

Glucose activation of the yeast plasma membrane H^+ -ATPase requires the ubiquitin–proteasome proteolytic pathway

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Abstract Glucose triggers transcriptional and post-transcriptional mechanisms that increase the level and activity of *Saccharomyces cerevisiae* plasma membrane H^+ -ATPase. We have studied the post-transcriptional activation of the enzyme by glucose and have found that Rsp5, a ubiquitin–protein ligase enzyme, Ubc4, a ubiquitin-conjugating enzyme, and the 26S proteasome complex are implicated in this activation. These results suggest that ATPase activation by glucose requires the ubiquitin–proteasome proteolytic pathway. This is supported by the fact that over-expression of the ubiquitin-specific protease Ubp2, which cleaves ubiquitin from its branched conjugates, inhibits this activation. We propose that glucose triggers degradation of an inhibitory protein resulting in enzyme activation.

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Key words: H^+ -ATPase; Plasma membrane; Glucose activation; Ubiquitin; *Saccharomyces cerevisiae*

1. Introduction

The plasma membrane H^+ -ATPase of yeast is a proton pump that plays a central role in the physiology of this organism [1,2]. This proton pump is regulated by a large number of environmental factors, among which glucose metabolism appears to be one of the most important. Glucose regulation takes place at two levels: at the transcriptional level, glucose increases the PMA1 mRNA synthesis [3,4] and at the post-transcriptional level, it induces ATPase activation. This activation results from a combined effect on the kinetic parameters of the enzyme, including K_m decrease, V_{max} increase and optimum pH shift to neutral values [5].

In an early study, we isolated mutations on seven genes affecting the regulation of the enzyme [4]. Here we show that one of these genes encodes Rsp5–ubiquitin ligase. The ubiquitin–protein ligase enzymes catalyze the last step in the formation of ubiquitin–protein conjugates, which is a signal for degradation of the tagged protein. [6,7]. Mutations at the *RSP5* locus disturb glucose ATPase activation by abolishing the K_m decrease that occurs during glucose activation. We also show that the Ubc4 ubiquitin-conjugating enzyme and an intact 26S proteasome complex are required for the glucose activation-triggered K_m decrease of the enzyme. The results suggest that a yet unidentified protein, which is a substrate for the ubiquitin–proteasome pathway, is involved in glucose activation of the ATPase.

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2. Materials and methods

2.1. Strains, plasmids and growth conditions

The *S. cerevisiae* strains used are listed in Table 1. Strain ANY03 was constructed by inserting a 2.0 kb *HindIII*–*KpnI* fragment containing the 5'-end of the *RSP5* gene at the genomic locus. The fragment was subcloned into the integrating plasmid YIp352 (URA3) [12] and digested with *Bam*HI prior to transformation of a *MAT α* derivative of BWG1-7A. Integration of the plasmid at the *RSP5* locus was confirmed by Southern blotting.

Strains NFL1-1A and NFL1-2C carrying the *ubc4- Δ 1::HIS3* or the *ubc5- Δ 1::LEU2* mutant alleles, respectively, are segregants of the cross MHY500 $\times$ MHY508.

Plasmid YEp::UBP1 carries the *UBP1* gene under the control of the *ADH1* promoter in the pRS423 vector (2 μ , *HIS3*) [13]. Plasmid YEp::UBP2 carries the *UBP2* gene under the control of its own promoter in the pRS426 vector (2 μ , *URA3*) [13]. Plasmid YEp::UBP3 carries the *UBP3* gene under the control of the *ADH1* promoter in the plasmid YEplac181 (2 μ , *LEU2*) [14].

Yeast strains were grown in medium with 2% glucose, 0.7% yeast nitrogen base without amino acids (US Biologicals, Swampscott, MA) and the appropriate requirements [15]. When indicated, medium was buffered with 50 mM MES, adjusted to pH 6.0 with Tris (SD6.0) or 50 mM succinic acid, adjusted to pH 3.0 with Tris (SD3.0).

