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Regulation of Trk-dependent potassium transport by the calcineurin pathway involves the Hal5 kinase

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ABSTRACT

The phosphatase calcineurin and the kinases Hal4/Hal5 regulate high-affinity potassium uptake in *Saccharomyces cerevisiae* through the Trk1 transporter. We demonstrate that calcineurin is necessary for high-affinity potassium uptake even in the absence of Na^+ stress. *HAL5* expression is induced in response to stress in a calcineurin–dependent manner through a newly identified functional CDRE (nt −195/−189). Lack of calcineurin decreases Hal5 protein levels, although with little effect on Trk1 amounts. However, the growth defect of *cnb1* cells at K^+-limiting conditions can be rescued in part by overexpression of *HAL5*, and this mutation further aggravates the potassium requirements of a *hal4* strain. This suggests that the control exerted by calcineurin on Hal5 expression may be biologically relevant for Trk1 regulation.

1. Introduction

The regulation of the intracellular concentrations of the major monovalent cations (H^+, K^+ and Na^+) is crucial to ensure proper function of many cellular systems. In most plants and fungi, K^+ is the major intracellular cation and it is largely responsible for the maintenance of appropriate cellular volume, electrical membrane potential and ionic strength. In contrast, most cells strive to maintain a relatively low concentration of sodium cations, particularly when K^+ supply is sufficient [1,2]. The concentration of K^+ in most natural environments is much lower than the intracellular concentration of this cation. Therefore, many organisms have developed high-affinity K^+ uptake systems able to accumulate potassium [1] .

In the model yeast *Saccharomyces cerevisiae*, high-affinity potassium transport is mediated by the partially redundant Trk1 and Trk2 plasma membrane proteins [3,4]. Trk1, by far the most active transporter, can work in a dual mode, showing low or high-affinity for the cation [1]. Work in the last few years has identified the existence of diverse regulatory factors for high-affinity potassium transport, such as the Hal3/Ppz system [5,6], the SR protein kinase Sky1 [7] or the osmo-induced protein Hal1 [8], although the precise regulatory mechanisms remain to be elucidated. A pair of partially redundant protein kinases, Sat4/Hal4 and Hal5, positively regulate Trk1 transporter function and confer salt tolerance when overexpressed [9]. Very recently, it has been shown that they are required for maintenance of Trk1 at the plasma membrane, particularly when K^+ levels in the medium are low [10]. Finally, an early report identified the calcium-activated protein phosphatase calcineurin as a necessary element for the transition of the transporter system to the high-affinity potassium state induced by Na^+ stress, which helps to discriminate K^+ over Na^+ [11]. However, in spite of this information, the molecular basis of the regulation of Trk1 function and the possible interconnection of the regulatory processes described above remain largely unexplored.

Calcineurin plays an important role in the response to different forms of stress, including cation stress [12]. In *S. cerevisiae*, the enzyme is a heterodimer composed of one catalytic subunit (encoded by *CNA1* or *CNA2*) and one regulatory subunit (encoded by *CNB1*). Its activation under stress conditions is triggered by a transient increase in intracellular calcium and results in changes in gene expression, essentially mediated through the Crz1/Tcn1/Hal8 transcription factor [13–16]. Upon dephosphorylation by calcineurin, Crz1 enters the nucleus [17] and binds to specific CDRE sequences

Abbreviations: CDRE, Calcineurin Dependent Response Element; DTE, 1,4-dithioerythritol

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(Calcineurin Dependent Response Elements), present in the promoter region of target genes.

Calcineurin exerts a complex influence on cation homeostasis. First, activation of the phosphatase upon sodium stress or alkaline pH increases the expression of the *ENA1* gene [11,18,19], which encodes a Na^+-ATPase required for sodium detoxification, by direct binding of Crz1 to the *ENA1* promoter [20–22]. Second, as mentioned above, in the presence of sodium stress, calcineurin affects potassium transport, presumably through regulation of the Trk1 transporter [11]. However, neither the possibility that calcineurin may play a role in the regulation of potassium transporters in the absence of stress nor the possible interaction between calcineurin and other pathways regulating Trk1 function have been investigated. In this work, we demonstrate that disruption of calcineurin signalling affects potassium uptake and increases potassium requirements even under standard growth conditions. In addition, we show that expression of the *HAL5* protein kinase gene is under the control of calcineurin and Crz1, thus establishing a functional link between two important regulators of Trk1 activity and potassium homeostasis.

2. Materials and methods

2.1. Yeast strains and growth conditions

Yeast cells were grown at 28 °C in YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose) or, when carrying plasmids, in synthetic complete medium [23] containing 2% glucose and lacking the appropriate selection requirements. Strains used in this work are listed in Table 1. Strain ESV212 was described in Ref. [24]. Strain MAR89 was constructed by transforming strain EDN92 with a previously described *cnb1::TRP1* disruption cassette [25]. Strain MAR91 was made by transforming MAR89 with a 2.44 Kbp *trk2::HIS3* cassette obtained from plasmid pRH-3 [26] by EcoRV/BamHI digestion. This strain was transformed with a 4.5 Kbp *trk1::LEU2* fragment, derived from plasmid pRH2.23 [26] digested with SacI, to yield strain MAR108. Construction of strains CCV31 and CCV32 is described in Supplementary data. Plasmid YEp-HAL5 contains the *HAL5* gene cloned into the multicopy vector YEp352, and includes 517 bp of 5′ and 201 bp of 3′ untranslated regions [10].

