cAMP- and RAS-independent Nutritional Regulation of Plasma-membrane H⁺-ATPase Activity in *Saccharomyces cerevisiae*

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(Received 30 August 1988; revised 9 January 1989; accepted 27 February 1989)

The plasma-membrane ATPase of *Saccharomyces cerevisiae* is a proton pump whose activity, essential for proliferation, is subject to regulation by nutritional signals. The previous finding that the *CDC25* gene product is required for the glucose-induced H⁺-ATPase activation suggested that H⁺-ATPase activity is regulated by cAMP. Analysis of starvation-induced inactivation and glucose-induced activation of the H⁺-ATPase in mutants affected in activity of the *RAS* proteins, adenylyl cyclase or cAMP-dependent protein kinase showed that nutritional regulation of H⁺-ATPase activity does not depend directly on any of these factors. We conclude that adenylyl cyclase does not mediate all nutritional responses. This also indicates that the specific *CDC25* requirement for the glucose-induced activation of the H⁺-ATPase identifies a new function for the *CDC25* gene product, a function that appears to be independent of *CDC25*-mediated modulation of the *RAS*/adenylyl cyclase/cAMP pathway.

INTRODUCTION

In the yeast *Saccharomyces cerevisiae* proliferation and differentiation are ultimately controlled by nutritional signals. Although the mechanism by which simple nutrients, such as glucose and NH₄⁺, regulate the transitions between vegetative growth and differentiation remains unknown, a number of recent studies suggest that the adenylyl cyclase/cAMP system is an essential element of such a control mechanism (for a summary of earlier work, see Matsumoto *et al.*, 1985). Adenylyl cyclase seems to be a primary target of systems that monitor the presence or absence of key nutritional compounds (Tatchell *et al.*, 1985; Tripp *et al.*, 1986; Boy-Marcotte *et al.*, 1987; Mbonyi *et al.*, 1988). The nutritional sensory systems whose existence is suggested by these observations do not appear to act directly on adenylyl cyclase but rather through intermediary proteins, three of which, encoded by the two *RAS* genes (Toda *et al.*, 1985), and the *CDC25* gene (Camonis *et al.*, 1986; Martegani *et al.*, 1986; Broek *et al.*, 1987; Daniel *et al.*, 1987), have been identified.

The plasma-membrane ATPase of *S. cerevisiae* is an H⁺-pump necessary for active nutrient transport (Serrano, 1984), and is an essential protein (Serrano *et al.*, 1986) whose activity, like that of adenylyl cyclase, is subject to nutritional regulation. H⁺-ATPase activity and cAMP levels are high in cells growing exponentially on glucose, and decrease rapidly when the glucose begins to be exhausted (François *et al.*, 1987). A rapid loss of H⁺-ATPase activity, as well as a rapid drop in cAMP levels, occurs when cells are harvested from growth medium and washed with water or a buffer. Addition of glucose to the washed cells results in a very rapid increase (3 to 8-fold) in cAMP levels (a peak is generally reached in less than a minute) (Van der Plaats,

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1974; Mazón *et al.*, 1982), followed by a slower activation of the H⁺-ATPase. Within 5 to 10 min the activity reaches a plateau value which generally approximates to the H⁺-ATPase activity present at the time of harvesting (Serrano, 1983). The mechanism of H⁺-ATPase activation and inactivation remains unknown, although some type of covalent modification is suggested by the fact that glucose activation of the H⁺-ATPase is accompanied by a change in the pH optimum and a lowering of the apparent K_m for ATP (Serrano, 1983).

The positive correlation between cAMP levels and H⁺-ATPase activity (François *et al.*, 1987) and the concomitant activation of H⁺-ATPase activity and increase in cAMP levels following a glucose pulse has implied a requirement for cAMP in the activation of the H⁺-ATPase. The recent finding that the *CDC25* gene product was required for the glucose-dependent activation of the H⁺-ATPase also implicated adenylyl cyclase as the effector system for the regulation of H⁺-ATPase activity (Portillo & Mazón, 1986). However, the relationship between the *CDC25* requirement and a possible cAMP requirement in H⁺-ATPase activation remained unclear. To re-examine the dependence of H⁺-ATPase activity on the adenylyl cyclase/cAMP pathway, we studied the starvation-induced inactivation and glucose-induced activation of the H⁺-ATPase in mutants that affect adenylyl cyclase, cAMP-dependent protein kinase (protein kinase A), and *RAS* activity.

