

# Hexose phosphorylation and the putative calcium channel component Mid1p are required for the hexose-induced transient elevation of cytosolic calcium response in *Saccharomyces cerevisiae*

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## Summary

*Saccharomyces cerevisiae* responds to environmental stimuli such as an exposure to pheromone or to hexoses after carbon source limitation with a transient elevation of cytosolic calcium (TECC) response. In this study, we examined whether hexose transport and phosphorylation are necessary for the TECC response. We found that a mutant strain lacking most of the known hexose transporters was unable to carry out the TECC response when exposed to glucose. A mutant strain that lacked the ability to phosphorylate glucose was unable to respond to glucose addition, but displayed a normal TECC response after the addition of galactose. These results indicate that hexose uptake and phosphorylation are required to trigger the hexose-induced TECC response. We also found that the TECC response was significantly smaller than normal when the level of environmental calcium was reduced, and was abolished in a *mid1* mutant that lacked a subunit of the high-affinity calcium channel of the yeast plasma membrane. These results indicate that most or all of the TECC response is mediated by an influx of calcium from the extracellular space. Our results indicate that this transient increase in plasma membrane calcium permeability may be linked to the accumulation of Glc-1-P (or a related glucose metabolite) in yeast.

## Introduction

A variety of hexose transporters of different affinity and specificity facilitate the uptake of glucose, galactose and other hexoses in *Saccharomyces cerevisiae* (Boles and Hollenberg, 1997; Ozcan and Johnston, 1999; Wieczorke *et al.*, 1999). After the entry of glucose into the cytosol, its sixth carbon is phosphorylated by either of two hexokinases or a glucokinase (Fraenkel, 1986; Walsh *et al.*, 1991; Clifton *et al.*, 1993). In contrast, the first carbon unit of galactose is phosphorylated by the unique galactokinase of yeast (Meyer *et al.*, 1991). The *GAL1* (galactokinase) and *GAL2* (galactose transporter) genes are effectively repressed by glucose, and metabolic adaptation is necessary before *S. cerevisiae* can use galactose as a carbon and energy source (Johnston, 1999).

The phosphorylation of hexoses also induces a number of transcriptional, translational and post-translational alterations within the cell (DeRisi *et al.*, 1997; Gancedo, 1998). Even brief exposure to glucose leads to cell cycle-related changes (Granot and Snyder, 1991). The regulation of gene expression by glucose is carried out by multiple mechanisms. One pathway involves the Mig1p transcriptional repressor, a likely target of the Snf1p protein kinase, and its regulators (Ostling *et al.*, 1996; Carlson, 1998; Hardie, 1999). However, the glucose signal that regulates Snf1p function is not known. Snf1p is related to mammalian AMP-activated protein kinases, and its activity may be regulated by AMP levels (Woods *et al.*, 1994; Johnston, 1999). Other proposed signalling mechanisms include glucose levels (Ozcan *et al.*, 1998), glucose transport (Reifenberger *et al.*, 1997) and the nucleocytosolic localization and glucose-regulated phosphorylation of hexokinase PII (Randez-Gil *et al.*, 1998a,b).

The addition of glucose to cells has also been shown to stimulate calcium uptake through the plasma membrane (Eilam and Othman, 1990; Eilam *et al.*, 1990), resulting in a transient elevation of the cytosolic calcium level (Nakajima Shimada *et al.*, 1991; Fu *et al.*, 2000). However, Granot and Snyder (1993) suggested that the glucose-induced physiological changes did not result from the entry of extracellular calcium. Since that time, a possible role for the calcium-calmodulin/calcineurin signal

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transduction pathway in glucose signalling has not been seriously explored.

Basal cytosolic calcium (50–200 nM) and total cellular calcium levels (1.5–4 mM) appear to be similar in *S. cerevisiae* and in nucleated mammalian cells (Nakajima Shimada *et al.*, 1991; Halachmi and Eilam, 1993; Batiza *et al.*, 1996; Miseta *et al.*, 1999a,b). In yeast, excess calcium is removed from the cytosol by the vacuolar H<sup>+</sup>/Ca<sup>2+</sup> antiporter Vcx1p and Ca<sup>2+</sup>-ATPase Pmc1p, as well as the Golgi-located Ca<sup>2+</sup>-ATPase Pmr1p (Rudolph *et al.*, 1989; Cunningham and Fink, 1994; Cunningham and Fink, 1996; Sorin *et al.*, 1997; Marchi *et al.*, 1999; Miseta *et al.*, 1999b; Wei *et al.*, 1999). The calcium-calmodulin/calcineurin signal transduction pathway stimulates the activities of both Pmc1p and Pmr1p, whereas it represses the activity of Vcx1p (Cunningham and Fink, 1996; Pozos *et al.*, 1996; Miseta *et al.*, 1999a).

The glucose-induced TECC response was first described by Nakajima Shimada *et al.* (1991) in *S. cerevisiae*. In the TECC response, the exposure of glucose-starved cells to glucose was found to mediate a transient increase in the cytosolic calcium level. Unlike the immediate rise in cytosolic calcium caused by either a sharp increase in extracellular calcium level or an osmotic shock, the glucose-induced TECC response occurs after a short delay, and the normal cytosolic calcium level is re-established within a few minutes. However, the events that occurred between the initial exposure to the hexose and the resulting cytosolic calcium response were not defined.

