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Multiple transduction pathways regulate the sodium-extrusion gene *PMR2/ENA1* during salt stress in yeast

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Abstract The yeast *PMR2/ENA1* gene encodes an ATPase involved in sodium extrusion and induced by NaCl. At low salt concentrations (0.3 M) induction is mediated by the HOG-MAP kinase pathway, a system activated by non-specific osmotic stress. At high salt concentrations (0.8 M) induction is mediated by the protein phosphatase calcineurin and is specific for sodium. Protein kinase A and Sis2p/Hal3p modulate *PMR2/ENA1* expression as negative and positive factors, respectively but Sis2p/Hal3p does not participate in the transduction of the salt signal. Salt stress decreases the level of cAMP and the resulting decrease in protein kinase A activity may contribute to HOG-mediated induction.

Key words: Signal transduction; Salt stress; MAP kinase; Calcineurin; cAMP; *Saccharomyces cerevisiae*

1. Introduction

Living cells can withstand a variety of stress conditions such as extreme temperatures, water deficit, high salt concentrations, starvation etc. Under these circumstances organisms modify their cellular machinery in order to counteract the deleterious effect of damaging agents. This constitutes a stress response and involves modulation of enzymatic activities as well as changes in gene expression [1,2]. In recent years considerable interest has been raised by the mechanisms of plant tolerance to drought and salinity, two major factors limiting agricultural productivity [3]. The signal transduction pathways operating during osmotic and salt stress are only partially understood and this gap of knowledge is a limiting factor for urgent biotechnological applications [2].

The yeast *Saccharomyces cerevisiae* can be utilized as a convenient model system in salinity studies [2]. Salt stress has two major harmful effects for cells: the loss of turgor pressure and the toxicity of Na^+ ions to cellular metabolism. The adaptation of yeast to these conditions requires the modification of the plasma membrane transport systems to exclude Na^+ ions from the cytosol and the onset of glycerol synthesis to restore turgor pressure. This adaptation is largely based on changes in the expression of key genes such as *PMR2/ENA1* (in the following referred to as *ENA1*), encoding an ATPase involved in sodium extrusion [4], and *GPD1*, encoding a dehydrogenase involved in glycerol synthesis [5]. Many other genes are also induced by salt stress whose contribution to the adaptation, if any, is not well understood [2].

In the present work we have investigated the environmental signals and transducing pathways mediating the induction of the *ENA1* gene by salt stress. Genetic analysis has recently disclosed several signal transduction pathways triggered by salt stress in yeast. A MAP kinase cascade (the so-called HOG pathway) is activated by the osmotic component of the salt [6] and it is involved in the induction of many genes [7], including *GPD1* [5], but its participation in *ENA1* expression was not investigated. The calcium-activated protein phosphatase calcineurin [8–10] and the novel regulatory protein Sis2p/Hal3p (in the following referred to as Hal3p) [10] have been implicated in the expression of *ENA1* but the actual signal transduced by these pathways was not clarified. Finally, protein kinase A (in the following referred as PKA) is a general modulator of stress responses in yeast [2,7,11] of unknown effects on *ENA1*. Our results indicate that *ENA1* responds to two different environmental signals contributed by salt stress: osmotic concentrations (via the HOG pathway) and sodium (via the calcineurin pathway). PKA and Hal3p also modulate *ENA1* expression. While Hal3p does not transduce the salt signal, a decrease in cAMP levels triggered by salt may implicate PKA in HOG-mediated induction.

2. Materials and methods

YPD and synthetic medium were prepared as described [12]. YPD contained 2% glucose, 1% yeast extract and 2% peptone. Synthetic medium contained 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco), 50 mM MES [2-(*N*-morpholino) ethanesulfonic acid] adjusted to pH 6.0 with Tris and the amino acids, purine and pyrimidine bases required by the strains.