METHODS

Strains and growth conditions. The strains used in this study are shown in Table 1. Cells were grown at 30 °C or at 24 °C (for the temperature-sensitive mutants) in (w/v) 1% yeast extract, 1% peptone, and 2% glucose broth. Growth was followed as the optical density at 660 nm. An OD₆₆₀ of 1 was equivalent to approximately 3.5 mg wet wt ml⁻¹.

Starvation-induced inactivation and glucose-induced activation of the H⁺-ATPase. To assay H⁺-ATPase activity in growing cells a volume of the culture calculated to give about 300 mg wet wt of cells was filtered rapidly and immediately frozen in liquid nitrogen without washing. H⁺-ATPase activities measured in cells taken in this manner are referred to as 'growth H⁺-ATPase levels'. To measure the response to removal from growth medium, vegetative cultures were harvested, washed twice with deionized water, resuspended in 0.1 M-MES buffer adjusted to pH 6.5 with Tris, and incubated at the desired temperature (generally 30 °C). After 15 min equilibration, two samples of 2 ml (about 300 mg wet wt cells) were removed to assay their H⁺-ATPase (basal) activity. The difference between the growth and basal H⁺-ATPase levels is referred to as starvation-induced inactivation. To measure the response of the washed cells to glucose (glucose-induced activation), 1 M-glucose was added to the rest of the suspension to give a final concentration of 0.1 M, and samples were taken at 5 and 10 min. For most strains

Table 1. *Yeast strains used*

Strain	Genotype	Source*
X2180-1A	<i>MATa Suc2 mal mel gal2 CUP1</i>	Yeast Genetic Stock Center
R146-11A	<i>MATa trp1 leu2 lys7 ura3-52 BCY1::URA3</i>	E. Martegani; Toda <i>et al.</i> , 1987
MSR1	<i>MATα bcy1-1 his7 ska1</i>	M. Behrens, M. Mazón, & R. Piñón
TK161-R2V	<i>MATa his3 leu2 ura3 trp1 ade8 can1 RAS2^{val19}</i>	J. Guinovart; Toda <i>et al.</i> , 1985
BR214-4a	<i>MATa ade1 arg4 his7 trp1 ura1 cdc35-1</i>	Yeast Genetic Stock Center
T27-25B	<i>MATa bcy1-1 ura3 leu2 his3 RAS1::HIS3 RAS2::LEU2</i>	O. Fassano; Toda <i>et al.</i> , 1985
TS1	<i>MATα ade2 can1-100 RAS1::URA3 ras2(ts)::SUP16 his3 leu2-3,112 lys1-1 ura3-52</i>	O. Fassano
TR4	<i>MATα ade2 can1-100 RAS1::URA3 ras2(ts)::SUP16 CRI4 his3 leu2-3,112 lys1-1 ura3-52</i>	O. Fassano
XL-1A	<i>MATα leu-2-112 his3-11,15 gal7</i>	Laboratory stock
RP11-19	<i>MATa leu2 his3 RAS2::LEU2</i>	This work, segregant of T27-25B × XL-1A

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tested under these conditions, the 5 and 10 min H⁺-ATPase activity levels were either equal or very similar; they represent the plateau levels and generally approximate the H⁺-ATPase activity the cells had at the time of harvesting, i.e., the growth levels. All samples were rapidly filtered through Millipore filters, and immediately frozen in liquid nitrogen and stored at -70 °C until use. When temperature-sensitive mutants were used, the cells were incubated in buffer at the permissive or restrictive temperature for 2 h before glucose was added.

