New aspects of the glucose activation of the H⁺-ATPase in the yeast \textit{Saccharomyces cerevisiae}

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The glucose-induced activation of plasma membrane ATPase from \textit{Saccharomyces cerevisiae} was first described by Serrano in 1983. Many aspects of this signal transduction pathway are still obscure. In this paper, evidence is presented for the involvement of Snf3p as the glucose sensor related to this activation process. It is shown that, in addition to glucose detection by Snf3p, sugar transport is also necessary for activation of the ATPase. The participation of the G protein, Gpa2p, in transducing the internal signal (phosphorylated sugars) is also demonstrated. Moreover, the involvement of protein kinase C in the regulation of ATPase activity is confirmed. Finally, a model pathway is presented for sensing and transmission of the glucose activation signal of the yeast H⁺-ATPase.

Keywords: signal transduction, nutrient signalling

INTRODUCTION

The plasma membrane ATPase of \textit{Saccharomyces cerevisiae} controls important physiological processes. By pumping protons, it regulates intracellular pH and provides the driving force for nutrient uptake. A remarkable characteristic of this enzyme is the fact that it is activated in the presence of glucose through transcriptional and post-transcriptional mechanisms that increase the level of ATPase activity in yeast cells (Serrano, 1983; Sychrova & Kotyk, 1985; Capieaux et al., 1989; Chang & Slayman, 1991; Rao et al., 1993; Garcia-Arranz et al., 1994; Eraso & Portillo, 1994; de la Fuente et al., 1997).

At the post-transcriptional level two different mechanisms seem to be involved: direct phosphorylation of the enzyme (Chang & Slayman, 1991; Eraso & Portillo, 1994) and/or proteolytic degradation of a putative inhibiting protein of the activation process (de la Fuente et al., 1997).

In spite of the fact that different putative phosphorylation sites present in this enzyme have been described in some detail (Eraso & Portillo, 1994), the identity of the protein kinase involved in ATPase phosphorylation is still not known. It was demonstrated that the RAS-cAMP-protein kinase A pathway is not involved in glucose-induced activation of ATPase (Becher dos Passos et al., 1992). On the other hand, based on the existence in the carboxyl terminus of the plasma membrane ATPase of phosphorylation consensus recognition sites for casein kinase I and calmodulin-dependent protein kinase I, these enzymes were proposed to be involved in this regulation process (Kolarov et al., 1988; Kemp & Pearson, 1990; Estrada et al., 1996). Recently, it was demonstrated that protein kinases involved in the regulation of other plasma membrane proteins would be related to the glucose-induced activation of the plasma membrane H⁺-ATPase (Goossens et al., 2000).

Furthermore, by working with different putative inhibitors for protein kinase C (PKC), calmodulin-dependent protein kinase and phosphatidylinositol turnover, we proposed that a phosphatidylinositol type signalling pathway could be involved in the glucose-induced activation of the plasma membrane ATPase (Brandão et al., 1994). Indeed, the involvement of phospholipids found in the plasma membrane in the control of ATPase activity had been described by Patton & Lester (1992). However, in spite of the fact that glucose stimulates phosphatidylinositol turnover in yeast (Francescotti et al., 1990), the identification of this hypothetical signalling pathway has not yet been achieved. Nevertheless, Coccetti et al. (1998) demon-
we demonstrate that the glucose-induced activation of phosphorylating and activating enzyme(s). In this paper receptors, G protein(s), internal signals and the acting in this process formed by specific glucose membrane ATPase, one could expect a complete system mechanism leading to the activation of the plasma membrane ATPase requires the presence of Snf3p (a glucose sensor), the Gpa2 protein (a G protein) and we confirm the involvement of PKC. We also show that phosphorylation of glucose is indeed the internal signal necessary to this activation process and that this signal is transduced via Gpa2p.

**METHODS**

**Strains and growth conditions.** The *Saccharomyces cerevisiae* strains used in this study are shown in Table 1. Yeast cells were maintained at 4 °C in the appropriate medium. They were grown in medium containing 2% peptone and 1% yeast extract (YP), supplemented with carbon sources and 1 M sorbitol (when indicated) for the mitogen-activated protein (MAP) kinase pathway strains. In all experiments the cells were grown in a rotary incubator (New Brunswick model G25; 200 r.p.m.) at 30 °C until the end of exponential phase. Cells were harvested and washed three times by centrifugation (approx. 2000 g) with 25 mM MES buffer, pH 6.0, with or without 1 M sorbitol, depending on the strain.