Strains YPH499 (*MATa ura3 leu2 his3 trp1 lys2 ade2*) and JBY10 (isogenic to YPH499 with *hog1-Δ1::TRP1*) [6] were obtained from Dr. M. Gustin (Houston). The *hog1 hal3* double mutant was obtained by generation of a *hal3::LEU2* genomic deletion in the JBY10 background as described [10]. Strains SP1 (*MATa ura3 leu2 trp1 ade8 can1*) and S13-58A (isogenic to SP1 with *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*) [13] were obtained from Dr. Francisco Portillo (Madrid). Strain RS-16 (*MATa ura3 leu2*) has been described [10]. Strains were transformed [14] with autonomous plasmid pFR70 and with integrative plasmid pFR70i [8] (kindly provided by Prof. A. Rodriguez-Navarro, Madrid), carrying an *ENA1-LacZ* promoter-reporter fusion. Integration of pFR70i was directed to the *URA3* locus by *Nco*I digestion.

For gene expression assays, NaCl was added to exponentially growing cells (absorbance at 660 nm 0.8–1.2) in YPD or YPD containing 2 μg/ml of the calcineurin inhibitor FK506 [10,15] and the culture incubated at 30°C for 90 min. β-galactosidase activity was assayed in permeabilized cells as described [16] and normalized to protein concentration. Values represent the average of at least three determinations with three independent transformants. Bars in the figures correspond to standard deviations. FK506 was a generous gift of Dr. Ihor Bekersky from Fujisawa Pharmaceutical Company, Deerfield, Illinois.

For CAMP measurements, NaCl was added to exponentially growing cells in YPD (absorbance at 660 nm 0.8–1.2) and the culture incubated at 30°C for 30 min. Aliquots of 10 ml were filtered (Whatman GF/C circles, 25 mm) and extracted in the cold with 1 ml 2 N perchloric acid. After neutralization with 1 ml of 1.8 N KOH and 0.4

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N $KHCO_3$ and centrifugation, samples were purified with Amprep SAX minicolumns (Amersham code RPN 1918), eluted with methanol-HCl and dried under vacuum. Assays were performed by a competitive binding method with the Amersham cAMP enzymeimmunoassay (EIA) system (code RPN 225).

For calculation of yeast concentrations, one unit of absorbance at 660 nm was equivalent to 2.5 mg fresh weight/ml and 0.25 mg protein/ml.

3. Results

Previous work has implicated calcineurin and Hal3p as regulators of *ENA1* expression [8–10]. However, we have realized that induction of this gene by low concentrations of NaCl (0.1–0.4 M) was independent of both regulatory proteins [10]. One possible transduction pathway operating under these conditions is the HOG-MAP kinase pathway, which displays maximal levels of activation at 0.3 M NaCl [6,17]. In order to test this hypothesis, an *ENA1-LacZ* promoter-reporter fusion was integrated into strains YPH-499 and JBY-10 [6], corresponding to wild type and *hog1* deletion mutant, respectively. Reporter expression was determined at low (0.3 M) and high concentration (0.8 M) of NaCl and in the absence and presence of the calcineurin inhibitor FK506. As shown in Fig. 1, the induction of *ENA1* at 0.3 M NaCl is abolished in the *hog1* mutant, indicating that regulation of this gene under these conditions is mediated by the HOG pathway. However, when the *hog1* mutant was treated with higher concentrations of salt (0.8 M NaCl) *ENA1* expression was still induced about four-fold. This remaining induction was abolished when the cells were grown in the presence of FK506, a specific inhibitor of calcineurin [10,15]. These results suggest that *ENA1* gene expression can be activated at high concentrations of NaCl by the calcineurin pathway in a HOG-independent manner. The fact that *ENA1* induction cannot be triggered by 0.3 M NaCl in a *hog1* mutant and that FK506 does not alter the level of expression in a wild-type strain at this salt concentration suggest that the calcineurin pathway requires concentrations higher that 0.3 M NaCl to be activated.