Homogenization and membrane preparation. Frozen samples were allowed to thaw in 1.5 ml 50 mM-Tris, 5 mM-EDTA, 5 mM-dithiothreitol, pH 8.5, and homogenized by vortex-mixing with 3 g glass beads (0.45 mm diameter) for 4 min with intermittent cooling on ice. The total membrane fraction was prepared as described by Serrano (1983).

H⁺-ATPase assay. The plasma membrane ATPase was assayed using between 15 and 40 µg protein as described by Serrano (1983), except that lysolecithin was omitted.

cAMP assay. cAMP measurements were done as described previously (Portillo & Mazón, 1986).

Protein determination. Protein was determined by a modified Bradford procedure (Read & Northcote, 1981) using bovine serum albumin as standard.

RESULTS

Role of protein kinase A

In *S. cerevisiae*, as in other eukaryotes, the effects of cAMP are thought to be mediated largely if not exclusively by cAMP-dependent protein kinase (protein kinase A). We might expect, therefore, that if cAMP has a role in the nutritional regulation of H⁺-ATPase activity, some aspect of that regulation would be altered in cells carrying mutations affecting protein kinase A activity. We tested the role of protein kinase A in a strain (R146-11A) in which protein kinase A activity is no longer dependent on cAMP (Toda *et al.*, 1987; M. Behrens, M. J. Mazón & R. Piñón, unpublished), and also in a strain (MSR1) with undetectable levels of protein kinase A activity in exponentially growing cells (M. Behrens, M. J. Mazón and R. Piñón, unpublished). The latter phenotype is very similar to that of *bcy1 tpk^{w1}* cells, in which *tpk^{w1}* suppresses the *bcy1* phenotype (Cameron *et al.*, 1988). In strain MSR1 suppression of the *bcy1-1* phenotype is due to the (probably different) *sk1* mutation. Table 2 shows that in strain R146-11A, as in wild-type strains, the H⁺-ATPase is inactivated by washing and incubation in buffer; following addition of glucose, the H⁺-ATPase is activated normally. Similarly, Table 2 shows that in strain MSR1, starvation-induced inactivation, as well as glucose-induced activation of the H⁺-ATPase, also take place normally. It is notable that in the latter strain, higher levels of H⁺-ATPase activity were found during growth and also after addition of glucose to washed cells.

In principle, a functionally equivalent way of testing for the effects of a constitutively active (i.e., independent of cAMP) protein kinase A is to use a strain with a constitutively active adenylyl cyclase. This is provided by strain TK161-R2V carrying the dominant *RAS2^{val19}* mutation which confers high intracellular cAMP levels (Toda *et al.*, 1985). The phenotype of the *RAS2^{val19}* strain should be the same as that of the strain carrying the *BCY1* disruption (R146-11A). This expectation was confirmed (Table 2).

Role of adenylyl cyclase.

The possibility that presence of sufficiently high cAMP levels is necessary, but not in itself sufficient for H⁺-ATPase activation, is consistent with the finding of Portillo & Mazón (1986) that in the mutant *cdc25-1*, adenylyl cyclase, but not H⁺-ATPase, was activated by glucose at the restrictive temperature. To test the cAMP requirement more specifically, we analysed the activation of the H⁺-ATPase in a thermosensitive mutant carrying a lesion in the gene *CDC35*, encoding adenylyl cyclase (Boutelet *et al.*, 1985). Under standard glucose-induction conditions (Portillo & Mazón, 1986), we found only a very slight increase in cAMP levels in cells carrying the *cdc35-1* mutation (strain BR214-4a) (Table 3). In contrast, under the same conditions, H⁺-ATPase was activated normally at both temperatures. At 24 °C, activity rose from a basal level of 90 to 670 mU (mg protein)⁻¹ within 10 min of the addition of glucose (7.4-fold stimulation); at 37 °C, activity rose from 120 to 787 mU (mg protein)⁻¹ within 10 min (6.6-fold stimulation). This shows that the H⁺-ATPase can be activated in the absence of the cAMP increase produced by glucose, and is consistent with the phenotype of strain MSR1 as described above.