**Measurement of proton-pumping activity.** The procedure used was described by Serrano (1980). Cells (around 100 mg, wet wt) were resuspended in 100 mM Tris/HCl buffer, pH 4.5, containing 100 mM KCl and incubated in a vessel in a total volume of 50 ml; the change in pH of the suspension was recorded before and after the addition of 100 mM glucose. Calibration pulses of 100 nmol HCl were also added. The maximal rate of proton pumping was calculated from the slope of the line, indicating the change in the pH in the medium.

**Measurement of H+ -ATPase activity.** For time-course measurements of ATPase activity, cells were incubated at a density of 75 mg ml⁻¹ (wet wt) in a reciprocating water bath shaker at 30 °C. Incubation was carried out in 100 mM MES/Tris buffer (pH 6.5) for 10 min before addition of glucose to a final

### Table 1. *Saccharomyces cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>W303</td>
<td>MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC</td>
</tr>
<tr>
<td>YSH813</td>
<td>W303-1A MATa bckA::URA3</td>
</tr>
<tr>
<td>YSH849</td>
<td>W303-1A MATa mpk1A::TRP1</td>
</tr>
<tr>
<td>YSH 850</td>
<td>W303-1A MATa pkc1A::HIS3</td>
</tr>
<tr>
<td>LK41</td>
<td>W303 gpr1(−1/ + 2869)Δ::URA3</td>
</tr>
<tr>
<td>SP1</td>
<td>MATa leu2 ura3 trp1 his3 ade8 can1</td>
</tr>
<tr>
<td>139</td>
<td>SP1 gpa2Δ::URA</td>
</tr>
<tr>
<td>MC9964</td>
<td>MATa URA3-52 bis11,15 leu2-3,112 MAL2 SUC2 GAL MEL10</td>
</tr>
<tr>
<td>RE800-A</td>
<td>MC9964+bxt1Δ::HIS3 bxt4 bxt5 Δ::LEU2 bxt2A::HIS3 bxt3Δ::LEU2 bxt6</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>FR801</td>
<td>RE800-A+gpa2Δ::URA3</td>
</tr>
<tr>
<td>YSH327</td>
<td>W303-1A bck1Δ::HIS3 bxt2Δ::LEU2</td>
</tr>
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<td>YSH753</td>
<td>W303-1A MATa glk1Δ::LEU2 bck1Δ::HIS3</td>
</tr>
<tr>
<td>YSH755</td>
<td>W303-1A MATa glk1Δ::LEU2 bck2Δ::LEU2</td>
</tr>
<tr>
<td>YSH757</td>
<td>W303-1A MATa glk1Δ::LEU2 bck1Δ::HIS bck2Δ::LEU2</td>
</tr>
<tr>
<td>YM6217</td>
<td>MATa ura3-52 bis3-200 ade2-101 bys2-801 trp1-903 leu2-3,112 tyr1-501 MET?</td>
</tr>
<tr>
<td>YM4817</td>
<td>YM6217 rgt2::HIS3</td>
</tr>
<tr>
<td>YM6175</td>
<td>YM6217 snf3::HIS3</td>
</tr>
<tr>
<td>YM6212</td>
<td>YM6217 snf3::HIS3 rgt2::HIS3</td>
</tr>
</tbody>
</table>
concentration of 100 mM. At different times, samples containing 375 mg cells (wet wt) were taken from the suspension and the cells collected as quickly as possible on glass fibre filters by vacuum filtration. The cells were quickly removed from the filters and immediately frozen in liquid nitrogen and stored until use.

The procedures used to obtain plasma membranes and to determine ATPase activity were described by Becher dos Passos et al. (1992). Protein was determined according to the Lowry method.

**Reproducibility of results.** The experiments were performed at least three times with consistent results. Standard deviations are indicated in each figure.

### RESULTS AND DISCUSSION

**Involvement of Gpr1p and Gpa2p in ATPase activation**

In previous studies (Becher dos Passos et al., 1992; Brandão et al., 1994) it was demonstrated that glucose-induced activation of *Saccharomyces cerevisiae* plasma membrane H⁺-ATPase not only occurs by an independent mechanism of cAMP pathway signalling, but apparently also depends on the stimulation of a pathway similar to that which exists in higher eukaryotes involving phosphatidylinositol metabolism. On the other hand, Kraakman et al. (1999) demonstrated that the Gpr1 receptor coupled to a G protein (Gpa2p) is important for the process of cAMP synthesis activation induced by glucose. However, this protein also seems to bind to the phospholipase C enzyme in yeast (Ansari et al., 1999). Since we demonstrated the involvement of phospholipase C (Plc1p) in the glucose-induced activation of plasma membrane ATPase (Coccetti et al., 1998), we have studied the involvement of this complex (Gpr1p/Gpa2p/Plc1p) in the process of H⁺-ATPase activation by glucose.