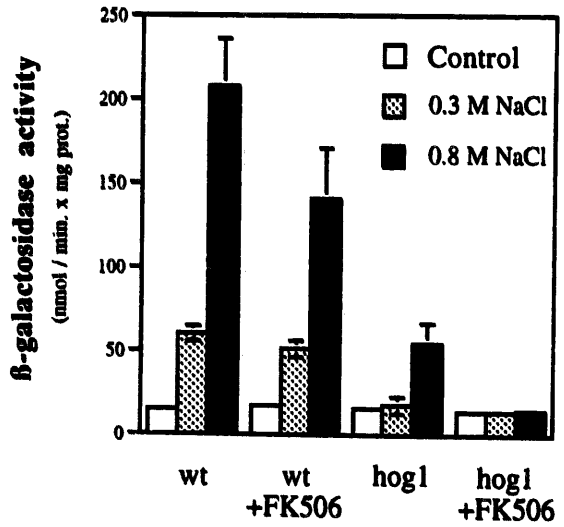

Fig. 1. Salt induction of *ENA1* is controlled by the HOG-MAP kinase and by calcineurin. Plasmid pFR70i (with an *ENA1-LacZ* fusion) was integrated into strains YPH-499 (wild type) and JBY-10 (*hog1* mutant). Cells growing exponentially in normal medium or in medium supplemented with the calcineurin inhibitor FK506 (2 μg/ml) were treated for 90 min with either 0.3 M NaCl or 0.8 M NaCl and β-galactosidase activity determined as described in section 2.

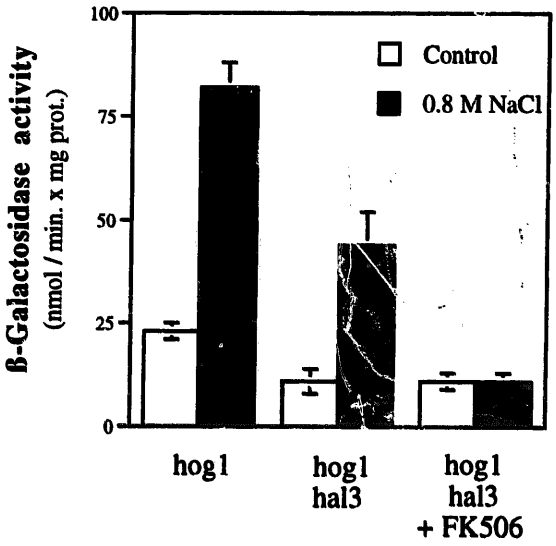

Fig. 2. *ENA1* expression is positively modulated by *HAL3*, which is dispensable for salt induction. Plasmid pFR70i (carrying an *ENA1-LacZ* fusion) was integrated into a *hog1* mutant and a *hog1 hal3* double mutant. Cells growing exponentially in normal medium or in medium supplemented with the calcineurin inhibitor FK506 (2 μg/ml) were treated for 90 min with 0.8 M NaCl and β-galactosidase activity determined as described in section 2.

It has been reported that the product of the *HAL3* gene (Hal3p) is required for full expression of *ENA1* and that it probably acts in parallel to calcineurin [10]. Our finding that the HOG pathway also regulates *ENA1* expression prompted a re-evaluation of the role of Hal3p in a *hog1* mutant. As shown in Fig. 2, although the level of *ENA1* gene expression is reduced in a *hal3* mutant, *ENA1* induction is still possible by 0.8 M NaCl in a *hog1 hal3* double mutant. In fact, the induction factor (4-fold) remains unchanged as compared to the *hog1* single mutant. This induction, however, is completely abolished by the addition of FK506. These results suggest that Hal3p acts as a constitutive element required for maximal expression of *ENA1*, rather than an element of a signal transduction pathway activated by salt stress.

The signal activating the HOG pathway is osmotic stress because NaCl and sorbitol have similar effects on tyrosine phosphorylation of the Hog1p MAP kinase [6] and on induction of HOG-dependent genes [7,17]. In Fig. 3 the specificity of the calcineurin pathway was investigated by measuring *ENA1* expression in a *hog1* mutant in the presence of different solutes The FK506-sensitive induction remaining in this mutant was specific for high sodium concentrations, because NaCl was a much better inducer than either KCl or sorbitol. Therefore the calcineurin pathway is not responsive to osmotic stress but specifically to high sodium concentrations.

Finally, the effect of the cAMP-dependent PKA on *ENA1* gene expression was investigated. An *ENA1-LacZ* promoter-reporter fusion was introduced into strains SP1 (wild type) and S13-58A (*bcy1*), displaying normal and high, unregulated levels of PKA activity, respectively [11,13]. As shown in Fig. 4, both the basal and the salt-induced levels of expression are negatively affected by PKA activity. However, the induction factor by high salt (8-fold) is maintained in the *bcy1* mutant.

