

Intracellular Glucose 1-Phosphate and Glucose 6-Phosphate Levels Modulate Ca^{2+} Homeostasis in *Saccharomyces cerevisiae**

Received for publication, August 27, 2002
Published, JBC Papers in Press, September 25, 2002, DOI 10.1074/jbc.M208748200

David P. Aiello‡, Lianwu Fu‡, Attila Miseta§, and David M. Bedwell¶¶

From the ‡Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294-2170 and the §Department of Clinical Chemistry, University Medical School, Pécs 7624, Hungary

The enzyme phosphoglucomutase plays a key role in cellular metabolism by virtue of its ability to interconvert Glc-1-P and Glc-6-P. It was recently shown that a yeast strain lacking the major isoform of phosphoglucomutase (*pgm2Δ*) accumulates a high level of Glc-1-P and exhibits several phenotypes related to altered Ca^{2+} homeostasis when D-galactose is utilized as the carbon source (Fu, L., Miseta, A., Hunton, D., Marchase, R. B., and Bedwell, D. M. (2000) *J. Biol. Chem.* 275, 5431–5440). These phenotypes include increased Ca^{2+} uptake and accumulation and sensitivity to high environmental Ca^{2+} levels. In the present study, we overproduced the enzyme UDP-Glc pyrophosphorylase to test whether the overproduction of a downstream metabolite produced from Glc-1-P can also mediate changes in Ca^{2+} homeostasis. We found that overproduction of UDP-Glc did not cause any alterations in Ca^{2+} uptake or accumulation. We also examined whether Glc-6-P can influence cellular Ca^{2+} homeostasis. A yeast strain lacking the β -subunit of phosphofructokinase (*pfk2Δ*) accumulates a high level of Glc-6-P (Huang, D., Wilson, W. A., and Roach, P. J. (1997) *J. Biol. Chem.* 272, 22495–22501). We found that this increase in Glc-6-P led to a 1.5–2-fold increase in total cellular Ca^{2+} . We also found that the *pgm2Δ/pfk2Δ* strain, which accumulated high levels of both Glc-6-P and Glc-1-P, no longer exhibited the Ca^{2+} -related phenotypes associated with high Glc-1-P levels in the *pgm2Δ* mutant. These results provide strong evidence that cellular Ca^{2+} homeostasis is coupled to the relative levels of Glc-6-P and Glc-1-P in yeast.

Ca^{2+} homeostasis in eukaryotic cells is a complex process involving the regulated internalization and sequestration of Ca^{2+} ions into a variety of intracellular compartments. The basic mechanisms that mammalian and yeast cells employ to carry out this process are similar, such that the cytosolic Ca^{2+} concentration in both cell types is normally maintained in the range of 50–200 nM (1–4).

In mammalian cells, the endoplasmic reticulum (ER)¹ serves

as a storage compartment that provides a readily mobilizable source of Ca^{2+} for use in Ca^{2+} signaling. Upon receiving an appropriate stimulus, ER Ca^{2+} stores can be released to generate a transient increase in the cytosolic Ca^{2+} concentration. This elevated Ca^{2+} level can then activate various signaling pathways in a tightly regulated manner (5). In a variety of non-excitatory cell types, the release of ER Ca^{2+} can lead to the generation of a store depletion signal that results in an influx of Ca^{2+} ions across the plasma membrane in a process termed capacitative Ca^{2+} entry (CCE). CCE amplifies the transient increase in cytosolic Ca^{2+} initiated by the release of ER Ca^{2+} and ultimately serves as a source of cytosolic Ca^{2+} for refilling the depleted ER Ca^{2+} store (6).

Mammalian ER Ca^{2+} stores are maintained by the activity of the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase pumps (7, 8). In addition, past work suggests that glucose metabolites also play a role in sequestering mammalian ER Ca^{2+} stores. Several studies have shown that both the rate and yield of ATP-dependent Ca^{2+} transport in microsomes isolated from a variety of tissues are stimulated by Glc-6-P (9–13). This effect appears to be mediated by a dedicated Glc-6-P transporter located in the ER membrane. The ER-localized Glc-6-P transporter has been cloned and is expressed as two distinct mRNA species (14–16). The longer isoform is highly enriched in heart, brain, and skeletal muscle, whereas the shorter isoform shows a more ubiquitous tissue distribution.

The vacuole, which is the major Ca^{2+} storage compartment in yeast cells, contains >95% of the total cellular Ca^{2+} (17). Until recently, this large store of vacuolar Ca^{2+} was thought to be relatively inert due to its association with polyphosphate. However, a recent study found that vacuolar Ca^{2+} can be released in a regulated manner through the action of Yvc1p, a TRP channel homolog (18). Ca^{2+} release through this channel was shown to be induced by hypotonic shock and may be responsive to the HOG/mitogen-activated protein kinase signaling cascade (19).

The Golgi apparatus has also been shown to play an important role in cellular Ca^{2+} storage in yeast through the action of the Golgi-localized Ca^{2+} -ATPase Pmr1p (3, 20–24). It has been shown that *pmr1Δ* mutants display a constitutively elevated cellular Ca^{2+} uptake phenotype that may be related to the CCE response of mammalian cells (25). This yeast version of the CCE response is mediated through the action of a high affinity Ca^{2+} channel in the plasma membrane containing at least the *MID1* and *CCH1* gene products as subunits (26). The increased Ca^{2+} accumulation that occurs in the *pmr1Δ* strain can stimulate the expression of a large number of genes by the transcription factor Tcn1p/Crz1p following its activation by the calmodulin-calcieneurin signaling pathway (27–30). These results suggest that the yeast Golgi apparatus may normally play a role that is functionally similar to that of mammalian ER because it has the potential to mediate a transient rise in

* This work was supported by Juvenile Diabetes Foundation Grant 99502 and American Heart Association (Southeast Affiliate) Grant 0255121B (to D. M. B.) and Hungarian National Science Foundation Grant OTKA T-038144 (to A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Microbiology, BBRB 432/Box 8, 1530 Third Ave., South, University of Alabama at Birmingham, Birmingham, AL 35294-2170. Tel.: 205-934-6593; Fax: 205-975-5482; E-mail: dbedwell@uab.edu.

¹ The abbreviations used are: ER, endoplasmic reticulum; CCE, capacitative Ca^{2+} entry; PGM, phosphoglucomutase; MES, 4-morpholineethanesulfonic acid.

