutant form of carboxypeptidase Y (CPY*) was normal, suggesting that the level of divalent cations in the ER

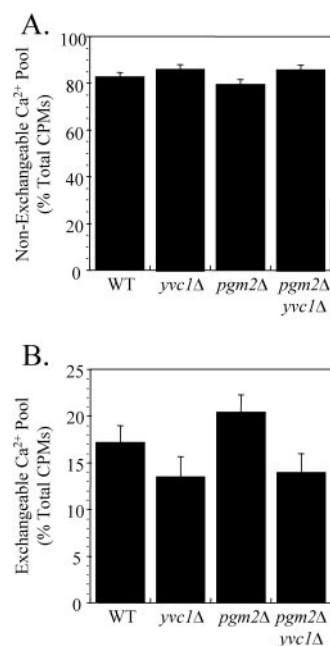


FIG. 4. Loss of the vacuolar Ca²⁺ channel Yvc1p reduces the size of the exchangeable Ca²⁺ pool in wild-type and *pgm2Δ* strains. Quantitation of the fraction of total cellular Ca²⁺ present in (the non-exchangeable pool (A) and the exchangeable pool (B)). The distribution of total cellular Ca²⁺ in each pool was determined as described under "Experimental Procedures."

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 Ca²⁺ or Mn²⁺. Complicating the use of these assays to estimate compartmental Ca²⁺ levels is the fact that Mn²⁺ can effectively replace the requirement for Ca²⁺ to promote the growth of yeast cells (40), and Mn²⁺ was found to suppress the defects in invertase glycosylation caused by the *pmr1Δ* mutation more effectively than Ca²⁺ (22). Thus, these results did not provide conclusive evidence that the Ca²⁺ level was normal in either compartment. In addition, our previous finding that a yeast strain carrying both the *pgm2Δ* and *pmr1Δ* mutations is unable to grow on media containing galactose as the carbon source (28) suggested that the *pgm2Δ* mutation may further reduce the depleted level of divalent cations in the secretory pathway caused by the *pmr1Δ* mutation. This led us to re-examine the level of divalent cations in the ER of the *pgm2Δ* 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 *pmr1Δ* and *cod1Δ* mutations, result in an elevated UPR (23). To further examine whether the level of divalent cations in the ER are affected in the *pgm2Δ* 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 β -galactosidase gene (26). Remarkably, we found that expression of the UPR reporter protein was 40-fold higher in the *pgm2Δ* 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 *pgm2Δ/pmc1Δ* 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

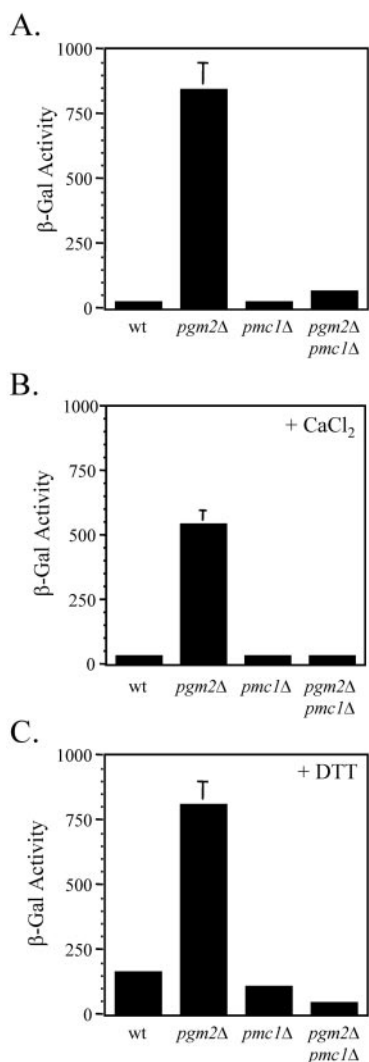


FIG. 5. The *pgm2Δ* mutant exhibits an elevated UPR. The indicated strains were grown in SMGal medium (A) to mid-log phase and harvested for β -galactosidase assays as described under “Experimental Procedures.” The magnitude of the UPR was also determined in strains grown in SMGal medium supplemented with 20 mM CaCl₂ (B) or 5 mM DTT (C). Data are represented as the mean \pm S.D. β -Gal, β -galactosidase.