As indicated in Fig. 4, the induction factor at low salt

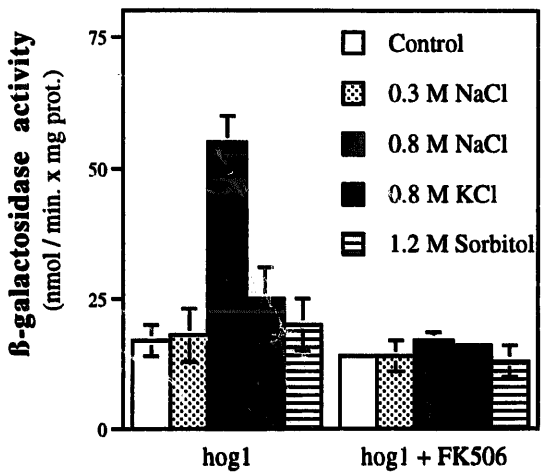

Fig. 3. The calcineurin pathway of *ENA1* induction is sodium-specific. Strain JBY-10 (*hog1* mutant) carrying the integrated *ENA1-LacZ* fusion was grown in YPD medium or in YPD containing the calcineurin inhibitor FK506 (2 μg/ml). Cells were treated for 90 min with 0.3 M NaCl, 0.8 M NaCl, 0.8 M KCl or 1.2 M sorbitol (equivalent osmotic concentration to 0.8 M salt) before determination of β-galactosidase activity.

concentration is reduced in the *bcy1* mutant (from 5-fold to 2-fold). This suggested some role of the cAMP-PKA pathway in the HOG-mediated induction operating under these conditions. As PKA activity has negative effects on the expression of stress genes [2,7,11], one plausible mechanism was that salt stress decreased CAMP levels and thereby inhibited PKA. As indicated in Fig. 5, this is actually the case. The decrease of cAMP reached its maximum value (a 2-fold reduction) at 0.5 M NaCl, with relatively smaller changes at higher salt concentrations.

4. Discussion

The major goal of the present work was to identify the environmental signals and transduction pathways regulating the expression of a gene which is crucial for salt tolerance in *Saccharomyces cerevisiae*: the sodium-extrusion ATPase gene *ENA1* [2,4]. Our results indicate that *ENA1* induction under salt stress requires the contribution of two different pathways: the HOG pathway, that controls expression at low concentrations of NaCl, and the calcineurin pathway, which is responsible for induction at high concentrations of NaCl. These two pathways respond to different environmental signals associated with salt stress. While the HOG pathway is responsive to non-specific osmotic stress [6,17], our results indicate that calcineurin-dependent induction can only be triggered by NaCl and not by KCl or sorbitol. This suggests that the calcineurin pathway is specifically activated by sodium ions. To our knowledge this is the first clue for a sodium-activated transduction pathway in yeast and the nature of the sodium sensor mediating activation of calcineurin deserves further investigation. As the only known regulation of calcineurin is calcium activation [15], it is plausible that high sodium concentrations increase intracellular free calcium in yeast. In this respect, it has been reported that salinity stress increases cytoplasmic calcium activity in maize root protoplasts [18]. Therefore the sodium sensor could correspond to a component of the calcium homeostasis machinery.

The only sodium-specific pathway characterized at the molecular level has been reported in *Escherichia coli*. In this organism, a gene coding for a Na^+/H^+ antiporter, *nhaA*, has been isolated which is indispensable for the adaptation to high salinity [19]. The expression of this antiporter is specifically induced by Na^+ and Li^+ and requires NhaR, a positive regulator that belongs to the OxyR-LysR family of transcription factors [20]. Mutations in this regulatory gene have been isolated that affect the Na^+ sensitivity of *nhaA* expression, suggesting that NhaR might represent a Na^+ sensor [21].

Genes regulated by the HOG pathway contain a positive stress promoter element (STRE) with core consensus sequence CCCCT or AGGGG [22,23]. A single STRE-like sequence is found in the *ENA1* promoter region (−651 from ATG) and it could mediate part of the effects observed in this work. However, genes containing functional STREs display several copies of the element [7,24]. In addition, a gene has been described that is HOG-dependent but lacks any STRE-related sequence [17]. Further work is required for the identification of the cis-acting elements of the *ENA1* promoter utilized by the different signal transducing pathways.

HAL3p is a positive factor for *ENA1* expression [10] but our results indicate that it does not participate in the salt induction mechanism. This is concluded from the fact that basal and salt-induced levels of *ENA1* expression are similarly affected by the *hal3* mutation. The nature of the signal transduced by Hal3p, if any, is presently unknown [10].

