Regulation of Monovalent Ion Homeostasis and pH by the Ser-Thr Protein Phosphatase SIT4 in Saccharomyces cerevisiae*

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A gene, SIT4, was identified as corresponding to a serine/threonine protein phosphatase and when overexpressed confers lithium tolerance in galactose medium to the budding yeast Saccharomyces cerevisiae. This gene has been previously identified as a regulator of the cell cycle and involved in nitrogen sensing. It is shown that the transcription levels of SIT4 are induced by low concentrations of Li⁺ in a time-dependent manner. Na⁺ and K⁺ at high concentrations, but not sorbitol, also induce transcription. As a response to Na⁺ or Li⁺ stress, yeast cells lower the intracellular K⁺ content. This effect is enhanced in cells overexpressing SIT4, which also increase ⁸⁶Rb efflux after the addition of Na⁺ or Li⁺ to the extracellular medium. Another feature of SIT4-overexpressing cells is that they maintain a more alkaline pH of 6.64 compared with 6.17 in the wild type cells. It has been proposed that the main pathway of salt tolerance in yeast is mediated by a P-type ATPase, encoded by PMR2A/ENA1. However, our results show that in a sit4 strain, expression of ENA1 is still induced by monovalent cations, and overexpression of SIT4 does not alter the amount of ENA1 transcript. These results show that SIT4 acts in a parallel pathway not involving induction of transcription of ENA1 and suggest a novel function for SIT4 in response to salt stress.

Lithium has been extensively used to treat manic bipolar disorder (1, 2). Its antimanic and antidepressant effects require days to weeks to appear, and several reports indicate that chronic administration of lithium affects gene expression (3). In this work we have used the yeast Saccharomyces cerevisiae as an eukaryotic cell model to study new possible targets of lithium action. Homeostasis of Li⁺ in yeast is maintained by multiple transport pathways that also transport Na⁺. The route of entry of both cations has not been clearly defined. It has been proposed to be through the K⁺ transporter Trk1p (4). When yeast cells are subjected to Na⁺ stress, its uptake is inhibited by increasing K⁺ import through Trk1p. However, this does not inhibit completely Na⁺ entry (4, 5). Two transport systems pump out Na⁺ that has entered the cell as follows: a cluster of up to five P-ATPases PMR2A/ENA1-4 (6, 7) and a Na⁺/H⁺ antiporter, encoded by NHA1 (8, 9). From the cluster of P-ATPases, Ena1p is the most highly expressed (6, 7). These two transport systems are differently regulated. ENA1 is induced by osmotic stress, starvation, or high extracellular pH (10). NHA1 increases sodium and lithium tolerance at an acidic or neutral pH of the external medium (11). The expression of PMR2A/ENA1 is regulated by several protein phosphatases and kinases. Deletion of the genes PPZ1 and PPZ2 coding for protein phosphatases increases expression of ENA1 (12), whose expression, on the contrary, is reduced by the deletion of the protein phosphatase calcineurin, causing hypersensitivity to sodium and high accumulation of lithium (13). Overexpression of a protein encoded by SIS2/HAL3 suppresses salt sensitivity in a calcineurin-deleted strain and stimulates transcription of the PMR2A/ENA1 gene (14). Hal3p acts as an inhibitory subunit of Ppz1p regulating its function on salt tolerance (15). Deletion of two protein kinase homologs YCR101c/SAT4/HAL4 (16) and YJL165c/HAL5 causes salt and pH sensitivity apparently as a result of deficient Trk1p and Trk2p activation (5).

Other regulatory pathways of ion homeostasis, which do not regulate expression of ENA1, have recently been described. A mutant strain lacking the transcriptional activator Imp2p was described as being hypersensitive to a variety of oxidative agents and also to Na⁺ and Li⁺. Imp2p does not increases ENA1 expression (17).

In this work we have searched for proteins that when overexpressed confer lithium tolerance to S. cerevisiae. We have identified SIT4, a type 2A/type 2A-related protein phosphatase, involved in the cell cycle. SIT4 is required for the late G₁ expression of cyclins and transcription factors essential for the execution of START and is also required for bud emergence (18, 19). SIT4 is also involved in the response to nitrogen starvation by controlling the compilation of transcription factors (20–22). In this work we have assigned a novel function to this protein. We demonstrate that its transcription is induced by monovalent cations and that it affects K⁺ homeostasis and cytoplasmic pH when overexpressed.