In the present work, we used an aequorin reporter system to examine whether glucose and galactose sensing, transport and phosphorylation are required to induce the TECC response in *S. cerevisiae*. We also found that the extracellular environment is responsible for the hexose-induced TECC response. Our results indicate that both hexose transport and phosphorylation are required for the TECC response, which is mediated predominantly (if not exclusively) by the uptake of extracellular calcium.

## Results

### *The cytosolic calcium concentration increases in wild-type S. cerevisiae cells after the addition of glucose or galactose*

We initially examined the TECC response in wild-type cells expressing the apoaequorin gene. Cultures were grown in synthetic medium (SM) containing 2% glucose or 2% galactose and harvested in the exponential growth phase (OD<sub>600</sub> ≈ 0.8). Cells were harvested, loaded with the apoaequorin cofactor coelenterazine and incubated in test medium (TM) without carbon source in the absence or presence of 10 mM CaCl<sub>2</sub> at 30°C for 3 h. An aliquot of

cells was transferred to a cuvette, and light emission (which is dependent upon the free cytosolic calcium level) was monitored (for further details, see *Experimental procedures*).

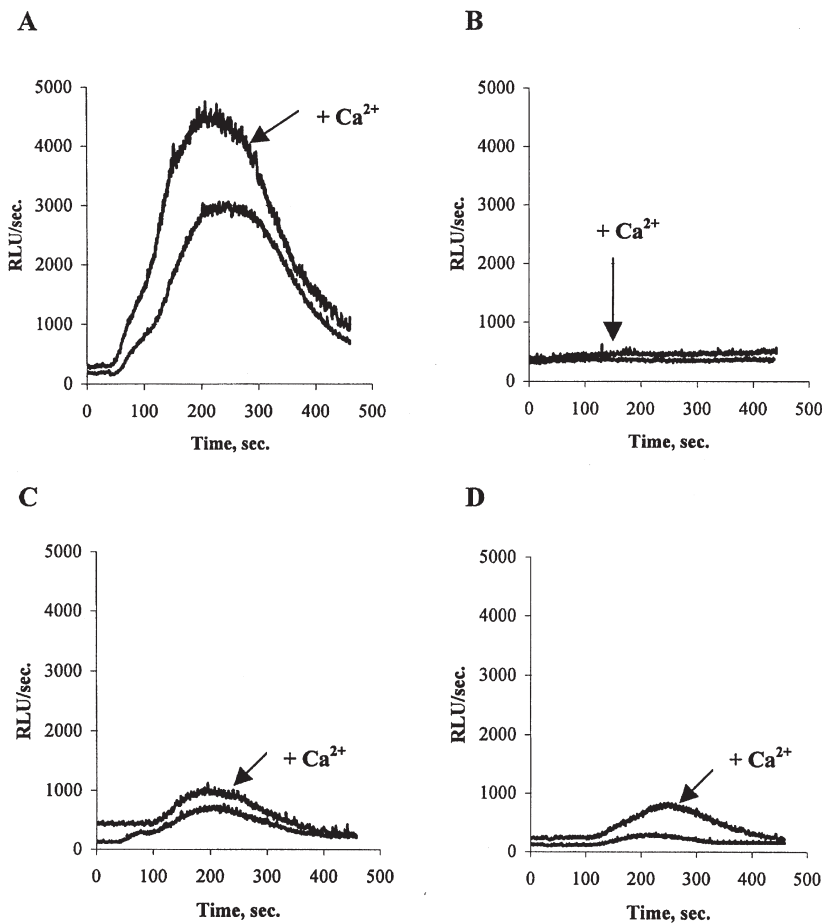
After measuring the basal light emission of cells starved for a carbon source, 100 mM glucose was injected into the cuvette (Fig. 1A). The cytosolic calcium level of glucose-grown cells started to increase about 40 s after the injection of glucose and peaked about 3 min later. The resting ≈ 55 nM calcium level increased to about 370 nM when the cells were starved in TM containing 10 mM CaCl<sub>2</sub>. A significantly smaller TECC response was observed when the glucose-grown cells were starved under low-calcium conditions in TM. In contrast to the response observed when 100 mM glucose was injected into the cuvette, no TECC response was observed when 100 mM galactose was added to glucose-grown cells (Fig. 1B).

In contrast to glucose-grown cells, cells grown with galactose as carbon source responded similarly to the additions of 100 mM glucose or 100 mM galactose, with peak levels of 200 nM and 185 nM respectively (Fig. 1C and D). Notably, the maximum levels of cytosolic calcium observed were significantly smaller than in glucose-grown cells, but the peak cytosolic calcium level was higher when cells were starved in test medium supplemented with 10 mM CaCl<sub>2</sub>.

Our results indicate that galactose does not produce an increase in the cytosolic calcium level in glucose-grown cells. As the transcriptional activation of the genes encoding both the galactose transporter (*GAL2*) and galactokinase (*GAL1*) takes several hours, our results suggest that the uptake and phosphorylation of galactose is needed to trigger the elevation of cytosolic calcium levels. In contrast, a subset of the genes involved in glucose transport and phosphorylation is expressed constitutively, which allows cells grown under diverse carbon sources to respond to glucose with an increase in the cytosolic calcium level.