Pmc1p function can largely suppress the UPR induction associated with the *pgm2Δ* mutation. Given the well characterized role of Pmc1p as a vacuolar Ca²⁺ transporter (3, 16, 39), these results suggest that this suppression of the UPR results from a specific reduction in Ca²⁺ sequestration into the vacuole.

We next sought to characterize in greater detail the UPR induction observed in the *pgm2Δ* strain. A recent report by Bonilla *et al.* examining the induction of UPR in the *pmr1Δ* mutant found that the addition of Ca²⁺ to the growth medium reduces the magnitude of UPR induction (23). Consistent with this finding, we found that the addition of 20 mM Ca²⁺ to the growth medium reduced the UPR response observed in the *pgm2Δ* mutant by ~35% (compare the magnitude of responses in Fig. 5, A and B). In addition, the 3-fold greater level of UPR induction observed with the *pgm2Δ/pmc1Δ* double mutant was completely eliminated in the presence of 20 mM extracellular Ca²⁺ (Fig. 5B). Several reports have also shown that the addition of DTT to the growth medium can cause an accumulation of unfolded proteins in the ER and activation of the UPR response (23, 41, 42). We found that the addition of 5 mM 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

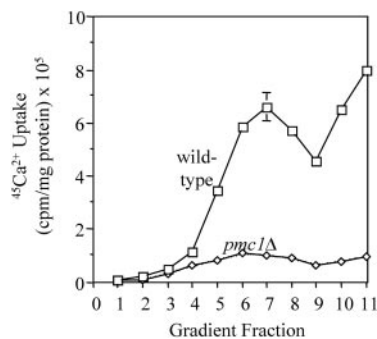


FIG. 6. Ca²⁺ sequestration into membrane vesicles isolated from wild-type cells correlates with Pmc1p activity. ⁴⁵Ca²⁺ sequestration into membrane fractions isolated from wild-type (*squares*) or *pmc1Δ* (*diamonds*) cells grown in SMGal medium. ATP-dependent ⁴⁵Ca²⁺ uptake assays were carried out in the presence of 25 μ M carbonyl cyanide *m*-chlorophenylhydrazone. The data are presented as mean \pm S.D. See “Experimental Procedures” for further details.

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 Ca²⁺ 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 Ca²⁺ becomes much less severe when vacuolar Ca²⁺ uptake is reduced.

Ca²⁺ Transport Activity in Cellular Membranes Is Increased in the *pgm2Δ* Strain—Our results indicate that yeast cells normally increase Ca²⁺ uptake and accumulation when shifted to a growth medium containing galactose as the carbon source. Furthermore, Ca²⁺ uptake is increased much more in the *pgm2Δ* strain under these conditions. To gain further insights into where this additional Ca²⁺ is sequestered inside the cell, we next assayed Ca²⁺ transport into isolated intracellular membrane vesicles after their fractionation on a sucrose step gradient as previously described (9, 14, 16, 36). We first examined ATP-dependent Ca²⁺ transport into membranes isolated from wild-type cells grown with galactose as the carbon source. As reported previously, we found ⁴⁵Ca²⁺ 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 ⁴⁵Ca²⁺ transport observed was dependent on the presence of Pmc1p. A *pmc1Δ* strain exhibited a large decrease in total ATP-dependent ⁴⁵Ca²⁺ 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 Ca²⁺ transport activity in yeast membranes.