EXPERIMENTAL PROCEDURES

Strains—Escherichia coli strain XLI-Blue was used for plasmid construction. S. cerevisiae strains R757 (MATα, his3-15, ura3-52, lys9, hol1) and FY833 (MATα, his3Δ200, ura3-52, leu2Δ1, lys2Δ200, trp1Δ63, GAL2Δ) kindly provided by Dr. M. Ghislain, were used. Isolation of Genes That Confer Lithium Resistance—Wild type strain R757 was transformed with an expression cDNA library under control of a galactose-inducible promoter (23). Transformed cells were plated in minimal medium (YNB-gal) containing YNB 6.7 g/liter, galactose 2%, His, Ura, Lys, and methionine 0.003% and 30 mM LiSO₄. Only six recombinants expressing a protein conferring lithium resistance were able to overcome the lithium stress. The plasmid from the selected
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colonies was isolated, and the DNA insert was subcloned in pBluescript SK+ and sequenced using AutoRead Sequencing Kit and ALF DNA Sequencer (Amersham Pharmacia Biotech). The sequence was compared with the known open reading frames from the SGD data base.

Characterization of Clones Resistant to Lithium—The DNA insert of the selected clone was subcloned in the expression vector pRN93 kindly donated by Dr. C. W. Slayman from Yale University), which contains a GALA promoter. This plasmid was named pRN93-SIT4. Both pRN93 and pRN93-SIT4 were used to transform strain FY833 by the lithium acetate method (24).

Disruption of SIT4—Disruption of the entire coding region of SIT4 in FY833 was carried out using the one-step gene replacement using the HIS3MX6 module (25). Genomic DNA from selected sit4 strain was examined for disruption by PCR1 and Southern blot analysis.

Quantitative RT-PCR—The relative quantity of mRNA was determined by quantitative RT-PCR. Quantitative PCR analyses have been used to study mRNA levels for different transcripts (26, 27). Yeast cells were grown in the indicated media to exponential phase and harvested. 10 μg of total RNA (28) were treated with 2.5 units of DNase I (Amersham Pharmacia Biotech) for 10 min at 37 °C and further incubated at 65 °C for 10 min. First strand cDNA was synthesized using the First Strand cDNA synthesis kit from Amersham Pharmacia Biotech, and oligonucleotide No1-(dT)18 was used as primer. The cDNA was diluted 40 times in water and analyzed in a flame photometer.

Measurement of Membrane Potential—Changes in membrane potential were estimated with the fluorescent dye di-thiocarbocyanine (DiSC3(5)); (30, 31), obtained from Molecular Probes. The fluorescence of the molecule at a concentration of 0.25 μM was recorded at room temperature at 540–600 nm in a spectrofluorometer with a magnetic stirrer in the sample compartment.

ATPase Activity—Plasma membrane of S. cerevisiae and the vane-date-sensitive ATPase activity were measured as described (32).

RESULTS

Screening for Recombinants with High Lithium Resistance—S. cerevisiae cells are able to adapt to a salt stress in a medium containing glucose as the carbon source. However, tolerance diminished when grown on galactose (Fig. 1). For strains R757 and FY833 the Li⁺ lethal growth dosage was 250 mM in glucose, and in galactose medium it was 30 mM. We took advantage of this observation and screened an expression cDNA library under the control of a galactose-inducible promoter for clones that conferred lithium resistance in YNB galactose medium. Among six lithium-tolerant clones from R757 strain, we identified the gene SIT4. This gene is involved in progression of the cell cycle from G1 into S phase (19). It encodes the catalytic subunit of a serine-threonine protein phosphatase (19) with homology to the Ser/Thr protein phosphatase PP6 from mammalian cells (33).

We isolated the open reading frame SIT4 from the library plasmid and subcloned it into plasmid pRN93 under control of the galactose promoter that was used to transform another wild type strain named FY833. As shown in Fig. 2A, overexpression of SIT4 also conferred resistance to lithium in this different strain. A characteristic effect of Li⁺ is that at sublethal concentrations growth is arrested after the first duplication. After a period of adaptation of about 10 h cells recover growth. This growth arrest was not seen when SIT4 was overexpressed (Fig. 2B).