### *Glucose-6-phosphate and glucose-1-phosphate levels increase shortly before the TECC response*

The above results show that an increase in cytosolic calcium begins 40–60 s after the readdition of glucose or galactose to wild-type cells. This observation suggests that the entry and phosphorylation of both hexoses may be required for the TECC response. To test this hypothesis, we grew wild-type yeast in SM plus 2% glucose or 2% galactose, then starved the cells for 3 h as described above. At the indicated times immediately before or after the addition of glucose or galactose, cells were transferred to a tube containing ice-cold perchloric acid (PCA), and extracts were prepared for the determination of



**Fig. 1.** The TECC response induced by the introduction of glucose or galactose to hexose-starved yeast cells. A wild-type strain from *S. cerevisiae* was grown in SM containing 2% glucose (A and B) or 2% galactose (C and D). Cells were loaded with coelenterazine and starved in TM ( $\pm 10$  mM  $\text{CaCl}_2$ ) for 3 h. The baseline luminescence was measured for 40 s, and 100 mM glucose (A and C) or 100 mM galactose (B and D) was added to the medium.

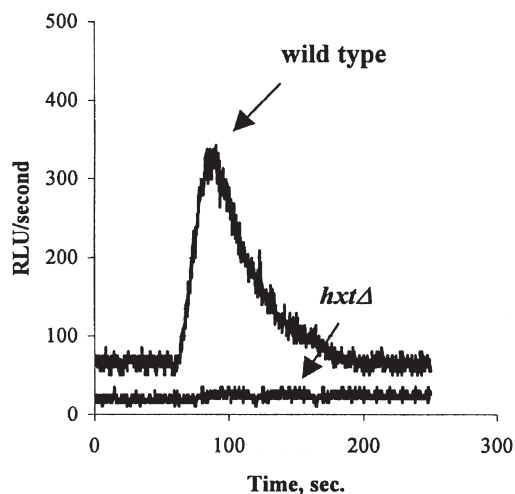
glucose-6-phosphate (Glc-6-P) and glucose-1-phosphate (Glc-1-P) concentrations.

Our results indicated that starved cells contain less than 0.15 mM Glc-6-P or Glc-1-P (Table 1). After the addition of 100 mM glucose or galactose to the starved cells, the hexose phosphate levels were found in the low millimolar range (a 20- to 40-fold increase). The highest increase was observed in glucose-grown cells after the addition of 100 mM glucose. An increase in the Glc-6-P or Glc-1-P levels was not observed when galactose was added to glucose-grown cells. However, an increase in the levels

of these metabolites was observed in galactose-grown cells after the addition of 100 mM glucose or galactose. The elevation in Glc-6-P and Glc-1-P levels comes before the TECC phenomena. Significant elevations in Glc-6-P and Glc-1-P were observed after 30 s incubation. The highest Glc-6-P and Glc-1-P levels were measured 1 min after the administration of glucose or galactose, and these levels slowly decreased by 1.5- to twofold over the next several minutes. The 10 min values were not significantly different from those measured after incubation for 20 or 30 min (data not shown). The fact that the concentrations

**Table 1.** Glucose-6-phosphate and glucose-1-phosphate levels in glucose- or galactose-grown *S. cerevisiae* cells after the readditions of 100 mM glucose or galactose.

Grown in Addition	2% glucose 100 mM glucose		2% glucose 100 mM galactose		2% galactose 100 mM glucose		2% galactose 100 mM galactose	
	G6P (mM)	G1P (mM)	G6P (mM)	G1P (mM)	G6P (mM)	G1P (mM)	G6P (mM)	G1P (mM)
0	0.06 $\pm$ 0.03	0.05 $\pm$ 0.03	0.04 $\pm$ 0.03	0.02 $\pm$ 0.01	0.12 $\pm$ 0.03	0.04 $\pm$ 0.02	0.11 $\pm$ 0.06	0.07 $\pm$ 0.04
0.5	3.42 $\pm$ 0.25	1.04 $\pm$ 0.20	0.03 $\pm$ 0.01	0.03 $\pm$ 0.02	2.27 $\pm$ 0.41	0.48 $\pm$ 0.14	2.36 $\pm$ 0.23	0.64 $\pm$ 0.12
1	5.97 $\pm$ 0.37	2.23 $\pm$ 0.30	0.04 $\pm$ 0.02	0.01 $\pm$ 0.01	3.92 $\pm$ 0.22	1.39 $\pm$ 0.31	3.66 $\pm$ 0.34	0.96 $\pm$ 0.10
2	3.97 $\pm$ 0.21	2.15 $\pm$ 0.28	0.00 $\pm$ 0.02	0.04 $\pm$ 0.02	2.90 $\pm$ 0.33	1.05 $\pm$ 0.27	3.34 $\pm$ 0.19	1.04 $\pm$ 0.13
4	2.84 $\pm$ 0.20	1.75 $\pm$ 0.33	0.04 $\pm$ 0.02	0.09 $\pm$ 0.06	2.59 $\pm$ 0.41	1.14 $\pm$ 0.14	2.77 $\pm$ 0.29	1.12 $\pm$ 0.08
10	2.39 $\pm$ 0.38	1.75 $\pm$ 0.25	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	2.62 $\pm$ 0.28	1.08 $\pm$ 0.23	1.67 $\pm$ 0.14	0.96 $\pm$ 0.17



**Fig. 2.** The TECC response in a wild-type strain and a hexose transporter-deficient strain. Wild-type and hexose transporter mutant strains were grown in SM containing 3% DL-lactate. Coelenterazine-loaded cells were starved in TM supplemented with 10 mM  $\text{CaCl}_2$  for 3 h. The baseline luminescence was measured for 40 s, and then 100 mM glucose was added to the medium.