We next compared ⁴⁵Ca²⁺ 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 ⁴⁵Ca²⁺ transport activity in Fraction 7 (Fig. 7). However, peak ⁴⁵Ca²⁺ 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

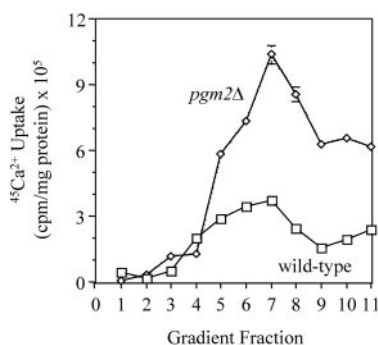


FIG. 7. Increased Ca²⁺ sequestration occurs in membrane vesicles harvested from the *pgm2Δ* strain metabolizing galactose as the carbon source. *In vitro* ⁴⁵Ca²⁺ uptake assay on membrane fractions isolated from wild-type (squares) or *pgm2Δ* (diamonds) cells grown in YMMG medium. The data are presented as the mean ± S.D. See “Experimental Procedures” for further details.

vacuolar Ca²⁺ sequestration is significantly increased in the *pgm2Δ* mutant.

Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ transport, we used the *in vitro* ⁴⁵Ca²⁺ transport assay to test whether Glc-1-P or Glc-6-P can directly stimulate Ca²⁺ 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 ⁴⁵Ca²⁺ 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 ⁴⁵Ca²⁺ transport into these membranes. Instead they decreased ⁴⁵Ca²⁺ transport in a dose-dependent manner. In the presence of 10 mM Glc-6-P, ⁴⁵Ca²⁺ accumulation decreased 33%. Similarly, the presence of 10 mM Glc-1-P decreased ⁴⁵Ca²⁺ accumulation 45%. To determine whether these decreases were specific for Glc-6-P and Glc-1-P, we measured ⁴⁵Ca²⁺ transport activity in the presence of 5 mM fructose 6-phosphate or mannose 6-phosphate. Interestingly, although no significant change was observed on ⁴⁵Ca²⁺ transport in the presence 5 mM fructose 6-phosphate, 5 mM mannose 6-phosphate led to a ~20% increase in ⁴⁵Ca²⁺ uptake that was reproducible in multiple experiments (Fig. 8C). Because previous reports predicted that Ca²⁺ is retained in the yeast vacuole by an interaction with polyphosphate (7), we also assayed ⁴⁵Ca²⁺ transport in the presence 5 mM sodium phosphate. Under these conditions, membranes from the wild-type strain reproducibly exhibited an increase in ⁴⁵Ca²⁺ accumulation of greater than 50% (Fig. 8C).

We also examined the effects of sugar phosphates on ⁴⁵Ca²⁺ 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 ⁴⁵Ca²⁺ accumulation into membranes from the *pgm2Δ* mutant in a dose-dependent manner. The addition of 10 mM Glc-6-P decreased ⁴⁵Ca²⁺ transport 37% (Fig. 8D), whereas the addition of 10 mM Glc-1-P decreased ⁴⁵Ca²⁺ 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 ⁴⁵Ca²⁺ 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 ⁴⁵Ca²⁺ 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 Ca²⁺ transport into the vacuolar compartment.

DISCUSSION

In the current study we found that cellular Ca²⁺ 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 Ca²⁺ uptake by Pmc1p plays a key role in the manifestation of the Ca²⁺ homeostasis defects observed in the *pgm2Δ* mutant. First, we found that the introduction of the *pmc1Δ* mutation reduced total cellular Ca²⁺ 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/pm1Δ* strain closely parallels the levels of Ca²⁺ uptake and accumulation observed in these strains. Further evidence that these Ca²⁺ homeostasis defects are largely attributable to Pmc1p function is provided by experiments in which the genes encoding other intracellular Ca²⁺ transporters are disrupted. Disruption of the vacuolar Ca²⁺/H⁺ exchanger Vcx1p did not significantly alter the Ca²⁺ homeostasis phenotype observed in the *pgm2Δ* mutant. Similarly, disruption of the gene encoding the ER Ca²⁺-ATPase Cod1p was unable to rescue the *pgm2Δ*-related Ca²⁺ 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 Ca²⁺ uptake and reduced ER/Golgi Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ into different intracellular compartments.