The entire coding region of SIT4 was deleted to abolish protein activity. Previous results showed that deletion causes a slow growth phenotype and larger cells (19). Our results showed that sit4 strain was able to grow on galactose, provided that rich medium was used. The sit4 strain did not grow in galactose nitrogen base minimal media. We tested if sit4 strain was able to adapt to Li⁺ stress. In contrast to overexpression, sit4 strain (Fig. 2B) recovered growth after the arrest period induced by lithium. This result indicates that SIT4 is not essential for Li⁺ adaptation, and other genes might perform overlapping actions in response to lithium stress. As shown in Fig. 2C we show the results of comparing the growth rate of the wild type, sit4-deleted cells and SIT4-overexpressing cells in the presence of different LiCl concentrations after the arrest period induced by Li⁺. We found that the latter is more resistant to Li⁺. It is interesting that resistance induced by overexpression of SIT4 was specific for Li⁺, as overexpression did not increase tolerance to Na⁺, K⁺, sucrose, or sorbitol (data not shown).

The abbreviations used are: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; MES, 4-morpholineethanesulfonic acid; DSC3, di-thiocarbocyanine.
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Expression of SIT4 Is Dependent on Monovalent Cations—
SIT4 is situated on chromosome IV. Its intergenic region contains 1558 base pairs, and a preliminary analysis of this region for putative consensus with the STRE sequence shows that it contains six STRE elements. This element mediates the activation of transcription in response to a wide type of stresses such as osmotic stress, heat shock, nitrogen starvation, oxidative stress, low external pH, and ethanol stress. Some of these responses are dependent on the high osmolarity glycerol pathway, but some are independent (34). We investigated whether SIT4 expression was induced by different monovalent cations. SIT4 was found to have a very low basal level of expression, and it was induced upon a stress by Li⁺, Na⁺, K⁺, and to a lesser degree by sorbitol (Fig. 3A). In order to test the dependence on sublethal concentrations of LiCl, we measured SIT4 induction in 15 mM LiCl. A 2-fold induction was observed after 5 h of Li⁺ stress (Fig. 3C). This time period correlates with the arrest period of growth. These results suggest that overexpression of SIT4 might be a physiological response to salt stress, and further work on the SIT4 promoter is required to identify the motifs that are responsible for induction of expression by salt.

Overexpression of SIT4 Does Not Increase PMR2A/ENA1 Expression—One of the main proteins controlling Na⁺/Li⁺ resistance is the P-type ATPase encoded by PMR2A/ENA1. The level of PMR2A/ENA1 transcription is modulated by a variety of phosphatases, and its promoter has been well studied (35). As shown in Fig. 3, A and B, the levels of PMR2A/ENA1 transcript were induced by Li⁺, Na⁺, K⁺, and to a lesser extent by sorbitol. These results agree with published results (6, 7, 36). The comparison of A and B show that SIT4 and PMR2A/ENA1 are concomitantly induced. With the aim to investigate whether SIT4 is a part of the mechanism by which PMR2A/ENA1 is induced, we measured by RT-PCR the relative quantity of PMR2A/ENA1 transcript on a strain where SIT4 is overexpressed and upon a Li⁺ or Na⁺ stress on a sit4 strain (Fig. 4). The relative quantity of PMR2A/ENA1 transcript was not altered by overexpression of SIT4 (Fig. 4B). However, PMR2A/ENA1 transcription level is still induced after a shock with 0.8 M NaCl or 0.8 M LiCl on a sit4 strain (Fig. 4C). These results show that induction of PMR2A/ENA1 expression by Li⁺, Na⁺, and K⁺ is independent of SIT4.

SIT4 Modulates K⁺ Homeostasis and pH—When yeast cells are subjected to a Na⁺ stress they undergo accumulation of osmotically active solutes such as glycerol and trehalose (37, 38). To determine whether overexpression of SIT4 changes the intracellular concentrations of the monovalent cations K⁺, Na⁺, and Li⁺, we determined their concentration after incubation for 30 min with increasing concentrations of NaCl or LiCl. In Fig. 5 we show the total monovalent cationic level; interest-
uptake did not change under these conditions by overexpression of SIT4 as shown in Fig. 6; however, the relative amount of K$^+$ inside the cell was diminished in relation to control conditions. These results indicate that overexpression of SIT4 causes a greater extrusion of K$^+$ upon Na$^+$ or Li$^+$ stress and that a K$^+$ transport system might be involved. In order to test this hypothesis we measured $^{86}$Rb$^+$ efflux in cells that had been previously starved and preloaded with $^{86}$RbCl. The results showed that in SIT4-overexpressing cells $^{86}$Rb$^+$ efflux in the presence of 500 mM NaCl or LiCl was highly increased (Fig. 7). sit4 strain showed the same kinetics as the wild type strain (data not shown).