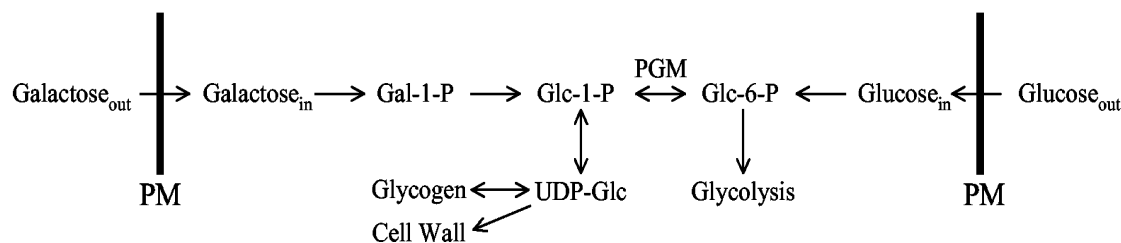


FIG. 1. Summary of glucose and galactose metabolism in yeast. PGM activity interconverts Glc-1-P and Glc-6-P and is required for the utilization of galactose as the carbon source. PM, plasma membrane.

cytosolic Ca^{2+} that can subsequently be amplified by a CCE-like mechanism.

The yeast ER appears to play a lesser role in cellular Ca^{2+} storage because the free Ca^{2+} concentration of this compartment has been reported to be only $\sim 10 \mu\text{M}$ (31). It was recently shown that the *COD1/SPF1* gene product is an ER-localized P-type Ca^{2+} -ATPase that sequesters cytosolic Ca^{2+} into the ER (32–34). A number of studies have also suggested that Pmr1p (20–23, 34) and the vacuolar Ca^{2+} -ATPase Pmc1p (23, 34) also play a role in maintaining ER Ca^{2+} stores under certain conditions. In contrast to mammalian cells, a Glc-6-P transporter capable of stimulating Ca^{2+} sequestration into the ER (or possibly the Golgi apparatus) has not been identified in yeast cells. However, it has been shown that Glc-1-P appears to play a role in yeast Ca^{2+} homeostasis (35).

Saccharomyces cerevisiae contains two genes that encode phosphoglucomutase (PGM), a key metabolic enzyme that interconverts Glc-6-P and Glc-1-P (see Fig. 1). The *PGM2* gene encodes the major isoform that accounts for $\sim 90\%$ of the total PGM activity in galactose-grown cells, whereas the *PGM1* gene product accounts for the remaining 10% (36). When glucose is utilized as the carbon source, a low level of PGM activity is required to maintain enough Glc-1-P for use as substrate in the synthesis of sugar nucleotides such as UDP-Glc. UDP-Glc is needed for anabolic reactions such as glycogen synthesis and cell wall biosynthesis. However, a much higher level of PGM activity is required during growth in medium containing galactose as the carbon source to provide the Glc-6-P used in both the glycolytic and pentose phosphate pathways. Consistent with this need for a greater metabolic flux from Glc-1-P to Glc-6-P, a *pgm2* Δ mutant accumulates a high level of intracellular Glc-1-P when grown in medium containing galactose as the sole carbon source (35). In addition, it was found that this high level of Glc-1-P is accompanied by a high rate of Ca^{2+} uptake and accumulation, suggesting a possible link between glucose metabolites and Ca^{2+} homeostasis in yeast.

The goal of this study was to further examine the hypothesis that the glucose metabolites Glc-1-P and/or Glc-6-P play a role in Ca^{2+} homeostasis in yeast. We show that Ca^{2+} homeostasis defects in the *pgm2* Δ mutant are specific to accumulated Glc-1-P using three strategies. First, we show that *Escherichia coli* PGM expression can rescue galactose-specific growth defects in *pgm2* Δ yeast strains. These data support the hypothesis that the loss of Pgm2p enzymatic activity is directly responsible for the observed alterations in cellular Ca^{2+} homeostasis. Second, we show that increased synthesis of UDP-Glc, a metabolite synthesized directly from Glc-1-P and UTP by the enzyme UDP-Glc pyrophosphorylase, has no effect on Ca^{2+} homeostasis. This result demonstrates that the increased synthesis of Glc-1-P, rather than sugar nucleotides derived from Glc-1-P, influences cellular Ca^{2+} homeostasis. Third, we show that the accumulation of Glc-6-P also has an effect on Ca^{2+} homeostasis that is distinct from Glc-1-P accumulation.

EXPERIMENTAL PROCEDURES

Strains Used—The strains used in this study include Sc252 (SJ21R) (*MATa ade1 leu2,3-112 ura3-52 MEL*) (36), YDB0171 (*MATa ade1 leu2,3-112 ura3-52 MEL pgm2 Δ ::LEU2*) (35), YDB0397 (*MATa ade2-101 his3- Δ 200 leu2,3-112 lys2- Δ 201 ura3-52 pfk2 Δ ::HIS3*), and YDB0395 (*MATa ade2-101 his3- Δ 200 leu2,3-112 ura3-52 pgm2 Δ ::URA3 pfk2 Δ ::HIS3*). Strain Sc252 was kindly provided by J. E. Hopper. The *pfk2* Δ strain YDB0397 was generated by disruption of the *PFK2* gene in YDB0355 (*MATa ade2-101 his3- Δ 200 leu2,3-112 lys2- Δ 201 ura3-52*) by insertion of *HIS3*. To do this, a 2.9-kb fragment containing the *PFK2* gene was amplified by PCR using yeast genomic DNA as template. The forward primer was DB732 (5'-ACG CGT CGA CCA TAC GCA ATG ACT GTT AC), which contains a *SalI* restriction endonuclease site (underlined). The reverse primer was DB733 (5'-GCT CTA GAC CAA ATG GTC AGC AAT GAG), which contains an *XbaI* site (underlined). The PCR product was digested with *SalI* and *XbaI* and subcloned into the same sites within plasmid pBluescript II KS(+) (Stratagene) to generate pDB0657. The 1.3-kb *BglIII/EcoRI* fragment in *PFK2* was removed from pDB0657 and replaced with an *EcoRI/XhoI* fragment from pJJ215 (37) to generate pDB0658. The *SalI/XbaI* fragment containing the disrupted *PFK2* gene was then used to transform strain YDB0355 by standard methods (38). The *pgm2 Δ /pfk2 Δ* strain YDB0395 was constructed by transforming the same *SalI/XbaI* fragment from pDB0658 into YDB0313 (*MATa ade2-101 his3- Δ 200 leu2,3-112 ura3-52 pgm2 Δ ::URA3*). In each strain constructed above, gene knockouts were confirmed by Southern blotting and enzyme assay for either PGM (35) or phosphofructokinase (39) activity.

Culture Media—Bacterial strains used for cloning and plasmid maintenance were grown in standard medium as described (40). Similarly, yeast media were prepared as described (38). Yeast extract/peptone (YP) medium and synthetic medium were supplemented with 2% glucose (dextrose) (YPD or SMD) or 2% galactose (YPGal). YPD and YPGal media were routinely buffered to pH 5.5 with 40 mM MES/Tris. Liquid cultures were grown for a minimum of six to seven generations to $\leq 0.8 A_{600}$ units/ml prior to harvesting unless otherwise indicated.