The results of *in vitro* Ca²⁺ transport assays carried out with membranes harvested from wild-type and *pgm2Δ* strains also suggest that the bulk of Ca²⁺ transport activity in intracellular membranes is attributable to the vacuolar Ca²⁺-ATPase Pmc1p. This finding is consistent with the previous conclusion that the vacuole contains >95% cellular Ca²⁺ in yeast (7). Other studies have also shown that *pmc1Δ* strains exhibit a reduced tolerance to high levels of extracellular Ca²⁺ and contain reduced amounts of total cellular Ca²⁺ (Refs. 3 and 39 and this study). However, a recent study using a similar Ca²⁺ uptake assay with isolated yeast membranes concluded that the Golgi Ca²⁺-ATPase Pmr1p rather than the vacuolar Pmc1p is the major intracellular Ca²⁺ 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 Ca²⁺ (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 Ca²⁺ 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 Ca²⁺ homeostasis rather than a defect in protein glycosylation. Based on this reasoning we propose the following model to explain how the *pgm2Δ* mutation alters Ca²⁺ 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

FIG. 8. Neither Glc-6-P nor Glc-1-P can directly stimulate Ca²⁺ uptake into membrane vesicles from the wild-type or *pgm2Δ* strains. Fractions showing maximal ⁴⁵Ca²⁺ uptake activity for each strain were assayed for ⁴⁵Ca²⁺ uptake in the presence of the indicated sugar phosphate at the indicated concentration. A–C, ⁴⁵Ca²⁺ uptake into membranes harvested from the wild-type strain. D–F, ⁴⁵Ca²⁺ uptake into membranes harvested from the *pgm2Δ* strain. The data are presented as the mean ± S.D. *G6P*, glucose 6-phosphate; *G1P*, glucose 1-phosphate; *F6P*, fructose 6-phosphate; *M6P*, mannose 6-phosphate. *ctrl.*, control.

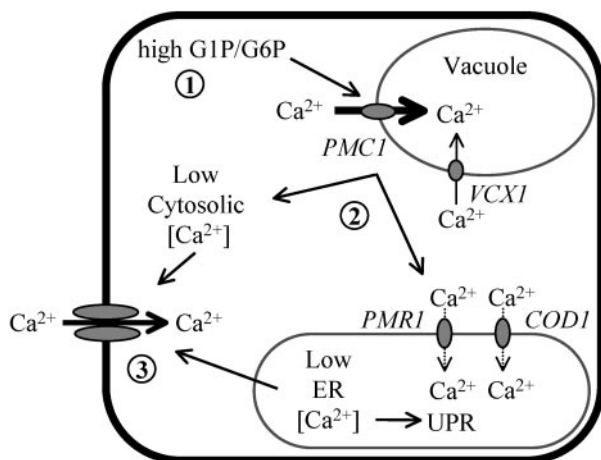
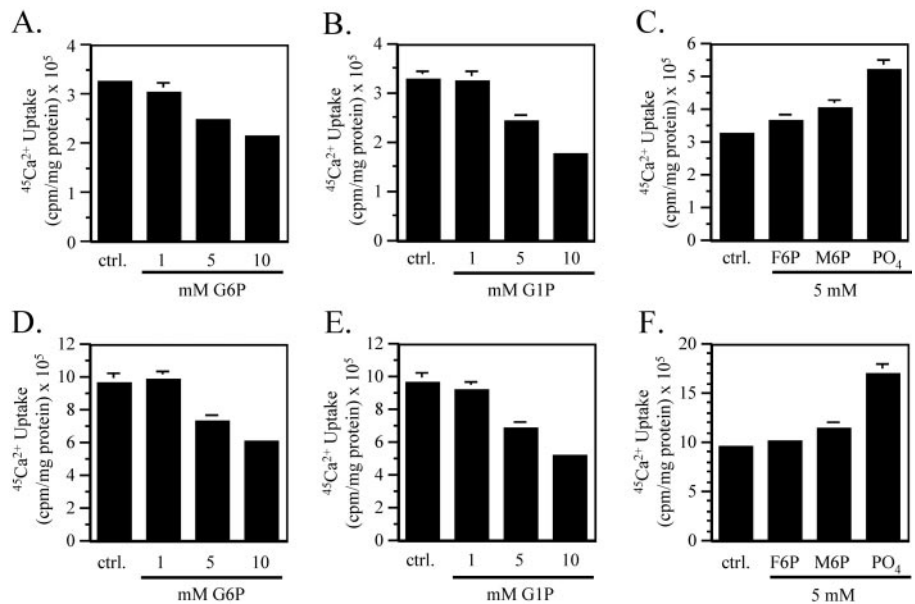