We explored if the change on K$^+$ homeostasis induced by overexpression of SIT4 could induce an alteration of the membrane potential or internal pH. We observed that overexpression of SIT4 did not alter the changes in membrane potential produced by the addition of Na$^+$ or Li$^+$ (Fig. 8), but the intracellular pH changed from 6.17 (± 0.04) in wild type cells to 6.64 (± 0.03) in SIT4-overexpressing cells. The sit4 strain did not show any significant alteration in pH as compared with the wild type strain. The alkalinization of the cytoplasm was not caused by an altered function of the plasma membrane H$^+$-ATPase, since the specific activity of this enzyme in purified plasma membranes from SIT4-overexpressing cells and wild type cells was the same (0.338 and 0.373 nmol/min/mg of protein at pH 5.5, respectively).

**DISCUSSION**

Cellular processes are highly regulated by a wide variety of signal transduction pathways. Protein kinases and phosphatases mediate these events. SIT4 encodes a protein with homology to the catalytic subunits of mammalian PP6 protein phos-
phathases (33) and regulates the cell cycle (19). Our sit4 strains are viable and show an increased abundance of un budded cells (21). However, sit4 is lethal in sds1 (a gene of unknown function) deletant, whereas temperature-conditional alleles of sit4 arrest in G2 when shifted to the non permissive temperature (19). It has been recently reported that sit4 mutants of the yeast Kluyveromyces lactis regulate drug resistance (39). In this work we demonstrate for the first time that sit4 plays a role in monovalent cation homeostasis. Our results show that growth was arrested by sublethal Li⁺ concentrations, but after an adaptation period wild type cells were able to recover growth. Diverse regulatory mechanisms must be turned on during the period of adaptation to Li⁺ stress. Our results point out that induction of expression of sit4 is a regulatory mechanism, because on overexpression, lithium did not cause this growth arrest. This idea is reinforced by the fact that sit4 transcription is induced by low lithium concentrations and that its time-dependent expression correlates with the arrest period induced by lithium. The results obtained with overexpression of sit4 might represent physiological changes during adaptation to Li⁺ stress.

ENA1 Is Induced by a SIT4-independent Pathway—ENA1 is a P-type ATPase that has been proved to be involved in the main pathway of Na⁺/Li⁺ tolerance (6, 7). A link has been suggested between sit4 and ENA1, mediated by SIS2/HAL3. Ferrando et al. (14) reported that SIS2/HAL3 suppressed salt sensitivity in a calcineurin-deleted background by stimulating transcription of the PMR2A/ENA1 gene. On the other hand, SIS2/HAL3 is also a suppressor of the lethal sit4-sds1 phenotype described above (19). These results led us to test if sit4 was essential for PMR2A/ENA1 induction of transcription. We found that overexpression of sit4 did not mimic the effect of SIS2/HAL3 as it does not increase PMR2A/ENA1 expression. We have also shown that PMR2A/ENA1 is still induced by Na⁺ and Li⁺ in a sit4 strain. These data indicate clearly that transcription of ENA1 is induced by salt in a pathway independent of sit4.

Overexpression of SIT4 Stimulates Rb⁺ Efflux Induced by Salt and Alters the Intracellular pH—To date the role of internal monovalent cations in cellular physiology is not known. K⁺ and Na⁺ regulate the activity of many enzymes, but the effect of altered monovalent cation homeostasis has not been studied. On the other hand, it has been shown that high extracellular K⁺ concentrations alleviate Na⁺/Li⁺ stress (5). Upon Na⁺ stress yeast cells undergo accumulation of solutes to overcome turgor pressure (37, 38) and also undergo an increase of intracellular Na⁺ and a decrease of intracellular K⁺ (40, 41). The fact that sit4 regulates total cation content upon an Na⁺ or Li⁺ stress by lowering the intracellular content of K⁺ indicates that the regulation of homeostasis by sit4 is mediated via a K⁺ efflux transporter. The increased K⁺ loss does not lead to a change of the electrical membrane potential, but it maintains the total monovalent cation content low after a Na⁺ or Li⁺ stress. One intriguing physiological response of overexpression of sit4 is the elevated cytoplasmic pF. Further work is necessary to test if this effect leads to altered K⁺ homeostasis or if it is involved in another function regulated by sit4, as cell cycle control (19). However, it is possible that the efflux may proceed through one of the cation/H⁺ exchange systems reported before (8, 42, 43).