In accordance with the findings with other HOG-regulated genes [2,7], PKA is a negative factor for *ENA1* expression. PKA does not participate in the induction of *ENA1* by high salt concentrations mediated by calcineurin because basal and induced levels of expression are similarly affected by the *bcy1* mutation. However, there is a complex interaction between the HOG and PKA pathways in the regulation of *ENA1* by low salt concentrations. Induction by 0.3 M NaCl is abolished in *hog1* mutants, pointing to a major role of the HOG pathway. But the *bcy1* mutation significantly decreases the induction factor (from 5-fold to 2-fold). In the *bcy1* mutant PKA is highly active and not regulated by cAMP [11,13]. Therefore,

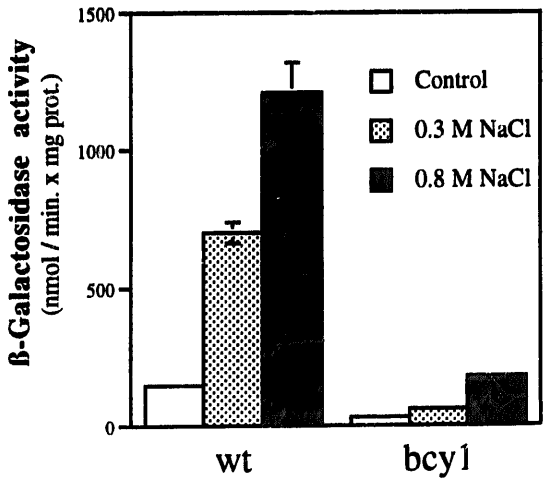

Fig. 4. *ENA1* expression is negatively modulated by PKA. Plasmid pFR70 (with an *ENA1-LacZ* fusion) was introduced into strains SP1 (wild type) and S13-58A (*bcy1* mutant). Cells growing exponentially in normal medium were treated for 90 min with 0.8 M NaCl and β-galactosidase activity was determined as described in section 2.

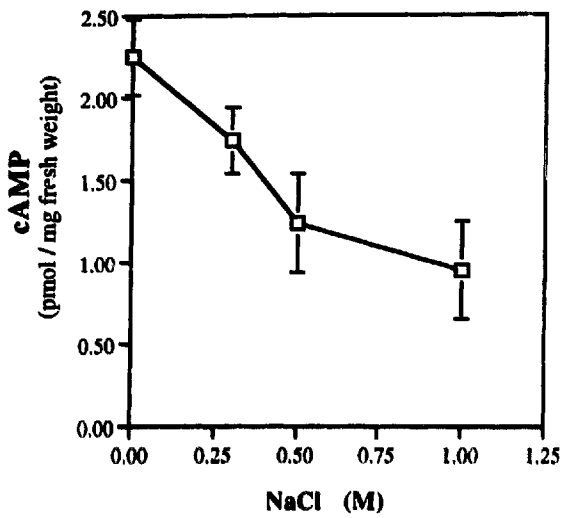

Fig. 5. Salt stress decreases cAMP levels. Cells (strain RS-16) growing exponentially in normal medium were treated for 30 min with the indicated concentrations of NaCl and intracellular cAMP concentrations were determined as described in section 2.

in addition to a general negative effect of PKA on the expression of stress genes [2,7,11], regulation of PKA during osmotic stress somehow contributes to HOG-dependent induction. Similar results have been found with another HOG-dependent gene, *CTT1*, where the *bcy1* mutation decreased the induction factor (0.3 M NaCl) from 12-fold to 5-fold [7]. Our finding of a decrease in cAMP during salt stress (Fig. 5) may provide an explanation for all these observations. As PKA is a negative factor for expression, the expected inhibition of PKA activity upon cAMP decrease may reinforce HOG-mediated induction. It would be interesting to determine if this interaction between the HOG and PKA pathways occurs at the level of either transcription factors or upstream signalling components.

The identification of transduction pathways and environmental signals mediating *ENA1* expression will facilitate the genetic dissection of salt tolerance in *Saccharomyces cerevisiae* and it may provide clues for the less tractable plant systems [2]. In addition, the multiplicity of pathways and signals affecting *ENA1* expression raises questions about the physiological role and specificity of stress responses.

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