Table 2. *Starvation-induced inactivation and glucose-induced activation of the H⁺-ATPase in wild-type and non-temperature-sensitive mutant strains*

The optical density at 660 nm and the H⁺-ATPase activity measured at the time of harvesting ('Growth'), after harvesting and resuspension in buffer ('Basal'), and 10 min after the addition of glucose to cells resuspended in buffer ['10 min(Glc)']. The ratio of the 10 min to basal value is entered under 'Activation'. Sampling and activity determination were done as described in Methods.

Strain	OD ₆₆₀	Growth	Basal	10 min(Glc)	Activation
X2180	1.1	1003	150	809	5.4
R146-11A*	2.2	724	167	919	5.5
MSR1	1.1	1607	137	1332	9.7
TK161-R2V*	1.5	1093	256	1258	4.9
T27-25B	0.9	1044	148	898	6.1
RP11-19	1.2	516	146	728	5.0

* In these strains H⁺-ATPase activity remained at the basal level after incubation in buffer for up to 2 h (R146-11A) or 40 min (TK161-R2V). Values reported here are taken from one experiment but represent typical results.

Table 3. *cAMP levels following stimulation by glucose*

For strain BR214-4a an exponentially growing culture was harvested, washed, resuspended in buffer, divided into two equal portions, and incubated at 24 °C and 37 °C. After 2 h, glucose was added to each and samples were removed at the times indicated. cAMP measurements were done as described previously (Portillo & Mazón, 1986). The cAMP levels during growth were determined in a sample removed from the culture just prior to harvesting. For strain T27-25B a standard glucose-induction assay was done on a sample removed in exponential phase (OD₆₆₀ = 0.9) and on the remainder of the same culture allowed to reach stationary phase (OD₆₆₀ = 3.3).

Strain	Growth	cAMP (μM)				
		Basal	15 s	1 min	3 min	10 min
BR214-4a (24 °C)	0.65					
(24 °C)		0.58	0.71	0.73	0.70	0.60
(37 °C)		0.60	0.73	0.80	0.65	0.59
T27-25B						
OD ₆₆₀ = 0.9		0.095	0.086		0.086	0.064
OD ₆₆₀ = 3.3		0.059	0.065	0.053	0.053	0.043

Role of RAS1 and RAS2

Since in *S. cerevisiae* the *RAS1* and *RAS2* proteins are positive activators of adenylyl cyclase activity (Toda *et al.*, 1985), we tested their role in H⁺-ATPase activation in strain T27-25B (Table 1). T27-25B cells carry the *bcy1-1* mutation, which bypasses the disruptions in both *RAS* genes; cAMP levels are thus very low, and glucose produces absolutely no change in cAMP levels (Table 3). However, as shown in Table 2, in spite of the barely detectable cAMP levels, H⁺-ATPase activity was regulated normally, both with respect to starvation-induced inactivation and glucose-induced activation. This suggests that the *RAS* proteins are not required for regulation of the H⁺-ATPase. This conclusion is consistent with the results described above that indicate that the adenylyl cyclase/cAMP pathway is not required for H⁺-ATPase activation. However, since *bcy1-1* bypasses the requirement for *RAS* function *bcy1-1* might also suppress *RAS*-mediated H⁺-ATPase activation. A more rigorous test of *RAS* function would have to be in a non-*bcy1-1* background.

We first tested a strain (RP11-19) with *RAS2* disrupted but *RAS1* intact. The H⁺-ATPase was activated normally (Table 2), suggesting that in the presence of a functional *RAS1* product, the *RAS2* product is not required for glucose-induced activation of the H⁺-ATPase. We then tested

Table 4. Glucose-induced H⁺-ATPase activation in strains TS1 and TR4

Cultures of TS1 and TR4 were harvested at the concentrations indicated, washed, resuspended in buffer, divided in equal portions, and incubated at 24 °C and 39 °C. After 2 h glucose was added and samples were removed at 5 min (data not shown) and 10 min and assayed for H⁺-ATPase activity. Basal values were those obtained within 15 min after resuspension in buffer. H⁺-ATPase activities after 1 and 2 h incubations in buffer at each temperature are also shown. At 24 °C, the H⁺-ATPase was activated 5.1- and 5.2-fold, and at 39 °C, 3.8- and 4.1-fold in strains TS1 and TR4, respectively.