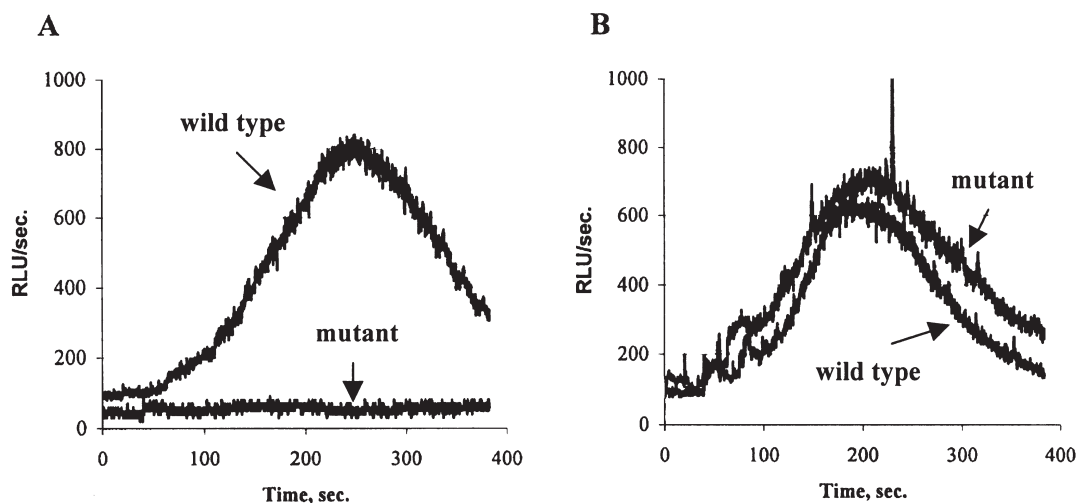
of Glc-6-P and Glc-1-P peak just before the TECC response suggests that these (or related) glucose metabolites may play a role in triggering this transient calcium increase.

*The TECC response is eliminated when hexose transport or glucose phosphorylation is blocked*

To examine further whether the entry and phosphorylation of hexoses are required to trigger the TECC response, we studied a mutant strain lacking the genes encoding the hexose transporter family (*HXT1-17*, *GAL2*) and

the maltose transporter family (*AGT11*, *YDL247W* and *YJR160C*). As the hexose transporter mutant cannot use glucose or galactose as a carbon source, mutant and control strains expressing the apoaequorin gene were maintained in SM containing lactate as carbon source. They were then incubated in TM lacking a carbon source (plus 10 mM  $\text{CaCl}_2$ ) for 3 h and tested for the TECC response. The addition of 100 mM glucose to wild-type cells grown under these conditions elicited an increase in cytosolic calcium as before (Fig. 2), although the peak cytosolic calcium level of 145 nM was smaller than that observed in cells grown with either glucose or galactose as carbon source. In contrast, the hexose transporter mutant cell was unable to elicit any glucose-mediated elevation in its cytosolic calcium concentration. These results indicate that glucose transport is necessary for the TECC response to occur.

We next examined the TECC response in a strain that is completely deficient in glucose phosphorylation. This strain lacks both isoforms of hexokinase (encoded by *HXK1* and *HXK2*) as well as the single glucokinase (encoded by *GLK1*). This strain is unable to grow on glucose as carbon source, but can use galactose. It has been shown that this strain can readily take up glucose, but cannot carry out its phosphorylation (Smits *et al.*, 1996). The glucose phosphorylation mutant strain and the wild-type control were grown in SM containing galactose as carbon source and then incubated in test media for 3 h without carbon source as described before. In the glucose phosphorylation mutant, the cytosolic calcium level remained unchanged after the addition of 100 mM glucose (Fig. 3). Normal TECC response was observed after the addition of 100 mM galactose. These results are consistent with the hypothesis that glucose transport and



**Fig. 3.** Cytosolic calcium levels in wild-type and hexose phosphorylation mutant strains. Wild-type and hexose phosphorylation null mutant (mutant) strains were grown in SM containing 2% galactose. Coelenterazine-loaded cells were starved in 10 mM  $\text{CaCl}_2$ -complemented TM for 3 h. The baseline luminescence was measured for 40 s, and 100 mM glucose (A) or galactose (B) was injected into the medium.

phosphorylation are required for the transient elevation of cytosolic calcium levels.

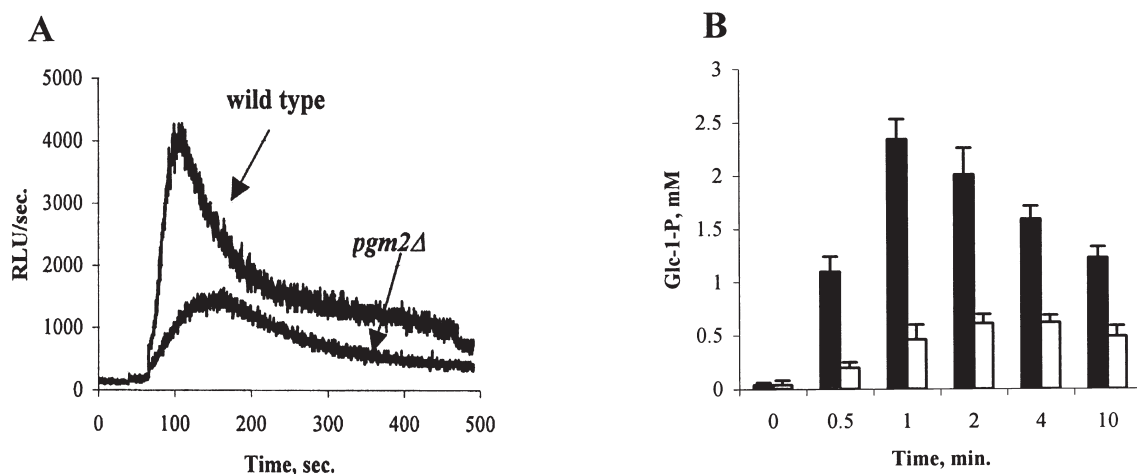
*The TECC response is reduced in a strain lacking the major isoform of phosphoglucomutase*