2.2. Cloning of the *RSP5* gene

The yeast strain H19 carrying the *apa2-4* mutant allele was transformed [16] with 50 μ g of DNA from a YCp50-based library [17]. Approximately 10 000 transformants were selected in SD6.0 medium. Transformed cells were pooled and plated in SD3.0 medium. After 4 days at 30°C, three colonies grew on acidic medium and exhibited wild-type ATPase levels. Plasmids were rescued from yeast [18] and amplified in *E. coli*. Restriction analysis and partial sequencing of these clones revealed that all three plasmids contained the same insert (Fig. 1).

To demonstrate linkage of the complementing DNA to the mutant phenotype, strain ANY03 (*rsp5* Δ ::YIp352:*RSP5*) was crossed with the H19 strain (*apa2-4*) and tetrads analyzed for segregation of the *apa2-4* mutation and the *URA3* marker.

2.3. Biochemical methods

Yeast plasma membrane was purified from glucose-starved and glucose fermenting cells by differential and sucrose gradient centrifugation [5]. ATPase activity was assayed at pH 6.5 with ATP concentrations from 0.8 to 6 mM [20]. The apparent K_m and V_{max} were extrapolated from double-reciprocal plots fitted using a standard least-squares method. Similar values (within 10%) were obtained with two different plasma membrane preparations isolated independently. Protein concentration was determined by the method of Bradford [21] with the Bio-Rad protein assay reagent, using bovine IgG as standard.

3. Results and discussion

3.1. Cloning of the *RSP5* gene

The *RSP5* gene was cloned by its ability to suppress the phenotype of the *apa2-4* mutant strain when carried on a low-copy plasmid. This strain exhibits slow growth in SD3.0 medium, as a consequence of a decreased plasma membrane H^+ -ATPase activity level [4]. After transformation of the recipient

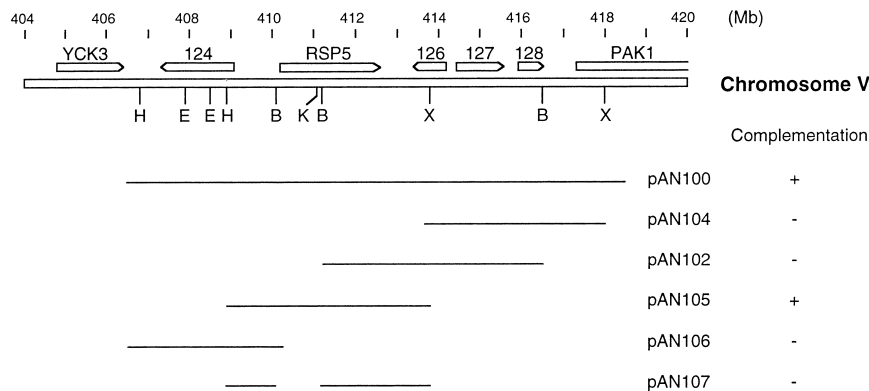


Fig. 1. Chromosomal location and subcloning of the *RSP5* gene. Restriction map of part of chromosome V [19]. Positions of the ORFs are indicated by arrows. DNA subclones used to test the ability to complement the *apa2* mutation are represented by thin horizontal lines. Restriction enzyme sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; X, *Xba*I.

strain with a YCp50-based yeast genomic library, we recovered one plasmid (pAN100) carrying a 12 kb insert (Fig. 1). This plasmid complements the slow growth phenotype of the mutant strain and restores wild-type levels of ATPase activity. Partial sequence analysis showed that the complementing insert corresponded to a region of chromosome V (Fig. 1). Subcloning experiments indicated that only the region containing gene *RSP5* is required for complementation; the *RSP5* gene is essential for growth [22,23] and encodes a ubiquitin–protein ligase enzyme. The possibility that *RSP5* was an extragenic suppressor of *apa2-4* was discarded by crossing strains ANY03 (*rsp5* Δ ::*YIp352::RSP5*) and H19 (*apa2-4*) and analyzing segregation of the *apa2-4* mutation and the *URA3* marker. In all 10 four-spore tetrads studied, the phenotype conferred by the *apa2-4* mutation and the *URA3* marker segregate independently, suggesting that the cloned gene corresponds to the *APA2* locus.