However, as can be seen in Fig. 1, deletion of the Gpr1p receptor does not decrease markedly the H⁺-ATPase activation. We also measured the glucose-induced H⁺ efflux: in the wild-type strain the H⁺-pumping rate was not statistically different (P > 0.05) from a gpr1Δ mutant [138 ± 30 μmol H⁺ h⁻¹ (g cell)⁻¹ and 111 ± 15 μmol H⁺ h⁻¹ (g cell)⁻¹, respectively]. Nevertheless, our results point to the participation of G protein in this activation process since deletion of GPA2 decreased by half the glucose activation of the H⁺-ATPase (Fig. 2). In this case the glucose-induced H⁺-pumping rate was reduced from 192 ± 47.9 μmol H⁺ h⁻¹ (g cell)⁻¹ in the wild-type strain to 95 ± 37.2 μmol H⁺ h⁻¹ (g cell)⁻¹ in the gpa2Δ mutant (values statistically different: P < 0.05). These results seem to suggest that the combined receptor–transducer system, Grp1p/Gpa2p, does not mediate ATPase regulation.

**The participation of Rgt2p and Snf3p glucose sensors**

We thus checked the possible involvement of other glucose sensors as participants in the activation mechanism of the plasma membrane ATPase. Snf3p and Rgt2p act as glucose receptors that bind glucose outside the cell and generate a signal inside the cell for induction of hexose transporter gene (*HXT*) expression (for a review, see Ozcan & Johnston, 1999). Snf3p seems to function as a sensor of low levels of glucose and because of that it is involved in the expression of high-affinity glucose carriers. On the other hand, Rgt2p seems to act as a sensor for high levels of glucose and is thus involved

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**Fig. 1.** Glucose-induced H⁺-ATPase activation in wild-type (W303, ○) and gpr1Δ mutant (LK41, ●) strains of *S. cerevisiae*. Results shown are means ± SD of triplicate experiments.

**Fig. 2.** Glucose-induced H⁺-ATPase activation in wild-type (SP1, ○) and gpa2Δ mutant (139, ●) strains of *S. cerevisiae*. Results shown are means ± SD of triplicate experiments.
in the control of the HXT1 expression (the principal low-affinity glucose carrier).

Surprisingly, as demonstrated in Fig. 3, only Snf3p seems to be required for a normal glucose-induced activation of the ATPase. Considering that we used 100 mM (±1.8%) glucose in all experiments, it might have been expected that the low-affinity glucose sensor, Rgt2p, would be the sensor. However, there is evidence that Snf3p can contribute to high-level glucose signalling, even taking into account that its own expression is repressed fivefold by high levels of glucose. In an rgt2Δ mutant the expression of HXT1 is only reduced about fivefold and it is completely absent in the snf3Δ rgt2Δ mutant (Ozcan & Johnston, 1995; Ozcan et al., 1996a).

In spite of the fact that Snf3p and Rgt2p are 60% identical, they differ in the long C-terminal segments. In these cytoplasmic tails, the sequences are only similar to one another over a stretch of 25 aa. Snf3p presents two of these 25 aa sequences and Rgt2p has only one (Ozcan et al., 1998). Apparently, these tails are involved in the generation of the signal responsible for HXT expression, but the signal itself is generated neither by glucose transport nor any of its metabolites. Thus, how the signal is transduced is an open question. It has been suggested that a conformational change induced by glucose binding to the transmembrane-spanning domain affects the C-terminal domain in such a way that it interacts with other component(s) of the pathway (Ozcan et al., 1996b; Ozcan & Johnston, 1999).

The nature of the internal signalling

Moreover, by using strains with deletions in all genes encoding functional glucose carriers (genes HXT1, 2, 3, 4, 5, 6, 7), and glucose or maltose as signalling molecules, we demonstrated that sugar transport is essential for the regulation of the H+-ATPase activation process. The glucose-induced efflux of protons was reduced from 243 ± 32.5 µmol H+ h−1 (g cell)−1 in the wild-type strain to 5 ± 0.1 µmol H+ h−1 (g cell)−1 in the hxt1-7Δ mutant (values statistically different: P < 0.05). On the other hand, by working with an hxt1-7Δ gpa2Δ strain, and using maltose as signalling molecule, we additionally demonstrated the importance of the G protein Gpa2p in the activation process of the plasma membrane H+-ATPase (Fig. 4). In the wild-type and in the hxt1-7Δ strains the maltose-induced efflux of protons was not statistically different: 28 ± 7.6 and 34 ± 12.9 µmol H+ h−1 (g cell)−1, respectively (P > 0.05). However, in the hxt1-7Δ gpa2Δ strain, the maltose-induced efflux of protons was almost zero. Since, in order to be metabolized, maltose should be hydrolysed and phosphorylated, these results seem to suggest that sugar phosphorylation is the internal signal for ATPase activation.