Recently, we reported that a mutant lacking the major isoform of phosphoglucomutase (encoded by *PGM2*) exhibited an increased rate of calcium uptake and accumulation (Fu *et al.*, 2000). The reduced phosphoglucomutase activity led to an eightfold increase in Glc-1-P in galactose-grown cells, but the Glc-1-P level was normal in glucose-grown cells. Strikingly, these differences in Glc-1-P level observed as a function of carbon source correlated well with the increased calcium uptake and accumulation measured in cells grown with galactose, but not with glucose, as carbon source. When the *pgm2Δ* mutant strain was grown in media containing galactose as carbon source, the TECC response elicited by either glucose or galactose was eliminated. This inability to increase cytosolic calcium was thought to result from the constitutive activation of intracellular calcium transporters by a calcineurin-mediated mechanism. Based upon the results of that study, it was suggested that the increased calcium uptake and accumulation observed in the *pgm2Δ* strain may be related to an elevated Glc-1-P level observed when it is grown in media containing galactose as carbon source.

Based on these results, it is possible that a metabolite produced by the Leloir pathway (such as Glc-1-P) may play an important role in regulating the calcium permeability of the plasma membrane. If this speculation were correct, the transient elevation of cytosolic calcium levels

seen after the addition of hexoses may be regulated by the same mechanism. Such a mechanism could be possible because the *PGM2* gene has a much higher basal level of activity in glucose-grown cells than the other enzymes of the Leloir pathway and contributes roughly half the total cellular phosphoglucomutase activity under those conditions (Fu *et al.*, 1995). To test this possibility, we next examined whether the addition of glucose to the *pgm2Δ* strain grown in SM glucose exhibited an altered TECC response. The *pgm2Δ* mutant and the isogenic wild-type strains were grown in SM 2% glucose and starved for carbon source. When 100 mM glucose was reintroduced, we found that the *pgm2Δ* mutant displayed a significantly smaller TECC response (peak cytosolic calcium level 228 nM) than the wild-type strain (peak cytosolic calcium level 350 nM) (Fig. 4A). Results of Glc-1-P measurement show that the *pgm2Δ* strain contains significantly lower levels of Glc-1-P compared with wild type (Fig. 4B). Accordingly, the maximum Glc-1-P level in the *pgm2Δ* strain is about four times less compared with wild type. In addition, the maximum Glc-1-P level was reached at 2 min incubation in the *pgm2Δ* mutant instead of 1 min, as is the case in wild type. Glc-6-P levels were not significantly different in the *pgm2Δ* mutant and wild-type strains (for wild-type Glc-6-P data, see Table 1).

These results indicate that the ability to convert Glc-6-P to Glc-1-P efficiently is necessary for a maximal TECC response. As the synthesis of the enzymes that carry out the subsequent steps of the Leloir pathway are tightly repressed in glucose-containing media, it is likely that Glc-1-P (or a derivative such as a sugar nucleotide) is responsible for this effect.

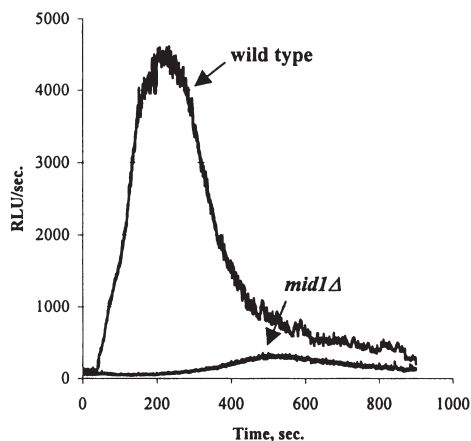


**Fig. 4.** A. Cytosolic calcium levels in wild-type and a phosphoglucomutase mutant (*pgm2Δ*) strains. Wild-type and *pgm2Δ* mutant strains were grown in SM containing 2% glucose. Coelenterazine-loaded cells were starved in 10 mM  $\text{CaCl}_2$ -complemented TM for 3 h. The baseline luminescence was measured for 40 s, and 100 mM glucose was injected into the medium. B. Corresponding Glc-1-P levels in both wild-type (filled columns) and the *pgm2Δ* (empty columns) strains.

### External calcium that enters through the high-affinity calcium channel mediates the TECC response

In the experiments described above, we found that the magnitude of glucose- and galactose-induced TECC responses was significantly larger when the test medium was supplemented with 10 mM  $\text{CaCl}_2$ . However, we still observed modest TECC responses in the absence of this extra calcium. As the TM is based on SM, the total calcium concentration is 1 mM. However, the 2 mM EGTA added to the test medium reduced the calculated free calcium level to  $\approx 34 \mu\text{M}$ . Although much lower than standard growth medium, this calcium concentration is still  $\approx 500$ -fold higher than the resting cytosolic calcium level of *S. cerevisiae*. Both low-affinity and high-affinity calcium uptake systems exist in the yeast plasma membrane (Locke *et al.*, 2000). The low-affinity calcium uptake system exhibits an apparent  $K_m$  of  $\approx 500 \mu\text{M}$ , whereas the high-affinity uptake system has an apparent  $K_m$  of  $\approx 10 \mu\text{M}$ . Given these values, the high-affinity channel would be required if external calcium mediated the TECC response in our test medium.