Potassium requirements on solid media was tested by spotting cells (5 μL) at initial OD_{600} of 0.05 on ammonium phosphate (AmP) medium agar plates containing different KCl concentrations. Doubling time was calculated as in [27]. In some cases (i.e. Fig. 4B and C), growth under limiting external potassium concentrations was carried out using a newly developed YNB-based liquid medium (Translucent K-free medium, [28]) whose final potassium content is negligible (~15 μM).

Table 1
Yeast strains used in this work.

Strain	Genotype	Source/ Reference
DBY746	*MAT α, trp1, leu2-3, ura3, his3*	Botstein et al.
MAR15	DBY746 *cnb1::KAN*	[19]
EDN92	DBY746 *crz1::KAN*	[19]
ESV212	DBY746 *trk1::LEU2 trk2::HIS3*	[24]
MAR89	DBY746 *crz1::KAN cnb1::TRP1*	This work
MAR91	DBY746 *crz1::KAN cnb1::TRP1 trk2::HIS3*	This work
MAR 108	DBY746 *crz1::KAN cnb1::TRP1 trk1::LEU2 trk2::HIS3*	This work
CCV31	DBY746 *hal4::HIS3*	This work
CCV32	DBY746 *cnb1::KAN hal4::HIS3*	This work

2.2. K^+ cellular content and Rb^+ transport experiments

The K^+ content of cells growing in AmP medium was determined essentially as in Ref. [29]. The kinetic constants for rubidium transport were determined in K^+-starved cells as described in Supplemental data.

2.3. LacZ reporters and β-galactosidase assays

Translational fusions of the *HAL4* and *HAL5* promoters to the LacZ gene were generated as follows. The regions −936/+45 of *HAL4* and −615/+33 of *HAL5* were amplified from genomic DNA using oligonucleotides pairs 5′-promHAL4/3′-promHAL4 and 5′-promHAL5/3′-promHAL5, respectively (see Supplementary data). Artificial EcoRI and XbaI ends were added to facilitate cloning into these same sites of plasmid YEp357, yielding pHAL4 and pHAL5. Mutagenesis of putative CDREs in the *HAL5* promoter was performed by two-step PCR as described in Supplementary data to yield constructs pHAL5-P^{CDRE1} and pHAL5-P^{CDRE2}, respectively. Assay of constructs was performed essentially as described [21]. Alkaline stress was carried out by resuspending cells in YPD buffered at pH 8.2 with 50 mM TAPS. For calcium stress, $CaCl_2$ was added to reach a final concentration of 0.2 M. In both cases cells were collected after 1 h.

2.4. Protein extractions and immunoblot analysis

For NaCl and $CaCl_2$ treatments, the indicated yeast strains were grown to an OD_{660} of 0.4 (5×10^6 cells/mL) in YPD media. Cells were treated with 0.8 M NaCl or 0.1 M $CaCl_2$ for 30 min, harvested by centrifugation and frozen at −70 °C. For K^+ starvation experiments, cells were grown to an OD_{660} of 0.4 in AmP media supplemented with 10 mM KCl. Cells were washed extensively in water and resuspended at the same density in ammonium phosphate media with or without K^+ supplementation (10 mM) and incubated for 2 h, harvested by centrifugation and frozen at −70 °C. For protein extraction, cells were resuspended in homogenization buffer (50 mM Tris pH 8.0, 0.1 M KCl, 5 mM EDTA, 5 mM 1,4-dithioerythritol (DTE), 20% sucrose (w/v), plus protease inhibitor cocktail (Roche)) and lysed by vortexing with glass beads. The lysate was collected after centrifugation for 5 min at 500×*g*. The crude extract was separated into soluble and particulate fractions by centrifugation for 30 min at 16 000×*g* at 4 °C. The particulate fraction was resuspended directly in Laemmli sample buffer, electrophoresed and immunoblots probed for Trk1 and Hal5 as described [30]. The relative intensity of each band was determined by quantifying digital images of the band corresponding to Hal5 (or Trk1) and a representative band in the same lane of the PonS-stained filter (loading control) using the Image Gauge V4.0 software (FUJIFILM). The local background was subtracted in each case. The value of the first control lane (defined as Hal5 intensity/loading control intensity) was set at 1.0 and the rest of the results are expressed as relative intensities in arbitrary units. Similar results were observed in two different experiments in all cases.

3. Results and discussion

Almost 15 years ago, a functional interaction between the Trk1 high-affinity potassium transporter and the calcineurin phosphatase was proposed [11]. The authors reported that the presence of functional calcineurin was important for proper Na^+/K^+ discrimination by the Trk1 transporter under conditions of salt stress. To investigate the mechanism of this regulation we performed, as a first step, a detailed characterization of the potassium requirements of mutant strains lacking the regulatory subunit of calcineurin, *CNB1*,