Plasmids Used—The centromeric plasmid pDB0608 was used to express *E. coli* PGM from the *PGM2* promoter in yeast strains SJ21R and YDB0171. Plasmid pDB0608 was constructed as follows. pDB0197, which contains a 3.3-kb *BamHI/XhoI* fragment with the yeast *PGM2* locus in pBluescript II KS(+), was digested with *HpaI/EcoRI* to remove the entire *PGM2* coding sequence. This sequence was replaced with an *HpaI/EcoRI* fragment from pNK3476 containing the 3'-coding sequence of *E. coli* PGM (41). The 5'-coding sequence was amplified by PCR using DB431 (5'-AAA GTT AAC ATA ACA TGG CAA TCC ACA ATC G) and DB432 (5'-CTG ATC GTT AAC GAT AGT CAG G), both of which contain an *HpaI* cut site (underlined). The resultant PCR product was cut and ligated into the *HpaI* site in the plasmid described above to yield full-length *E. coli* PGM under the control of the yeast *PGM2* promoter in pDB0540. A 3.6-kb *EcoRI/BamHI* fragment containing the entire *E. coli* PGM gene under the control of the yeast *PGM2* promoter was removed from pDB0540 and ligated into the same sites of pSEYC58 (42) to give pDB0608. Finally, the multicopy plasmid pYJM1 (a kind gift from Jean François) was used to overexpress the *UGP1* gene. This plasmid contains the *UGP1* gene on a 4.7-kb *SphI/BamHI* fragment inserted into the same site of plasmid YE

351 (43).

Measurement of Metabolite Levels—Glc-6-P and Glc-1-P levels were measured as described previously (35). Glc-6-P was measured in acid-soluble cell extracts using a coupled enzyme assay with Glc-6-P dehydrogenase (Sigma) (44). Glc-1-P was measured similarly with the addition of PGM (Sigma). UDP-Glc was measured in acid-soluble extracts of mid-log phase cells grown in YPD medium using a coupled enzyme assay with UDP-Glc dehydrogenase (45).

RESULTS

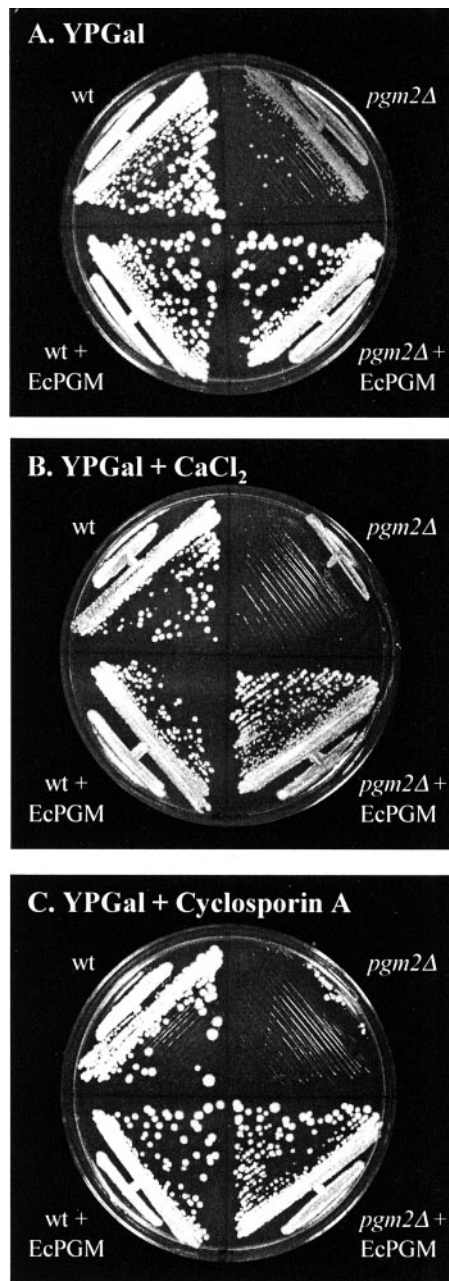


FIG. 2. *E. coli* PGM complements galactose-specific growth defects in the *pgm2Δ* mutant. 300 mM CaCl_2 or 10 $\mu\text{g/ml}$ cyclosporin A was added to YPGal medium as indicated. Plates were incubated for 6 days at 30 °C. *wt*, wild-type strain; *EcPGM*, *E. coli* PGM.

Measurement of Ca^{2+} Uptake and Total Cellular Ca^{2+} — Ca^{2+} uptake measurements were carried out as described previously (22, 35). Cells were harvested at a cell density of $<0.8 A_{600}$ units/ml, washed, and resuspended at a cell density of 1 A_{600} unit/ml in uptake buffer (40 mM MES/Tris (pH 5.5) and 20 mM D-glucose). Cells were incubated in uptake buffer for 10 min at 30 °C, and $^{45}\text{Ca}^{2+}$ uptake was initiated by the addition of $^{45}\text{Ca}^{2+}$ to a final concentration of 1 $\mu\text{Ci/ml}$. At the indicated times, 1-ml aliquots of cells were filtered through a 0.45- μm Gelman GN-6 Metrical filter prewashed with buffer containing 20 mM MgCl_2 and 0.2 mM LaCl_3 . The filters were washed three additional times with the same buffer, and the amount of cell-associated $^{45}\text{Ca}^{2+}$ was determined by liquid scintillation counting. Total cellular Ca^{2+} in unlabeled cells was determined by flame photometry as previously described (3). Briefly, 50–100 A_{600} units of yeast grown in YPD or YPGal medium as indicated were harvested and washed with YP medium. The cell pellets were lyophilized and then resuspended in 1 M HCl. Total Ca^{2+} in this acid-soluble extract was measured by flame photometry and is expressed in mmol of Ca^{2+}/kg of cells (dry mass).

Expression of *E. coli* PGM in the *pgm2Δ* Strain Suppresses Galactose-specific Growth Defects—Previous work has shown that disruption of the *PGM2* gene in *S. cerevisiae* leads to high Ca^{2+} uptake and accumulation when the *pgm2Δ* strain is grown in medium containing galactose as the carbon source (35). This phenotype may be related to an inability to efficiently convert Glc-1-P to Glc-6-P, a step required for cells to utilize galactose as the carbon source (Fig. 1). However, it is also possible that Pgm2p has another cellular function that leads to alterations in Ca^{2+} homeostasis when the gene encoding this protein is deleted. This alternative function could be related to the previous finding that Pgm2p undergoes a post-translational modification in the form of a glucosyl residue attached by a phosphodiester linkage to one or more O-linked mannose residues (46). The extent of the post-translational modification of Pgm2p is regulated by both the carbon source and heat shock; however, it is unclear what role this modification plays in Pgm2p function (47, 48). Because cytosolic proteins are not glycosylated in bacteria and because *E. coli* PGM shares only limited homology (31% sequence identity) with yeast Pgm2p, we asked whether the expression of *E. coli* PGM can suppress the Ca^{2+} homeostasis defects associated with the *pgm2Δ* mutation. As shown in Fig. 2, the *pgm2Δ* strain exhibited several phenotypes, including slow growth in YPGal medium, sensitivity to high extracellular Ca^{2+} levels, and sensitivity to cyclosporin A (an inhibitor of calcineurin function). However, the *pgm2Δ* strain expressing *E. coli* PGM grew normally under each of these conditions. These results suggest that the Ca^{2+} homeostasis defects observed when the *pgm2Δ* mutant was grown in YPGal medium are directly related to the loss of PGM enzymatic activity, and not other putative functions of Pgm2p.