To test this hypothesis, we constructed a mutant strain lacking Mid1p, which associates with Cch1p, a homologue of the mammalian high-affinity voltage-gated calcium channel component (Iida *et al.*, 1994; Paidhungat and Garrett, 1997; Locke *et al.*, 2000). Wild-type and *mid1* $\Delta$  mutant cells expressing the apoaequorin gene from a plasmid were grown in SM plus 2% glucose medium and tested as described above. After the starvation period, we found that the addition of 100 mM glucose induced a normal TECC response in the wild-type strain, but did not elicit a TECC response in the *mid1* $\Delta$  mutant (Fig. 5). Glc-6-P and Glc-1-P levels appeared to be similar



**Fig. 5.** Cytosolic calcium levels in wild-type and *Mid1p*-deficient *S. cerevisiae* strains. Wild-type and *mid1* $\Delta$  mutant strains were grown in SM containing 2% glucose. Coelenterazine-loaded cells were starved in 10 mM  $\text{CaCl}_2$ -complemented TM for 3 h. The baseline luminescence was measured for 40 s, and 100 mM glucose was injected into the medium.

in wild-type and the *mid1* $\Delta$  mutant strains (data not shown), indicating that the lack of Mid1p did not interfere with glucose uptake and phosphorylation. We did observe a small, delayed elevation of the cytosolic calcium level in this mutant about 2 min after the addition of 100 mM glucose. We found that this small, delayed increase was dependent upon external calcium. However, it was not sensitive to the addition of 10 mM  $\text{MgCl}_2$  (data not shown), which has been shown to inhibit the low-affinity calcium channel (Locke *et al.*, 2000). In other experiments, we found that the stretch-activated calcium channel blocker lanthanum also blocked the TECC response in the wild-type strain (data not shown). In addition, we prepared media with ion-exchanged water that had been treated with EGTA beads for maximum elimination of divalent cations. We found that the glucose-induced TECC response was not observed in this medium, but was restored upon the readdition of  $\text{Ca}^{2+}$ . When taken together, these results indicate that the TECC response is mediated primarily (if not exclusively) by the uptake of extracellular calcium.

It appeared to be a remote, but not entirely impossible, scenario that the differences in TECC signals relate to the intracellular–organellar sequestration of calcium ions. We reported earlier that strains lacking the vacuolar plasma membrane-type calcium ATPase Pmc1p, the vacuolar  $\text{Ca}^{2+}/\text{H}^+$  antiporter Vcx1p or the Golgi–endoplasmic reticulum (ER)-localized SERCA-type calcium ATPase Pmr1p react differently to short-term and long-term environmental calcium challenge (Miseta *et al.*, 1999a,b). However, the initial phase of the glucose-induced TECC response is not significantly different from wild-type *S. cerevisiae* in *pmc1* $\Delta$ , *vcx1* $\Delta$  and *pmr1* $\Delta$  strains. In addition, the subsequent elimination of surplus cytosolic calcium from the cytosol follows the characteristics we reported earlier for these mutant strains (Miseta *et al.*, 1999a,b).

## Discussion

In this study, we examined the relationship between glucose transport, the initial steps of glucose and galactose metabolism and the hexose-mediated transient elevation of cytosolic calcium levels in *S. cerevisiae*. Our results indicate that wild-type cells starved of a carbon source respond to the reintroduction of glucose with a TECC response as reported previously (Nakajima Shimada *et al.*, 1991; Fu *et al.*, 2000). Similarly, galactose triggers the TECC response in cells grown with galactose as carbon source, but fails to induce this response in cells grown with glucose as carbon source. As the synthesis of enzymes required for galactose uptake and phosphorylation is repressed in the presence of glucose (Nehlin *et al.*, 1989), this result suggests that the presence of galactose

in the extracellular compartment (galactose sensing) is not sufficient to induce the TECC response.

*Saccharomyces cerevisiae* adjusts to environmental hexose levels by altering the expression of its arsenal of hexose transporters (Ozcan and Johnston, 1999). We found that the addition of 100 mM glucose induced maximal elevations in Glc-6-P and Glc-1-P levels in glucose-grown cells and smaller increases in the abundance of these metabolites in galactose-grown cells. Similarly, the TECC response was maximal in glucose-grown cells and smaller in cells grown in galactose as carbon source. This strong correlation suggests that the size of the pools of one (or both) of these glucose metabolites may play a direct role in determining the magnitude of the TECC response.

The elevations in both Glc-6-P and Glc-1-P were faster than the corresponding rise in cytosolic calcium levels. This may indicate that these metabolites must reach a threshold level to activate calcium entry. Alternatively, the rise in the metabolite, which is ultimately responsible for the opening of plasma membrane calcium channels, is somewhat delayed when compared with the elevation in Glc-6-P and Glc-1-P levels. Measurements of metabolites derived from Glc-1-P may help us to elucidate this question.

A TECC response could not be induced by either glucose or galactose in a yeast strain lacking the capacity to transport these hexoses, indicating that they must enter the cytosol before a TECC response can be triggered. In addition, a strain that lacked the ability to phosphorylate glucose but retained an intact galactose metabolic pathway did not display a glucose-induced TECC response, but could be stimulated by galactose. When taken together, these observations provide powerful evidence that the TECC phenomenon that follows the addition of glucose or galactose requires the entry and phosphorylation of one of these hexoses.