In fact, sugar phosphorylation has already been suggested as being necessary to the glucose-induced activation of the plasma membrane ATPase (Becher dos Passos et al., 1992). Now, by working with strains with single and different combinations of deletions in the genes encoding different sugar kinases (HXK1, HXKII and GLK1) and by using glucose (Fig. 5a, c) and fructose (Fig. 5b, d) as signalling molecules it was possible to demonstrate that sugar phosphorylation is an essential step in this regulatory mechanism. From the measurements of both H+-ATPase activity as well as the determination of the H+ -pumping rates, it can be concluded that for the activation of the plasma membrane ATPase to occur, at least one appropriate sugar kinase must also be active.
Plasma membrane ATPase in yeast cells

Fig. 5. Glucose-induced (a) and fructose-induced (b) H⁺-ATPase activation, and glucose-induced (c) and fructose-induced (d) extracellular acidification in wild-type (W303, ○, ■), hxk1Δ hxk2Δ (YSH327, ●, □), hxk1Δ glk1Δ (YSH753, ▽, △), glk1Δ hxk2Δ (YSH755, ▽, △) and glk1Δ hxk1Δ hxk2Δ (YSH757, ●, □) strains of S. cerevisiae. It is important to note that the enzymes hexokinase I and II are able to phosphorylate both glucose as well as fructose, while glucokinase I only phosphorylates glucose. Results shown are means ± SD of triplicate experiments.

Involvement of the PKC MAP kinase pathway

It is already known that in yeast, PKC1 participates in the regulation of the biochemical pathway responsible for the activation of the glucano-synthase enzyme (involved in cell wall synthesis). This PKC MAP kinase pathway consists of Bck1, Mkk1/2 and Mpk1 enzymes that are activated by phosphorylation. Loss of the PKC functions results in a cell lysis defect due to a deficiency in cell wall construction. Its mutant phenotype was used to identify the downstream components of the PKC1 pathway. Four genes were discovered that, when over-expressed, suppressed the cell lysis defect associated with pathway inactivation. From epistatic analysis and structural relatedness to kinases in other pathways, it was proposed that PKC phosphorylates MAPKKK Bck1 that, also by phosphorylation, activates two redundant MAPKKs, Mkk1 and Mkk2, which phosphorylate the MAPK Mpk1. One of the targets of this pathway seems to be a glucan synthase complex, an enzyme that is controlled by MPK1 phosphorylation in a PKC-dependent way. Addition of osmotic stabilizing agents (sorbitol or NaCl) to the growth medium compensates for the defect and allows for cell proliferation. Moreover, the mutant phenotype is less severe in the last four components of the pathway. This fact is consistent with the idea that there is a bifurcation after PKC, suggesting that this protein kinase has several important functions in yeast (Levin et al., 1994), but hypothetical additional targets of PKC1p have not been clearly identified.

Due to the fact that in a previous study we demonstrated...
the involvement of PKC in the glucose-regulated activation of the plasma membrane ATPase (Brandão et al., 1994), we decided to clarify this situation by using strains with mutations in the PKC MAP kinase pathway found in yeast cells. In the present study we confirmed the involvement only of PKC1 and not the other MAP kinases of the pathway in H⁺-ATPase activation (Fig. 6a, b). These data confirm that there is a bifurcation after PKC and one of its alternative targets seems to be the plasma membrane ATPase.

**A model pathway for plasma membrane ATPase regulation**

If we admit the involvement of the Snf3p sensor in the glucose-induced activation of plasma membrane ATPase, and taking into account that sugar phosphorylation is essential for the activation of the enzyme, we can imagine that the regulation of the activity of this ATPase is a result of at least two different actions. First, glucose binding would induce the interaction of the C-terminal domain of Snf3p, probably through the second stretch of 25 aa (absent in Rgt2p), with the next component of the system (Gpa2p?). Second, the internal signal generated by sugar phosphorylation would be transmitted to Gpa2p. Gpa2p and Plc1p are part of a complex present in the plasma membrane which could generate secondary messengers that would activate PKC and then the plasma membrane ATPase would be phosphorylated and activated. However, by using the mutants described here it will be possible to verify whether PKC is involved in the phosphorylation of Ser-899 and/or Thr-912, known to be involved in the glucose-induced post-transcriptional activation of the plasma membrane ATPase in yeast.

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Plasma membrane ATPase in yeast cells


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