An Elevated Level of UDP-Glc Is Not Responsible for Altered Ca^{2+} Homeostasis in the *pgm2Δ* Mutant—The results described above support the hypothesis that a loss of Pgm2p enzymatic activity and the resultant accumulation of cellular Glc-1-P are responsible for the observed alterations in cellular Ca^{2+} homeostasis. We next asked whether these effects are attributable specifically to the accumulation of Glc-1-P or to a metabolic derivative of this molecule. Other than the formation of Glc-6-P by PGM, the major way to consume Glc-1-P is through the action of the enzyme UDP-Glc pyrophosphorylase, which uses Glc-1-P and UTP to catalyze the formation of UDP-Glc (Fig. 1). If the cellular level of either UDP-Glc or a downstream metabolite was increased in the *pgm2Δ* mutant in medium containing galactose as the carbon source, it could be responsible for the observed Ca^{2+} homeostasis defects in the *pgm2Δ* null mutant, rather than the high level of Glc-1-P. To explore this possibility, we overexpressed UDP-Glc pyrophosphorylase to determine whether an elevated level of UDP-Glc can affect Ca^{2+} homeostasis in yeast.

UDP-Glc pyrophosphorylase is encoded by the *UGP1* gene. It was previously reported that introduction of *UGP1* into a multicopy plasmid leads to an increase in both UDP-Glc pyrophosphorylase activity and the cellular UDP-Glc concentration in glucose-grown cells (43). Consistent with that report, we observed a 10-fold increase in the cellular UDP-Glc concentration in wild-type cells carrying a multicopy plasmid expressing *UGP1* when this strain was grown in YPD medium (Fig. 3A). However, this strain exhibited normal $^{45}\text{Ca}^{2+}$ uptake when grown in YPD medium (Fig. 3B) or YPGal medium (data not shown). These results demonstrate that an increased level of UDP-Glc is not responsible for the observed alterations in Ca^{2+} homeostasis when the *pgm2Δ* mutant is grown with galactose as the carbon source.

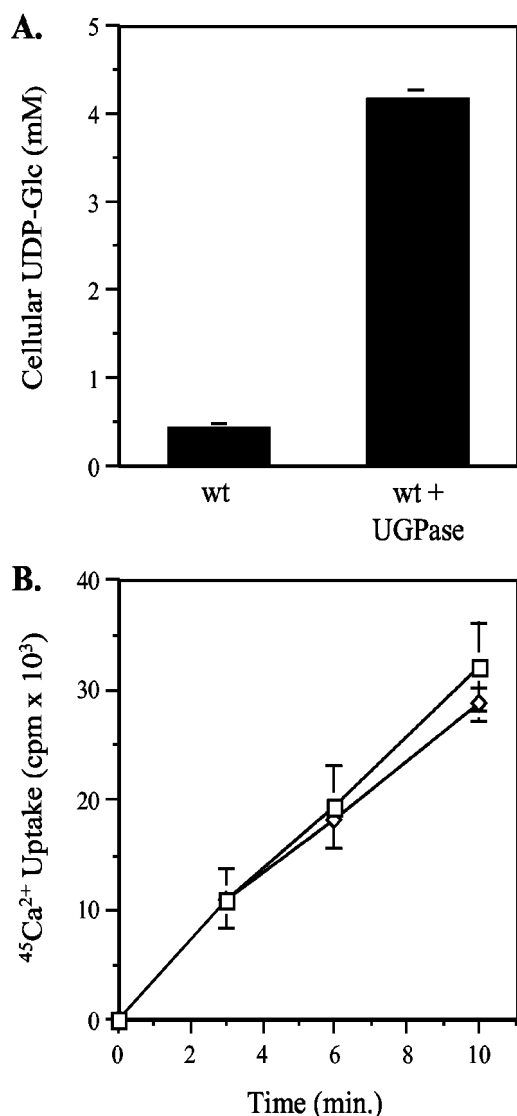


FIG. 3. Increased levels of UDP-Glc do not cause changes in cellular Ca^{2+} homeostasis. **A**, overexpression of UDP-Glc pyrophosphorylase (*UGPase*) leads to a 10-fold increase in the cellular UDP-Glc concentration. Cultures were initially grown to mid-log phase in SMD medium (minus uracil) and then diluted back to $0.05 A_{600}$ units/ml in YPD medium and harvested at $0.6\text{--}0.8 A_{600}$ units/ml. Cell lysates were made and assayed for UDP-Glc as described under "Experimental Procedures." **B**, cells overexpressing UDP-Glc pyrophosphorylase (\diamond) show $^{45}\text{Ca}^{2+}$ uptake rates similar to those of wild-type yeast cells (\square). Cultures were grown as described for **A**, and cells were harvested and assayed for $^{45}\text{Ca}^{2+}$ uptake as described under "Experimental Procedures."

Disruption of PFK2 Increases Cellular Glc-6-P in both Glucose- and Galactose-grown Cells—When grown in medium containing galactose as the carbon source, the *pgm2* Δ mutant accumulates an elevated level of Glc-1-P and exhibits a reduced growth rate, as well as sensitivity to cyclosporin A and high levels of extracellular Ca^{2+} (35). Because the related metabolite Glc-6-P has been shown to influence Ca^{2+} sequestration into the ER of mammalian cells (9–13), we next asked whether an elevated level of Glc-6-P could mediate similar phenotypes in yeast. Previous studies have shown that strains containing a disruption of the *PFK2* gene, which encodes the β -subunit of phosphofructokinase, accumulate a high level of Glc-6-P (49). To determine whether Glc-6-P also plays a role in yeast Ca^{2+} homeostasis, we compared the Ca^{2+} -related phenotypes of the wild-type, *pgm2* Δ , *pfk2* Δ , and *pgm2* Δ /*pfk2* Δ strains.