Recently, we found that a yeast strain lacking the major isoform of phosphoglucomutase maintained an elevated level of total cellular calcium when grown with galactose, but not with glucose as a carbon source (Fu *et al.*, 2000). The *pgm2Δ* mutant also maintained an eightfold increase in Glc-1-P while maintaining a normal level of Glc-6-P. Although this mutant demonstrated a normal basal cytosolic calcium level when grown in media containing galactose as carbon source, the glucose- and galactose-induced TECC response was eliminated in galactose-grown cells. The *pgm2Δ* mutant has no apparent calcium-related phenotype when maintained in glucose-containing media. However, as the results of the current study suggest that glucose- and galactose-induced TECC may be coupled to a transient elevation of Glc-1-P or Glc-6-P, the high level of Glc-1-P observed when the *pgm2Δ* mutant is grown in galactose-containing media may also

be responsible for its increased calcium uptake. Phosphoglucomutase activity is significantly higher in glucose-grown cells than the other enzymes of the Leloir pathway. This relatively high basal level of phosphoglucomutase activity is necessary to provide the Glc-1-P used in the production of sugar nucleotides and, ultimately, cell wall biosynthesis (Fu *et al.*, 1995). Of this activity, the *PGM2* gene contributes roughly half the total cellular phosphoglucomutase activity under those conditions (Fu *et al.*, 1995). This provided the opportunity to test whether a decreased TECC response could be correlated with the reduced phosphoglucomutase activity present in glucose-grown *pgm2Δ* cells after starvation and the reintroduction of glucose. Our finding that the glucose-induced TECC response is significantly reduced when the *pgm2Δ* mutant is grown in SM glucose suggests that the level of Glc-1-P (or a related metabolite) may play a key role in controlling calcium uptake during the TECC response.

It has been demonstrated recently that lithium inhibits phosphoglucomutase in *S. cerevisiae* (Masuda *et al.*, 2001). Earlier, we described how decreased phosphoglucomutase activity results in elevated total cellular calcium levels in *S. cerevisiae* maintained in galactose-containing media (Fu *et al.*, 2000). Taken together, these observations may suggest that lithium, a widely used drug in the treatment of manic-depressive psychosis, may alter cellular calcium homeostasis through the inhibition phosphoglucomutase. However, lithium inhibits a variety of other enzymes (Masuda *et al.*, 2001) including inositol phosphatases, which are linked to cellular calcium signalling (Berridge, 1989). The elucidation of this important question requires careful and detailed studies.

It is not yet known how calcium uptake across the plasma membrane is regulated by glucose metabolism. Several mechanisms are possible. First, there is evidence that Glc-6-P can stimulate the uptake of calcium into the ER in mammalian cells (Chen *et al.*, 1998). It is possible that Glc-1-P functions in an analogous manner in yeast. If this were the case, calcium sequestration into an intracellular compartment could possibly cause a drop in cytosolic calcium that stimulates the uptake of exogenous calcium. It is also possible that the hexose-dependent TECC response results from a transient increase in the concentration of a molecule derived from Glc-1-P that increases the calcium permeability of the plasma membrane. A candidate for such a molecule is calcium influx factor (CIF), a hypothetical molecule with a molecular mass of  $\approx 700$  Da (Randriamampita and Tsien, 1993) that was reported to have properties similar to a sugar phosphate or sugar nucleotide (Kim *et al.*, 1996). It has been proposed that CIF synthesis or release is induced by calcium store depletion during a mechanism called capacitative calcium entry (CCE). The depletion of calcium

stores in the endoplasmic or sarcoplasmic reticulum of mammalian cells can be induced by thapsigargin, an inhibitor of the SERCA-type calcium ATPase. When this occurs, CIF induces the uptake of calcium across the plasma membrane. Consistent with the suggestion that CIF is a soluble factor, the injection of a soluble extract from thapsigargin-treated mammalian cells can rapidly induce calcium uptake in *Xenopus* oocytes. Similarly, the injection of a similar extract prepared from a yeast strain lacking Pmr1p, the SERCA-type calcium ATPase located in the Golgi apparatus, also increased the calcium permeability of *Xenopus* oocytes (Csutora *et al.*, 1999; Locke *et al.*, 2000).

In a previous study, it was shown that the TECC response did not occur after the addition of glucose to glucose-starved cells when the assay was carried out in a buffer that lacked calcium (Nakajima Shimada *et al.*, 1991). This led to the suggestion that the glucose-induced TECC response was dependent upon extracellular calcium. Consistent with this result, we found that deletion of the *MID1* gene, a postulated yeast homologue of the mammalian voltage-gated calcium channel, significantly reduced the hexose-induced TECC response. In other experiments, we also found that the addition of 10 mM  $\text{LaCl}_3$ , which blocks stretch-activated calcium channels in the plasma membrane of mammalian cells, also effectively inhibited the hexose-induced TECC phenomenon in yeast. These results suggest that the entry of extracellular calcium, rather than its mobilization from internal stores, is responsible for the hexose-induced TECC response. As the extracellular calcium concentration is normally several orders of magnitude higher than the resting cytosolic calcium concentration, this appears to be a reasonable mechanism to mediate transient changes in the cytosolic calcium level of wild-type *S. cerevisiae*. Further studies are required to determine how the level of these glucose metabolites can regulate this unique calcium signalling mechanism.