Strain	OD ₆₆₀	Basal	H ⁺ -ATPase activity [mU (mg protein) ⁻¹]					
			24 °C			39 °C		
			Buffer		Glucose	Buffer		Glucose
			1 h	2 h	10 min	1 h	2 h	10 min
TS1	0.9	242	235	205	1154	180	179	685
TR4	1.5	212	271	209	1200	276	131	840

strains TS1 and TR4. Strain TS1 carries a disruption of *RAS1*, and a lesion in *RAS2*, which permits growth at 24 °C, but not 39 °C (De Vendittis *et al.*, 1986). The mutant *RAS2* gene in TS1 cells has recently been shown to encode a protein in which glycine residues in positions 82 and 84 have been replaced by serine and arginine, respectively (Fasano *et al.*, 1988). Strain TR4, isolated as a suppressor of TS1, carries, in addition, a dominant mutation, *CRI4*, in the gene encoding adenylyl cyclase; this renders adenylyl cyclase constitutively active, bypassing the need for *RAS*-mediated activation of adenylyl cyclase (De Vendittis *et al.*, 1986). The results of standard glucose-induction assays of the H⁺-ATPase activity of TS1 and TR4 cells at 24 °C and 39 °C are shown in Table 4. Since at the permissive temperature, the H⁺-ATPase is activated 5.1-fold in strain TS1 and 5.2-fold in strain TR4 (Table 4), we conclude that in the presence of a functional *RAS2* protein, the *RAS1* protein is not required for H⁺-ATPase activation. At 39 °C, which leads to a tight block to growth in strain TS1, the extent of H⁺-ATPase activation in strain TS1 is reduced to 3.8-fold. However, in *CRI4* cells (strain TR4), in which cAMP levels and growth at 39 °C are restored, the extent of H⁺-ATPase activation is 4.1-fold, which does not differ significantly from that in TS1 cells. Hence, restoration of cAMP levels in *CRI4* cells does not affect H⁺-ATPase activation. Inactivation of the *RAS2* protein at the restrictive temperature reduces the extent of H⁺-ATPase activation, but this reduction is not decisive with respect to growth.

DISCUSSION

The plasma-membrane ATPase of *S. cerevisiae* is a protein whose activity as an H⁺-pump appears to be essential for vegetative growth (Serrano *et al.*, 1986). Circumstantial evidence had previously indicated that the regulation of H⁺-ATPase activity might be mediated by cAMP. We tested this hypothesis by analysing the starvation-induced inactivation and glucose-induced activation of the H⁺-ATPase in mutants that inactivate or otherwise affect the activity of the *RAS* proteins, adenylyl cyclase, or cAMP-dependent protein kinase (protein kinase A). Since the effects of cAMP are thought to be mediated by protein kinase A, we first sought evidence for the participation of protein kinase A in the regulation of H⁺-ATPase activity. This analysis showed that in cells with a constitutively active protein kinase A, the result of either the absence of its regulatory subunit (strain R146-11A) or constitutively high levels of cAMP (strain TK161-R2V), was that starvation-induced H⁺-ATPase inactivation took place normally. We concluded, therefore, that neither cAMP nor protein kinase A have a role in the starvation-induced inactivation of the H⁺-ATPase. Also, normal H⁺-ATPase activation in strain MSR1,

characterized by the absence of detectable protein kinase A activity *in vitro*, suggests that protein kinase A does not play a direct role in the glucose-induced activation of the H⁺-ATPase.

To test the role of cAMP more specifically, we analysed mutants of adenylyl cyclase and mutants in *RAS1* and *RAS2*. We found that in the temperature-sensitive adenylyl cyclase mutant *cdc35-1*, the H⁺-ATPase activity is regulated normally irrespective of the cAMP levels.