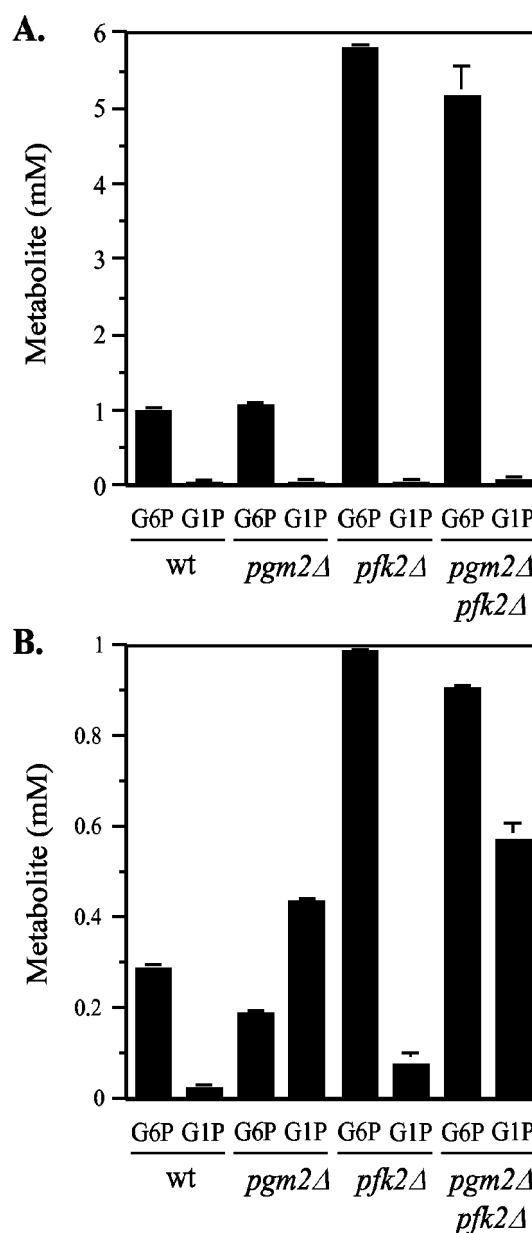


FIG. 4. The *pgm2* Δ and *pfk2* Δ mutations increase Glc-1-P and Glc-6-P levels, respectively. Cells were grown in YPD (**A**) or YPGal (**B**) medium to mid-log phase and then harvested for Glc-6-P (G6P) and Glc-1-P (G1P) measurements as described under "Experimental Procedures." *wt*, wild-type strain.

We first determined the cellular concentrations of Glc-6-P and Glc-1-P in strains carrying these mutations. As previously reported (35), the levels of these two metabolites were similar in the wild-type and *pgm2* Δ strains when grown in YPD medium (Fig. 4A). In contrast, the cellular level of Glc-6-P was increased by 5–6-fold in the *pfk2* Δ and *pgm2* Δ /*pfk2* Δ strains grown in YPD medium. When grown in YPGal medium, the *pgm2* Δ mutant accumulated 10–15-fold more Glc-1-P than the wild-type strain (Fig. 4B), as previously reported (35). The *pfk2* Δ strain accumulated 3-fold more Glc-6-P than the wild-type strain when grown under these conditions, whereas the *pgm2* Δ /*pfk2* Δ strain accumulated high levels of both Glc-6-P and Glc-1-P when grown in YPGal medium. Thus, the double mutant accumulated Glc-1-P only when utilizing galactose as the carbon source, but accumulated Glc-6-P when utilizing either glucose or galactose.

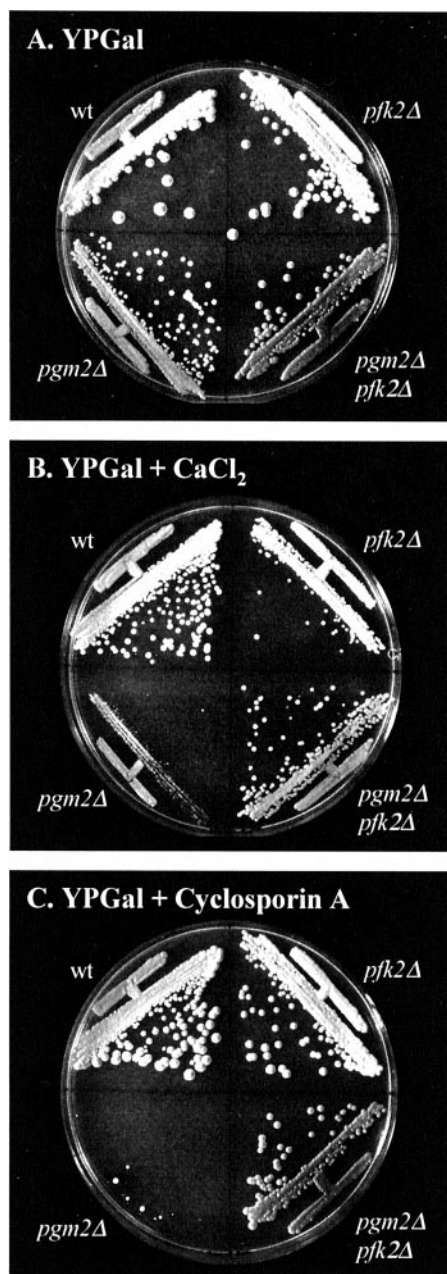


FIG. 5. Deletion of *PFK2* suppresses galactose-specific growth defects in the *pgm2Δ* mutant. 300 mM CaCl_2 or 10 $\mu\text{g/ml}$ cyclosporin A was added to YPGal medium as indicated. Plates were incubated for 6 days at 30 °C. *wt*, wild-type strain.

Loss of *PFK2* Suppresses Galactose-specific Growth Defects Observed in the *pgm2Δ* Strain—We next compared the growth phenotypes of the *pgm2Δ*, *pfk2Δ*, and *pgm2Δ/pfk2Δ* mutants. We found that the *pfk2Δ* mutant exhibited only a slightly reduced growth rate compared with the wild-type strain on both YPD medium (data not shown) and YPGal medium (Fig. 5A). In addition, this strain was able to tolerate high extracellular Ca^{2+} levels (Fig. 5B) or cyclosporin A (Fig. 5C) under these conditions. These results indicate that an increased level of Glc-6-P does not cause the same growth defects seen with the *pgm2Δ* mutant on YPGal plates.

Like the *pfk2Δ* single mutant, we found that the *pgm2Δ/pfk2Δ* strain also showed a slightly reduced growth rate in both YPD and YPGal media. However, it grew better than the *pgm2Δ* single mutant on YPGal plates (Fig. 5A). Surprisingly, combining the *pfk2Δ* and *pgm2Δ* mutations also suppressed the