Two homologs of sit4 have been found in mammalian tissue. PMR2A/ENA1 was essential for type 2 diabetes (19). These results led us to test if SIT4 also functions as a suppressor of the lethal sit4 mutant. It has been recently reported that sit4 growth was arrested by sublethal Li⁺ concentrations (14). K⁺ is known to be important in the main pathway of Na⁺/Li⁺ tolerance (6, 7). A link has been shown between sit4 and ENA1, mediated by SIS2/HAL3. Ferrando et al. (14) reported that SIS2/HAL3 suppressed salt sensitivity in a calcineurin-deleted background by stimulating transcription of the PMR2A/ENA1 gene. On the other hand, SIS2/HAL3 is also a suppressor of the lethal sit4-sds1 phenotype described above (19). These results led us to test if sit4 was essential for PMR2A/ENA1 induction of transcription. We found that overexpression of sit4 did not mimic the effect of SIS2/HAL3 as it does not increase PMR2A/ENA1 expression. We have also shown that PMR2A/ENA1 is still induced by Na⁺ and Li⁺ in a sit4 strain. These data indicate clearly that transcription of ENA1 is induced by salt in a pathway independent of sit4.

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REFERENCES
1. Schou, M. (1997) Arch. Gen. Psychiatry 4, 9–13
2. Jope, R. S. (1999) Mol. Psychiatry 2, 117–128
3. Manji, H. K., Potter, W. Z., and Lenox R. H. (1995) Arch. Gen. Psychiatry 52, 531–543
4. Gómez, M. J., Luyten, K., and Ramos, J. (1996) FEMS Microbiol. Lett. 135, 157–160
5. Mulet, J. M., Leube M. P., Kron, S. J., Rios, G., Fink, G. R., and Serrano, R. (1999) Mol. Cell. Biol. 19, 3298–3337
6. Haro, R., García-del-Caballero, B., and Rodríguez-Navarro, A. (1991) FEBS Lett. 291, 189–191
7. Wieland, J., Noseke, A. M., Strayle, J., Steiner, H., and Rudolph, H. K. (1995) EMBO J. 14, 3870–3876
8. Prior, C., Potier, S., Souciet, J. L., and Sychrová, H. (1996) FEBS Lett. 387, 89–93
9. Sychrová, H., Ramirez, J., and Peña, A. (1999) FEMS Microbiol. Lett. 171, 167–172
10. García-del-Caballero, B., Rojo, F., Quintero, F. J., Baniuelos, M. A., Haro, R., and Rodríguez-Navarro, A. (1993) Mol. Gen. Genet. 236, 363–368
11. Baniuelos, M. A., Sychrová, H., Bleykasten-Grosshans, C., Souciet, J. L., and Potier, S. (1998) Microbiology 144, 2749–2758
12. Sychrová, H., Ramirez, J., and Peña, A. (1999) FEBS Lett. 14865–14872
13. Mendoza, I., Rubio, F., Rodríguez-Navarro, A., and Pardo, J. M. (1994) EMBO J. 13, 9577–9582
14. Garcia-del-Caballero, B., Rosas, G., and Calahorra, M. (1995) J. Bacteriol. 177, 1017–1022
15. Peña, A., Uribe, S., Pardo, J. P., and Berbolla, M. (1984) Arch. Biochem. Biophys. 231, 217–225
16. Chen, X. J., Bauer, B. E., Kuchler, K., and Clark-Walker, G. D. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 8892–8896
17. Reed, R. H., Chudek, J. A., Foster, R., and Gadd, G. M. (1987) J. Bacteriol. 161, 893–898
18. Proft, M., and Serrano, R. (1999) J. Bacteriol. 181, 5860–5865
19. Ueda, H., Kuroda, K., and Nishikawa, K. (1999) J. Bacteriol. 181, 175–182
20. Reed, R. H., Chudek, J. A., Foster, R., and Gadd, G. M. (1987) J. Bacteriol. 167, 1097–1102