3.2. The *rsp5* mutation affects glucose-triggered plasma membrane ATPase activation

Since the plasma membrane ATPase is a long-lived and metabolically very stable protein [24] that is not ubiquitinated under physiological conditions ([25]; de la Fuente and Portillo, unpublished results), and the *rsp5* mutation does not affect PMA1 mRNA or protein levels (García-Arranz and Portillo

unpublished results), we explored the effect of the *rsp5* mutation on glucose activation of the enzyme (Fig. 2). In the wild-type strain, after 15 min incubation with glucose, a 7-fold increase in ATPase activity was observed, while under the same conditions, glucose caused a 3.5-fold activation in the *rsp5* mutant strain.

To further characterize the effect of the *rsp5* mutation on the activation of the enzyme by glucose, we determined the kinetic properties of the enzyme in purified plasma membrane of the wild-type and *rsp5* mutant obtained from glucose-starved and glucose-fermenting cells (Fig. 3). In the enzyme from the *rsp5* mutant, the typical *K_m* decrease caused by glucose was not observed, while the *V_{max}* increase was not significantly different from that of the enzyme from wild-type cells.

These results suggest that the glucose-triggered *K_m* decrease in this enzyme is regulated directly or indirectly by the ubiquitin pathway.

3.3. Activation of the plasma membrane ATPase in a ubiquitin-conjugating deficient mutant

Ubiquitin-conjugating enzymes transfer activated ubiquitin from the ubiquitinating-activating enzymes to ubiquitin–protein ligases [26]. This prompted us to test the effect of ubiquitin-conjugating enzymes on glucose activation of the ATPase. To

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Reference
BWG1-7A	<i>MATa adel-100 his4-519 leu2-3,112 ura3-52</i>	[8]
W303-1B	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	[9]
H19	<i>BWG1-7A apa2-4</i>	[4]
ANY03	<i>BWG1-7A rsp5Δ::YIp352::RSP5</i>	this work
MHY500	<i>MATa his3-200 leu2-3,112 ura3-52 lys2-801 trp1-1</i>	[10]
MHY508	<i>MHY500 MATa ubc4-Δ1::HIS3 ubc5-Δ1::LEU2</i>	[10]
MHY552	<i>MHY500 ubc6-Δ1::HIS3 ubc7-Δ1::LEU2</i>	[10]
NLF1-1A	<i>MHY500 ubc4-Δ1::HIS3</i>	this work
NLF1-2C	<i>MHY500 ubc5-Δ1::LEU2</i>	this work
WCG4a	<i>MATa his 3-11,15 leu2-3,112 ura3-52</i>	[11]
WCG4A-11/21a	<i>WCG4a pre1-1 pre2-1</i>	[11]
YMTAB	<i>WCG4a pra-Δ1::HIS3 prbl Δ</i>	[11]
FPY693	<i>W303-1B YEpl:UBP1 YEpl:UBP2 YEpl:UBP3</i>	this work
FPY681	<i>W303-1B YEpl:UBP1</i>	this work
FPY683	<i>W303-1B YEpl:UBP2</i>	this work
FPY685	<i>W303-1B YEpl:UBP3</i>	this work

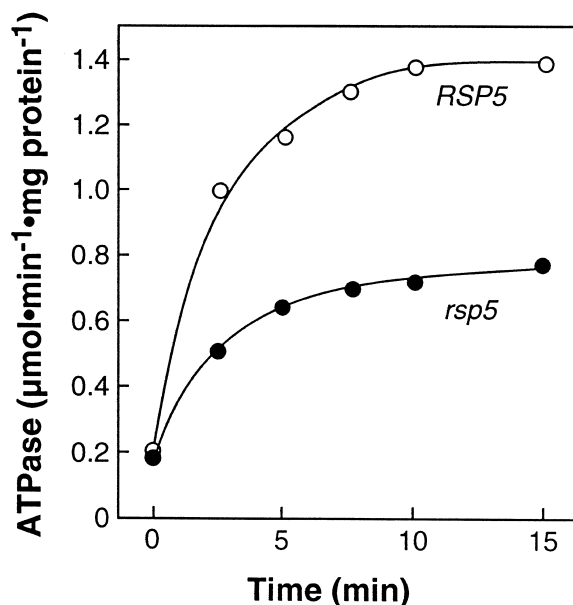


Fig. 2. Glucose activation of ATPase from a wild-type and an *rsp5* mutant strain. At time zero, glucose was added to either wild-type (○) or to the *rsp5* mutant strain (●), samples were taken at the indicated times and plasma membrane was purified. The ATPase activity of the purified membrane fraction was determined at pH 6.5 with 2 mM ATP. Similar values (within 10%) were obtained in two independent experiments.