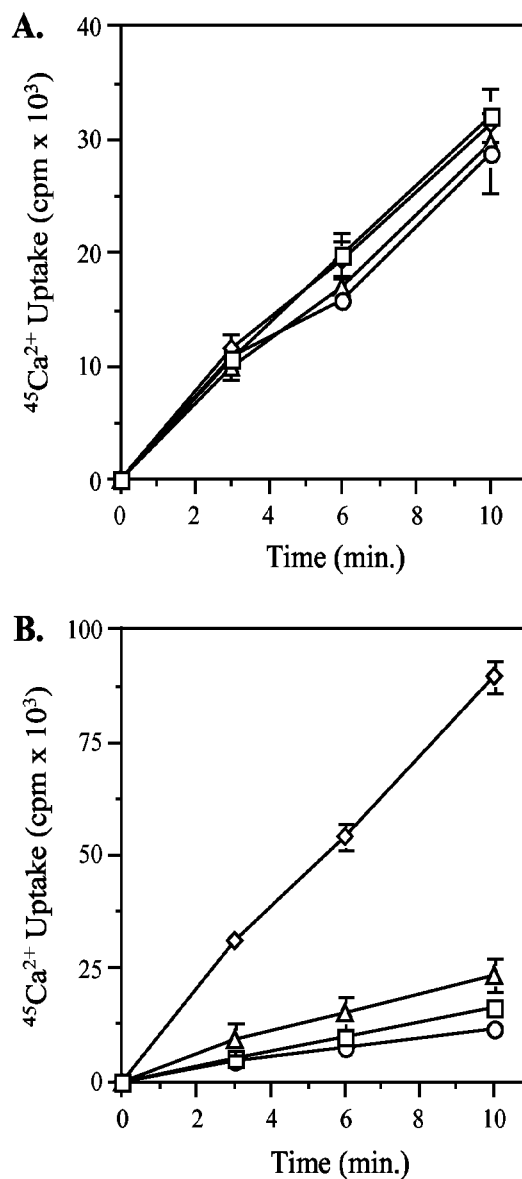


FIG. 6. The *pfk2Δ* mutation suppresses the high Ca^{2+} uptake phenotype associated with the *pgm2Δ* mutant. The wild-type (□), *pgm2Δ* (◇), *pfk2Δ* (○), *pgm2Δ/pfk2Δ* (Δ) strains were grown to mid-log phase in YPD (A) or YPGal (B) medium and harvested for $^{45}\text{Ca}^{2+}$ uptake as described under “Experimental Procedures.”

galactose-specific growth defects associated with the *pgm2Δ* mutation in medium with high extracellular Ca^{2+} levels (Fig. 5B) or cyclosporin A (Fig. 5C). These results indicate that the elevated level of Glc-6-P in the *pgm2Δ/pfk2Δ* strain can suppress the growth defects associated with a high level of Glc-1-P.

Loss of *PFK2* Suppresses the High Ca^{2+} Uptake and Accumulation Phenotypes Observed in the *pgm2Δ* Mutant Grown in Galactose-containing Medium—The data presented above indicate that an elevated level of Glc-6-P can suppress the growth defects observed when a strain carrying the *pgm2Δ* mutation is grown in YPGal medium. To determine whether this is due to an alleviation of the Ca^{2+} homeostasis defects associated with the *pgm2Δ* mutation, we measured $^{45}\text{Ca}^{2+}$ uptake and total cellular Ca^{2+} accumulation in strains carrying various combinations of the *pgm2Δ* and *pfk2Δ* mutations. In glucose-grown cells, $^{45}\text{Ca}^{2+}$ uptake measured in the *pfk2Δ*, *pgm2Δ*, and *pgm2Δ/pfk2Δ* strains was comparable to that in the wild-type strain (Fig. 6A). When the strains were grown in YPGal medium, the *pgm2Δ* strain exhibited a 6-fold higher rate of $^{45}\text{Ca}^{2+}$

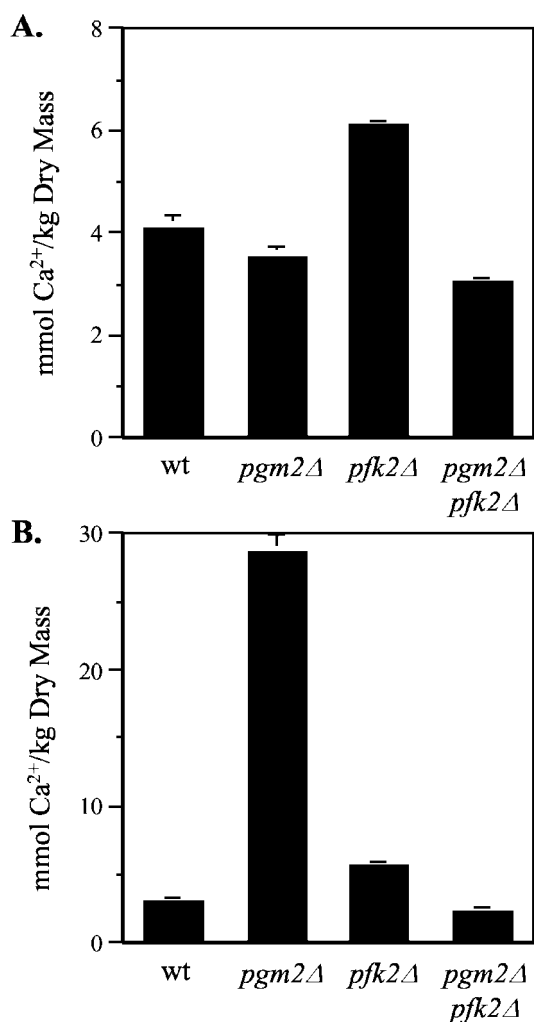


FIG. 7. The *pfk2Δ* mutation suppresses the high Ca²⁺ accumulation phenotype associated with the *pgm2Δ* mutation. The strains were grown in YPD (A) or YPGal (B) medium to mid-log phase and harvested for total cellular Ca²⁺ determination by flame photometry as described under "Experimental Procedures." wt, wild-type strain.

uptake (Fig. 6B), as previously reported (35). In contrast, the *pfk2Δ* single mutant displayed a rate of ⁴⁵Ca²⁺ uptake that was comparable to that of the wild-type strain. Finally, in close agreement with the growth phenotypes shown in Fig. 5, the introduction of the *pfk2Δ* mutation into the *pgm2Δ* background completely suppressed the high ⁴⁵Ca²⁺ uptake phenotype seen in galactose-grown cells.

We next determined total cellular Ca²⁺ levels by flame photometry of extracts from both glucose- and galactose-grown cells. In multiple experiments, we found that the *pfk2Δ* strain accumulated 1.5–2-fold more total cellular Ca²⁺ than the wild-type strain when grown in YPD medium (Fig. 7A). In contrast, the *pgm2Δ* and *pgm2Δ/pfk2Δ* mutants exhibited a slight decrease in total cellular Ca²⁺ when grown under these conditions. When these strains were grown in YPGal medium, we found that the *pgm2Δ* mutant exhibited a 9–10-fold increase in total cellular Ca²⁺ (Fig. 7B), as previously reported (35). The *pfk2Δ* mutant again exhibited a smaller 1.5–2-fold increase in total cellular Ca²⁺. In agreement with its reduced rate of ⁴⁵Ca²⁺ uptake, the excess Ca²⁺ accumulation associated with the *pgm2Δ* mutation was eliminated when the *pgm2Δ/pfk2Δ* strain was grown in YPGal medium. In fact, the level of total cellular Ca²⁺ in the *pgm2Δ/pfk2Δ* strain was slightly lower than that observed in the wild-type strain. These results dem-

onstrate that an increased level of Glc-6-P does not cause the same large increase in Ca²⁺ uptake and accumulation that is observed when the *pgm2Δ* strain is grown in galactose-containing medium. However, a small increase in Ca²⁺ accumulation was observed that was independent of the carbon source. Finally, we found that the accumulation of Glc-6-P associated with the *pfk2Δ* mutation completely suppressed the Ca²⁺ homeostasis defects associated with the accumulation of Glc-1-P in the *pgm2Δ* strain.