## Experimental procedures

### Strains and culture media

The control strain CEN.PK2-1C (*MATa ura3, leu2, trp1, his3, MAL<sup>+</sup>, GAL<sup>+</sup>*) and the hexose transport mutant EB.YVW4000 (*MATa ura3, leu2, trp1, his3 MAL<sup>+</sup>, hxt1-17, gal2, stil1, agt1, mph2, mph3*) were kindly provided by Dr E. Boles. The DFY-632 (*MATa ura3*) strain was a gift from Dr D. Fraenkel. The YDB0311 (*Mata, ade2, leu2, his3, lys2, ura3 pgm2Δ::URA3*) and YDB0384 (*Mata, ade1, leu2, ura3, mid1Δ::LEU2*) strains were created by standard, targeted gene deletion methods. These strains were transformed with the plasmids pEVP11 or pDB617; both contain the apoaequorin gene under *ADH1* promoter control. The strains were maintained in synthetic media (SM) lacking either leucine (strains containing pEVP11) or uracil (strains containing pDB617). SM was

complemented with one of the following carbon sources: 2% D-glucose, 2% D-galactose, 3% DL-lactate.

### Measurements of cytosolic and total cellular calcium concentrations

The strains used were grown in SM-glucose, SM-galactose or SM-lactate in the absence of leucine or uracil depending on the properties of the plasmid-carrying strain. Thereafter, the cells were lysed, and the level of aequorin expression relative to total protein concentration was measured as described earlier (Batiza *et al.*, 1996; Allen *et al.*, 1977; Miseta *et al.*, 1999a). The light emission of 1 mg of protein containing lysate from our control strain (maintained in the presence of the relevant carbon source containing SM) was considered as 1. Relative light emissions measured in lysates from other strains fall into the 0.72–1.15 range. Experimental results were corrected according to the level of actual apoaequorin expression throughout this report. In addition, we found that no more than about 8% of the total reconstituted aequorin was discharged in any of the experiments described here.

For *in vivo* cytosolic calcium measurements, we used a Berthold 9050 Lumat luminometer equipped with two injectors. Cultures were grown until the cell density ( $\text{OD}_{600}$ ) was between 0.7 and 1.0  $\text{OD}_{600}$  units. Usually, 10  $\text{OD}_{600}$  units of cells were harvested by centrifugation, resuspended in 0.2 ml of test medium (TM = SM containing 2 mM EGTA and 40 mM MES-Tris, pH 6.5), loaded with 10  $\mu\text{l}$  of 590  $\mu\text{M}$  coelenterazine at room temperature for 20 min (excess coelenterazine in the supernatant was removed by centrifuging and washing the cells once with 0.5 ml of fresh test medium). The cells were then resuspended in 1 ml of TM (without carbon source) and incubated for 3 h. Two  $\text{OD}_{600}$  units of cells were used for a single measurement. Cells were preincubated in the dark in TM ( $\pm 10$  mM calcium) at room temperature for 3 h. Considering the ionic composition, pH and temperature of the TM, the free calcium concentration in TM was calculated to be 34  $\mu\text{M}$ , whereas in TM supplemented with 10 mM calcium, the free calcium concentration was calculated to be 8 mM. Calculations of calcium concentration were done using the MAX-CHELATOR program. After the preincubation period, cells were transferred into sample holders, and measurement was initiated. After measuring the baseline light emission for about 40 s, 100 mM glucose or galactose was injected directly into the sample holder. In some experiments, the preincubation medium was supplemented with 10 mM  $\text{LaCl}_3$ . Every experiment was repeated at least three times before data were accepted. Results of representative experiments are displayed.

Total cellular calcium levels were measured by an Eppendorf Efox 5053 flame photometer. Briefly, cells were grown to a cell density of 0.8–1.2  $\text{OD}_{600}$  units and then harvested by centrifugation at room temperature (5 min at 10 000 *g*). A single sample contained about 100  $\text{OD}_{600}$  units. Measurements were routinely carried out in triplicate. The samples were transferred into microcentrifuge tubes of known weight and centrifuged at room temperature at 15 000 *g* for 10 min. The supernatants were carefully removed, and the sample was measured gravimetrically on an analytical balance. Each sample was dried in a Speed Vac (Savant) vacuum refriger-

ator for 3 h, and the dry weight of the samples was measured gravimetrically. An aliquot of 0.6 ml of 1 M HCl was added to the dry samples, and the samples were vortexed. The samples were incubated on a rocker table for 24 h and then centrifuged at 15 000 *g* for 5 min. The supernatants were removed and the calcium measurements carried out.

#### Measurements of cellular glucose-6-phosphate and glucose-1-phosphate levels

For the measurement of glucose metabolite levels, the strains were grown as indicated above, harvested by centrifugation and resuspended in SM (100 OD<sub>600</sub> units ml<sup>-1</sup>). The volume of cells was measured by haematocrit capillary centrifugation. The cells were incubated in TM (without carbon source) for 3 h, and the experiment was initiated by the addition of 100 mM glucose or 100 mM galactose while continuously agitating the cells in an environmental shaker incubator (200 r.p.m. min<sup>-1</sup>). Samples were collected by pipetting 1 ml of culture directly into microcentrifuge tubes containing 0.11 ml of 6.67 M perchloric acid (PCA) and ≈ 200 mg of glass beads. After sample addition, the microcentrifuge tubes were incubated on wet ice for 20 min. Subsequently, samples were vortexed vigorously five times for 1 min, centrifuged at 10 000 *g* for 5 min, and the supernatant collected. After the neutralization of the supernatant with 5 M KOH (final pH 6.0–6.5), the Glc-6-P and Glc-1-P measurements were carried out according to the method of Bergmeyer (1984). Results obtained were multiplied by dilution factors, corrected for the net volumes of samples and expressed in mM.

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