this end, we analyzed the kinetic properties of the enzyme in purified plasma membrane obtained from glucose-starved and glucose-fermenting cells of the wild-type and ubiquitin-conjugating mutant strains (Table 2). The results showed that only the *ubc4Δ* mutant strain is defective in the K_m change induced by glucose. This reinforces the idea that a ubiquitin-dependent protein is involved in the K_m change of the ATPase upon glucose activation.

3.4. Activation of ATPase is altered in a proteasome-deficient mutant

Biochemical and genetic evidence suggests that the enzyme responsible for the ATP-dependent degradation of many ubiquitinated proteins is the 26S proteasome complex [27]. It has also been suggested that many ubiquitinated proteins are down-regulated by endocytosis and degraded by the vacuolar proteases [25,28–31]. To test whether ATPase activation by glucose requires the degradation of a ubiquitinated protein via the 26S proteasome complex or the vacuolar pro-

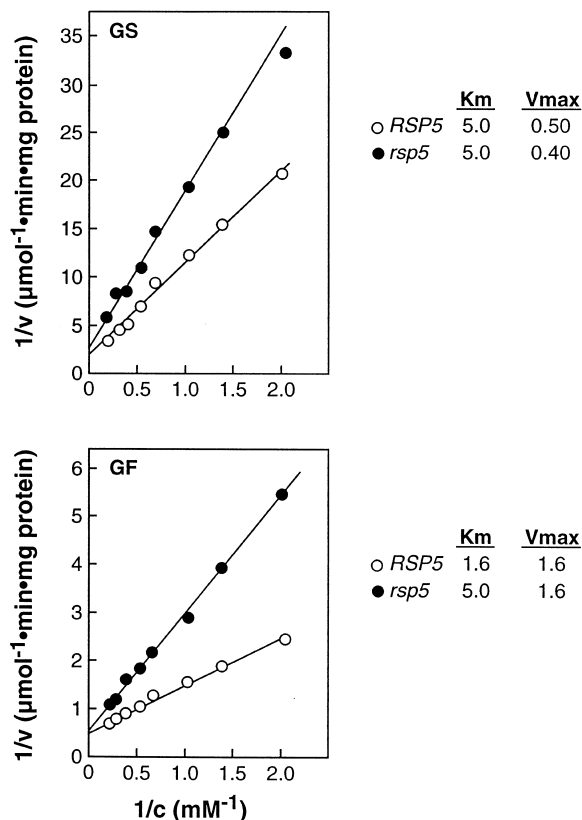


Fig. 3. Effect of glucose on the kinetics of plasma membrane ATPase from a wild-type and an *rsp5* mutant strain. Strains BWG1-7A (wild-type; ○) and H19 (*rsp5*; ●) were incubated with (GF) or without glucose (GS) before homogenization. The ATPase activity of purified plasma membrane was assayed at pH 6.5 with the indicated concentration of ATP. The apparent K_m (mM) and V_{max} ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$) calculated from the figure are indicated. Similar values (within 10%) were obtained with two different plasma membrane preparations obtained independently.

teolytic system, we analyzed the kinetic properties of the enzyme in purified plasma membrane from glucose-starved and glucose-fermenting cells of the wild-type, *prelpre2* and *pralprb1* strains. The *prelpre2* mutant strain is impaired in the chymotrypsin-like activity of the proteasome [11] and the *pralprb1* mutant strain lacks vacuolar proteinases A and B [11]. The enzyme from the proteasome-deficient mutant shows no change in K_m (Table 2). This suggests that degradation of a ubiquitinated protein via the 26S proteasome complex is a requisite for the K_m change caused by glucose-induced activa-

Table 2

Kinetic parameters of ATPase from glucose-starved and glucose fermenting wild-type and mutant cells