DISCUSSION

In this study, we found that cellular Ca²⁺ homeostasis in yeast is responsive to the relative levels of the glucose metabolites Glc-1-P and Glc-6-P. Our results indicate that both the slow growth phenotype and the large increase in Ca²⁺ uptake and accumulation observed when the *pgm2Δ* strain is grown in galactose-containing medium are specific to the increased level of Glc-1-P in the cell, rather than metabolism of the Glc-1-P to UDP-Glc or another downstream metabolite. In addition, the slow growth phenotype of the *pgm2Δ* mutant is not due to ATP limitation in the cell. The most sensitive way to measure the availability of high energy phosphate bonds available for use in ATP-dependent reactions is to determine the cellular energy charge (defined as the sum of the ATP concentration plus one-half of the ADP concentration divided by the sum of the concentrations of ATP, ADP, and AMP in the cell). A previous study found that the cellular energy charge is not reduced in the *pgm2Δ* mutant grown in YPGal medium (35). Consistent with this result, we found that the cellular energy charge was not significantly different from that in the wild-type strain in either the *pfk2Δ* or *pgm2Δ/pfk2Δ* strain when grown in YPGal medium (data not shown). We also observed that the slow growth phenotype of the *pgm2Δ* strain was partially suppressed in the *pgm2Δ/pfk2Δ* strain. Because the combination of these mutations was also shown to normalize Ca²⁺ homeostasis, we conclude that the slow growth of the *pgm2Δ* strain in galactose-containing medium is partially attributable to Ca²⁺ stress, rather than a limitation of metabolic flux from Glc-1-P to Glc-6-P.

The intracellular concentration of Glc-6-P was more than an order of magnitude higher than the Glc-1-P concentration when wild-type cells were grown in either YPD or YPGal medium. The ratio of these two metabolites was unchanged when the *pgm2Δ* strain was grown in YPD medium, and Ca²⁺ homeostasis remained indistinguishable from that in the wild-type strain. However, the ratio of these two metabolites was significantly altered when the *pgm2Δ* mutant was grown in YPGal medium, such that Glc-1-P became ~2-fold more abundant than Glc-6-P. This large change in the relative levels of these metabolites coincided with a drastic change in cellular Ca²⁺ homeostasis. These results suggest that either the increase in the absolute level of Glc-1-P or the accompanying large change in the ratio of Glc-6-P to Glc-1-P is responsible for the observed changes in Ca²⁺ uptake and accumulation. The hypothesis that the altered ratio of Glc-6-P to Glc-1-P is responsible for the changes in Ca²⁺ homeostasis observed in the *pgm2Δ* mutant is strongly supported by our results with the *pgm2Δ/pfk2Δ* strain. This strain, like the *pgm2Δ* strain, contained a high level of Glc-1-P when grown in medium containing galactose as the carbon source. However, the increased level of Glc-6-P in the *pgm2Δ/pfk2Δ* strain again made it the more abundant of these two metabolites. Remarkably, this "normalization" of the relative levels of Glc-6-P and Glc-1-P in the *pgm2Δ/pfk2Δ* strain was accompanied by a return to nearly normal levels of Ca²⁺ uptake and accumulation in galactose-containing medium. This strain also regained the ability to grow in the presence of high extracellular Ca²⁺ levels or cyclosporin A under these

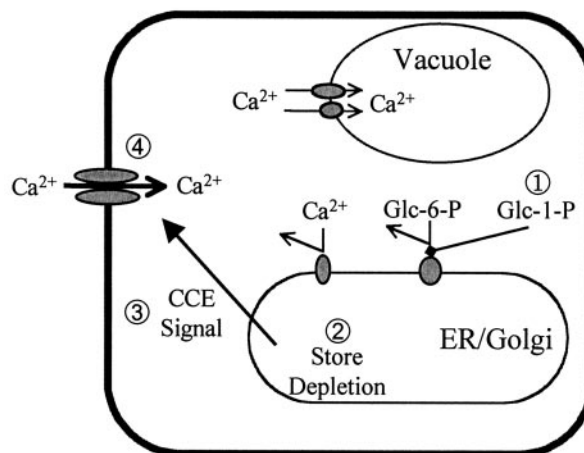
growth conditions. Taken together, these results suggest that the high Ca^{2+} uptake and accumulation observed in the *pgm2Δ* strain are directly attributable to the change in the relative levels of Glc-6-P and Glc-1-P.

It was previously shown that the increased level of Glc-1-P observed when the *pgm2Δ* strain is grown in medium containing galactose as the carbon source correlates with a large increase in Ca^{2+} uptake and accumulation (35). In contrast, the increased level of Glc-6-P observed in the *pfk2Δ* strain was accompanied by a much more subtle 1.5–2-fold increase in the level of total cellular Ca^{2+} . Furthermore, unlike the *pgm2Δ* strain, this change in the cellular Ca^{2+} level was observed when the *pfk2Δ* mutant was grown in medium containing either glucose or galactose as the carbon source. These findings suggest that Glc-6-P also plays a role in yeast Ca^{2+} homeostasis that has not been reported previously. A number of studies have shown that Glc-6-P can stimulate the ATP-dependent sequestration of Ca^{2+} into the ER of several mammalian cell types (9–12). Consistent with these findings, an isoform of the ER-localized Glc-6-P transporter is ubiquitously expressed in mammalian cells. To date, a dedicated Glc-6-P transporter has not been reported in yeast. However, a BLAST search of the GenBankTM/EBI Data Bank using the sequence of the cloned mammalian Glc-6-P transporter indicated that several yeast open reading frames share limited sequence homology with this mammalian protein.² Some of these potential candidates are members of the hexose transporter gene family and have yet to have definitive subcellular locations assigned. Future work should be directed at determining whether any of these proteins possess the ability to transport Glc-6-P into an intracellular compartment in a manner that influences cellular Ca^{2+} homeostasis.

Currently, we do not know the mechanism by which Glc-6-P and Glc-1-P influence Ca^{2+} homeostasis in yeast. However, the results obtained to date are consistent with at least two models. The first model (Fig. 8A) is based on previous studies showing that Glc-6-P can stimulate Ca^{2+} uptake into mammalian microsomes (9–13). By analogy with this mechanism, the excess intracellular Glc-1-P that accumulates in the *pgm2Δ* mutant may act as a competitive inhibitor of Glc-6-P transport, resulting in a reduction of Ca^{2+} sequestration into an intracellular compartment (depicted for illustrative purposes as the ER/Golgi apparatus). If this inhibition of Ca^{2+} transport ultimately leads to Ca^{2+} depletion within the affected intracellular compartment, it may result in an increased rate of cellular Ca^{2+} uptake and accumulation by a CCE-like mechanism. There is evidence that a CCE-like mechanism exists in yeast because it was recently reported that cellular Ca^{2+} uptake and accumulation are increased when Ca^{2+} sequestration into the ER/Golgi apparatus is blocked by a *pmr1Δ* mutation (26). This model could also account for the suppression of Ca^{2+} homeostasis phenotypes in the *pgm2Δ* mutant by the *pfk2Δ* mutation because the elevated level of Glc-6-P caused by the *pfk2Δ* mutation would largely restore the normal Glc-6-P/Glc-1-P ratio and thus relieve the Glc-1-P-mediated inhibition of Glc-6-P transport.