Analysis of the role of *RAS* showed that neither *RAS1* nor *RAS2* are required for the glucose-induced activation of the H⁺-ATPase. Our observation that in strain T27-25B the H⁺-ATPase is activated normally, while adenylyl cyclase is not, confirmed that the absence of the *RAS* proteins prevents the glucose-induced activation of adenylyl cyclase (Mbonyi *et al.*, 1988), and suggested strongly that the *RAS* proteins are not required for H⁺-ATPase activation. To eliminate the possibility that H⁺-ATPase activation had occurred in strain T27-25B because of the *bey1-1* mutation, we analysed the requirement for *RAS* function in a non-*bey1-1* background. We found that in strain TS1 (carrying a *RAS1* disruption and the double mutation in *RAS2* that results in temperature-sensitive growth), H⁺-ATPase activation at the restrictive temperature was reduced by about 25% compared to that at the permissive temperature (Table 4). However H⁺-ATPase activation in strain TR4, which carries a dominant mutation (CR14) in adenylyl cyclase that restores cAMP levels and suppresses the *RAS2*-dependent temperature-sensitive phenotype of TS1, did not differ from that in TS1 cells. The similarity of H⁺-ATPase activation in strains TR4 and TS1 suggests that H⁺-ATPase activation is not dependent on cAMP. This conclusion is further strengthened by the finding that although adenylyl cyclase activity in plasma membranes from TS1 cells is almost absent, the cells grow efficiently on glucose at 30 °C (Fasano *et al.*, 1988). Nevertheless, the phenotypic similarity between TS1 and TR4 cells with respect to H⁺-ATPase activation does suggest that the perturbation produced by the mutant *RAS2* protein on H⁺-ATPase activation is probably real, but that such a perturbation is not the reason for the temperature-sensitive growth phenotype of TS1. More likely, perhaps, is that the mutant *RAS2* protein contained in TS1 and TR4 cells interferes with or affects other elements in the signalling mechanism that activates the H⁺-ATPase. A comparable difference between the phenotypic consequences of null and missense mutations has also been observed in an analysis of the role of *SNF3* in the expression of invertase (Neigeborn *et al.*, 1986).

The clear differences in the response of adenylyl cyclase and the H⁺-ATPase to glucose stimulation between the mutant *cdc25-1* (Portillo & Mazón, 1986) and the mutants studied in this report suggest that the glucose-induced activation of the H⁺-ATPase is a *CDC25*-mediated function independent of the adenylyl cyclase/cAMP system. This conclusion is consistent with other observations that hint at a complex role for the *CDC25* product (for a discussion, see Tripp & Piñón, 1988). Our finding that a mutant *RAS2* protein perturbed the glucose-induced activation of the H⁺-ATPase may suggest that the *CDC25* and the *RAS2* products interact directly.

What might be the role of *CDC25* with respect to H⁺-ATPase activation? A complete answer will likely require that the *CDC25* requirement be placed in the context of the other known requirements for H⁺-ATPase activation, namely glucose transport and metabolism. Glucose metabolism is required, since non-metabolizable analogues of glucose fail to activate the H⁺-ATPase (Serrano, 1983). In contrast, activation of adenylyl cyclase by glucose does not require glucose metabolism (Eraso & Gancedo, 1985). The requirement for glucose metabolism suggests that the primary signal for H⁺-ATPase activation is generated inside the cell. One possibility is that the *CDC25* protein might be a sensor (or be coupled to a sensor) of signals generated by glucose metabolism, such as transient acidification (Caspani *et al.*, 1985; Valle *et al.*, 1986), or perhaps a key glycolytic intermediate. It will be of interest to determine whether the *CDC25* protein is functionally coupled to the H⁺-ATPase.

Thanks are due to Juana Maria Gancedo for the critical reading of the manuscript. This work was supported by the Comisión Asesora de Investigación Científica y Técnica and from the Fondo de Investigaciones Sanitarias. R.P. was supported by a grant from the US Spanish Joint Committee for Scientific and Technological Cooperation.

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