Strain	Relevant genotype	GS ^a		GF ^a	
		K_m (mM)	V_{max}^c ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg prot.}^{-1}$)	K_m (mM)	V_{max}^c ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg prot.}^{-1}$)
MHY500	wild-type	5.0	0.5	1.6	2.0
MHY508	<i>ubc4Δ ubc5Δ</i>	4.5	0.4	5.0	1.8
MHY552	<i>ubc6Δ ubc7Δ</i>	5.0	0.6	1.5	1.7
NLF1-1A	<i>ubc4Δ</i>	5.0	0.5	5.0	2.0
NLF1-2C	<i>ubc5Δ</i>	4.5	0.5	1.7	1.8
WCG4a	wild-type	4.0	0.4	1.2	1.3
WCG4a-11/21a	<i>pre1 pre2</i>	4.0	0.4	4.0	2.0
YMTAB	<i>praΔ prb1</i>	4.0	0.3	1.3	1.0

^aGS, glucose-starved cells; GF, glucose-fermenting cells. Cells were grown, collected and treated with glucose as described under Section 2.

Table 3

Kinetic parameters of ATPase from yeast strains overexpressing ubiquitin-specific proteases (*UBP*)

Strain	UBP over-expressed	GS ^a		GF ^a	
		K_m (mM)	V_{max} ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg prot.}^{-1}$)	K_m (mM)	V_{max} ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg prot.}^{-1}$)
W303-1B	none	4.0	0.4	1.0	1.3
FPY693	YEp: <i>UBP1</i> YEp: <i>UBP2</i> YEp: <i>UBP3</i>	4.0	0.3	4.0	1.6
FPY681	YEp: <i>UBP1</i>	4.2	0.3	1.1	1.5
FPY683	YEp: <i>UBP2</i>	4.0	0.4	4.0	2.0
FPY685	YEp: <i>UBP3</i>	4.0	0.5	1.1	1.4

^aGS, glucose-starved cells; GF, glucose-fermenting cells. Cells were grown, collected and treated with glucose as described under Section 2.

tion of the ATPase. The vacuolar proteinases, in contrast, do not appear to be involved in the process.

3.5. Activation of the plasma membrane ATPase in yeast strains overexpressing ubiquitin-specific proteases

Ubiquitin-specific, ATP-independent proteases are capable of cleaving ubiquitin from its linear or branched conjugates; overexpression of ubiquitin-specific proteases would thus be expected to perturb the degradation of ubiquitinated substrates and presumably to revert the physiological consequences of such degradation. In fact, overexpression of the yeast ubiquitin-specific protease Ubp2 results in a stabilization of short-lived substrates and as a consequence cells are hypersensitive to stress [32]. In the case of ATPase activation by glucose it would be expected that overexpression of ubiquitin-specific proteases would alter the steady-state ubiquitination of the regulatory protein, thus disturbing ATPase activation. When activation of the ATPase was analyzed in yeast strains expressing different ubiquitin-specific proteases (Table 3) it was found that Ubp2 overexpression hinders a modification of ATPase which alters its glucose-triggered K_m decrease.

Since ATPase itself is not ubiquitinated [25] it must be assumed that a protein which could be degraded via the ubiquitin–proteasome proteolytic pathway is implicated in this regulation. That overexpression of the Ubp2 ubiquitin-specific protease abolishes the K_m decrease suggests that the postulated protein has an inhibitory role on the K_m decrease produced by glucose addition to yeast cells.

ATPase activation by glucose is mediated by Ser/Thr phosphorylation [33]. Genetic studies suggest that amino acids Ser⁸⁹⁹ and Thr⁹¹², located at the carboxyl-terminus, define two independent regulatory sites involved in the K_m decrease and V_{max} increase, respectively. These two residues define potential phosphorylation sites for casein-kinase II and calmodulin-dependent protein kinase II [34].

Taking into account the above-mentioned findings, it is tempting to propose a model based on the existence of a protein that acts as inhibitor of the putative protein kinase which phosphorylates Ser⁸⁹⁹ to induce the K_m decrease. Upon glucose addition, this inhibitory protein becomes ubiquitinated and is degraded by the 26S proteasome complex, allowing phosphorylation of Ser⁸⁹⁹ by the putative protein kinase. Nevertheless the present results cannot dismiss an alternative model in which the postulated inhibitory protein would interact directly with the ATPase, blocking the phosphorylatable site that would be exposed to the kinase after ubiquitination and degradation of the protein.

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