The second model (Fig. 8B) proposes that the high level of Glc-1-P that accumulates in the *pgm2Δ* strain acts to stimulate the transport of Ca^{2+} out of the cytosol. Again using the analogy of Glc-6-P-stimulated Ca^{2+} uptake into mammalian microsomes (9–13), Glc-1-P may stimulate the sequestration of Ca^{2+} into an intracellular compartment such as the vacuole, ER, or Golgi apparatus in yeast. The imbalance in the Glc-6-P/Glc-1-P ratio in the *pgm2Δ* mutant may enhance this sequestration to

A. Model 1: Ca^{2+} Store Depletion



B. Model 2: Decreased Cytosolic Ca^{2+}

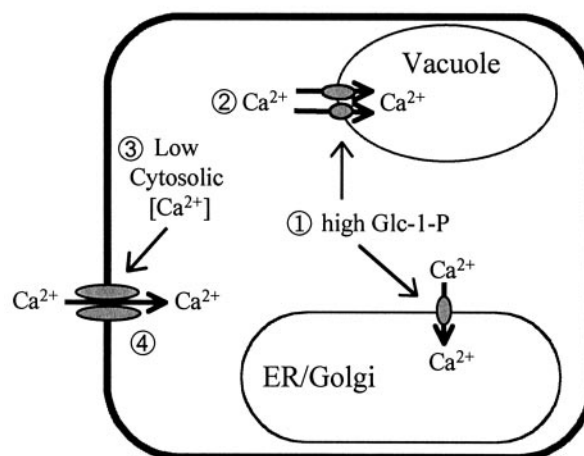


FIG. 8. Two models explaining how the accumulation of Glc-1-P may disrupt cellular Ca^{2+} homeostasis in yeast. A, a Glc-1-P-mediated block in the sequestration of Ca^{2+} into an intracellular compartment induces Ca^{2+} uptake and accumulation. *Step 1*, Glc-1-P competitively inhibits Glc-6-P-mediated Ca^{2+} sequestration into an intracellular compartment (indicated here as ER/Golgi). *Step 2*, the block in compartmental Ca^{2+} uptake leads to store depletion. *Step 3*, store depletion leads to the generation of a CCE-like uptake signal. *Step 4*, Ca^{2+} channels in the plasma membrane are opened to allow the influx of Ca^{2+} in an attempt to refill the depleted internal stores. B, an elevated level of Glc-1-P increases the sequestration of cytosolic Ca^{2+} into an intracellular compartment, leading to induction of Ca^{2+} uptake and accumulation. *Step 1*, Glc-1-P accumulates in the *pgm2Δ* mutant. *Step 2*, increased Glc-1-P levels induce sequestration of intracellular Ca^{2+} . *Step 3*, reduced cytosolic Ca^{2+} levels induce a signal for Ca^{2+} uptake through the plasma membrane. *Step 4*, Ca^{2+} channels in the plasma membrane open to allow the influx of Ca^{2+} to restore a normal cytosolic Ca^{2+} level. See “Discussion” for further details.

the point that the free cytosolic Ca^{2+} level drops below a critical threshold level, thus leading to the induction of a signal to take up more Ca^{2+} from the external environment. Inherent in this model is a mechanism by which the Glc-6-P/Glc-1-P ratio is monitored by an intracellular sensor that couples the relative abundance of these metabolites to Ca^{2+} sequestration. Under normal conditions, an external stimulus such as a change in availability of a carbon source may alter the Glc-6-P/Glc-1-P ratio, causing this sensor to activate a signaling pathway that alters either the activity or expression of intracellular Ca^{2+} transporters. In this scenario, the high Glc-1-P levels present

² D. P. Aiello, L. Fu, A. Miseta, and D. M. Bedwell, unpublished data.

in the *pgm2Δ* mutant would inappropriately (and constitutively) activate this Glc-6-P/Glc-1-P sensor. This would lead to a much greater increase in cytosolic Ca^{2+} sequestration than normally occurs, leading to the induction of Ca^{2+} uptake from the extracellular environment. Such a model is supported by the observation that the altered Glc-6-P/Glc-1-P ratio is the key factor controlling Ca^{2+} homeostasis in the *pgm2Δ* mutant, and not the high absolute level of Glc-1-P.

Several mechanisms could be used to monitor the Glc-6-P/Glc-1-P ratio in the second model. Snf3p is known to be a high affinity glucose receptor located at the plasma membrane and has been shown to regulate the expression of a variety of downstream target genes via Mth1p and Std1p (50–52). Snf3p has a large C-terminal domain that is thought to transduce a signal indicating the presence of extracellular glucose, but the detailed mechanism by which this occurs is not clear (53, 54). It was recently reported that Snf3p can signal via the C-terminal domain in the absence of extracellular glucose, and it was hypothesized that Snf3p may be responsive to intracellular glucose metabolites such as Glc-6-P (55). Inappropriate signaling via Snf3p, which might occur when excess Glc-1-P is present, could lead to the increased sequestration of cytosolic Ca^{2+} . It is not currently known whether Mth1p or Std1p regulates any intracellular Ca^{2+} transporters. However, the global changes in gene expression in response to glucose derepression make this an intriguing possibility. A second related possibility is inappropriate signaling via the Ras-cAMP pathway. Import and phosphorylation of glucose to Glc-6-P can induce the Ras-cAMP signaling cascade (56). A clear role for Gpr1p and Gpa2p has been established in both sensing extracellular glucose and transmitting this signal downstream via the Ras-cAMP pathway (56–58). Furthermore, Gpr1p and Gpa2p have been reported to be required for the Ca^{2+} influx that occurs in response to glucose addition to nutrient-starved cells (2, 59, 60). The altered level of Glc-1-P in the *pgm2Δ* mutant could upset the normal regulatory mechanisms that control this pathway and thus lead to the inadvertent increased activity or expression of transporters that sequester intracellular Ca^{2+} .

The models presented above present several intriguing avenues for further study. Determining an intracellular compartment that has altered Ca^{2+} levels and sequestration properties in the *pgm2Δ* mutant will represent an important step in elucidating the mechanisms that couple Ca^{2+} homeostasis and glucose metabolism. Ultimately, gaining a better understanding of these processes could lead to advances in the treatment of various diseases.

Acknowledgments—We thank Drs. Jean François and Nancy Kleckner for providing strains and reagents and Dr. Richard Kellermayer for helpful discussions.

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