FIG. 9. Model; excessive vacuolar Ca²⁺ uptake in the *pgm2Δ* mutant leads to the depletion of ER Ca²⁺ and induction of the UPR and CCE responses. Step 1, increased ratio of Glc-1-P/Glc-6-P stimulates vacuolar Ca²⁺ sequestration by Pmc1p. Step 2, increased vacuolar Ca²⁺ sequestration results in a decrease in the cytosolic Ca²⁺ level, which leads to Ca²⁺ store depletion in the ER. Step 3, the low cytosolic Ca²⁺ level and decreased luminal Ca²⁺ level in the secretory pathway induces the UPR and activates a signal for Ca²⁺ uptake across the plasma membrane. See “Discussion” for further details. *G1P*, glucose 1-phosphate; *G6P*, glucose 6-phosphate.

Glc-6-P (29) that leads to an increase in Pmc1p activity (this study). The increased rate of vacuolar Ca²⁺ uptake reduces the level of cytosolic Ca²⁺, which in turn causes a depletion of free Ca²⁺ in the ER. This decrease in the level of free Ca²⁺ 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 Ca²⁺ uptake across the plasma membrane by a CCE-like mechanism. Evidence that excessive vacuolar Ca²⁺ uptake can deplete ER Ca²⁺ stores was previously provided by Cunningham and co-workers (21), who found that increased vacuolar Ca²⁺ sequestration could effectively out-compete the secretory pathway Ca²⁺ transporters for free Ca²⁺ in the cytosol. Notably, this led to an induction of a CCE-like response due to the depletion of ER Ca²⁺ stores (21). According to our model, the suppression of these Ca²⁺ homeostasis defects by the *pmc1Δ* mutation occurs by moderating the excessive vacuolar Ca²⁺ sequestration, thus

allowing the Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 *pgm2Δ* strain, since the *MID1/CCH1* gene products were previously shown to be required for a CCE-like mechanism in a *pmr1Δ* strain (21). However, we found that the introduction of a *mid1Δ* mutation into the *pgm2Δ* strain was unable to suppress the Ca²⁺ homeostasis defects associated with the *pgm2Δ* mutation.² Because both the rate of cellular Ca²⁺ uptake and UPR induction are much larger in the *pgm2Δ* mutant than was previously shown to be associated with the *pmr1Δ* mutation (21, 23), it is possible that the more robust CCE response observed in this strain is mediated by either the low affinity Ca²⁺ uptake pathway or a combination of both.

Although we found that cellular Ca²⁺ uptake in the *pgm2Δ* 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 Ca²⁺ uptake. This finding is consistent with our hypothesis that an increased Glc-1-P/Glc-6-P ratio stimulates cellular Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ uptake correlated with a ~1.5-fold increase in *PMCI* mRNA levels in both wild-type and *pgm2Δ* strains (data not shown). The much larger increase in Ca²⁺ uptake observed in the *pgm2Δ* strain suggests that a post-transcriptional mechanism may complement this modest increase in *PMCI* 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 Ca²⁺ signaling mechanisms. Our finding that neither Glc-

² D. Aiello and D. Bedwell, unpublished results.

6-P nor Glc-1-P can enhance Ca²⁺ transport into intracellular membranes *in vitro* suggests that these metabolites do not directly stimulate the activity of a Ca²⁺ transporter such as Pmc1p *in vivo*. However, it remains a formal possibility that a metabolite derived from one of these compounds *in vivo* 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 UTP 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 Ca²⁺ homeostasis like those observed in the *pgm2Δ* strain (28, 29).

If Glc-1-P and Glc-6-P do not stimulate intracellular Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Hxk2p (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 Ca²⁺ 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 Ca²⁺ 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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