Protein Phosphatase Type 1 Regulates Ion Homeostasis in *Saccharomyces cerevisiae*

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Manuscript received September 18, 2001
Accepted for publication January 25, 2002

**ABSTRACT**

Protein phosphatase type 1 (PP1) is encoded by the essential gene *GLC7* in *Saccharomyces cerevisiae*, *glc7-109* (K259A, R260A) has a dominant, hyperglycogen defect and a recessive, ion and drug sensitivity. Surprisingly, the hyperglycogen phenotype is partially retained in null mutants of *GAC1*, *GIP2*, and *PIG1*, which encode potential glycogen-targeting subunits of Glc7. The R260A substitution in *GLC7* is responsible for the dominant and recessive traits of *glc7-109*. Another mutation at this residue, *glc7-R260P*, confers only salt sensitivity, indicating that the glycogen and salt traits of *glc7-109* are due to defects in distinct physiological pathways. The *glc7-109* mutant is sensitive to cations, aminoglycosides, and alkaline pH and exhibits increased rates of l-leucine and 3,3'-dihexyloxacarbocyanine iodide uptake, but it is resistant to molar concentrations of sorbitol or KCl, indicating that it has normal osmoregulation. KCl suppresses the ion and drug sensitivities of the *glc7-109* mutant. The CsCl sensitivity of this mutant is suppressed by recessive mutations in *PMA1*, which encodes the essential plasma membrane H^+*-ATPase*. Together, these results indicate that Glc7 regulates ion homeostasis by controlling ion transport and/or plasma membrane potential, a new role for Glc7 in budding yeast.

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al. 1994; Ozer et al. 1998). However, the mRNA and protein levels of Pma1 do not change significantly during activation by glucose, suggesting that in this case post-translational modulation of ATPase activity may be the primary mechanism for Pma1 regulation (Eraso et al. 1987).

The activity of Pma1 changes in response to extracellular glucose (Portillo et al. 1991; Garcia-Arranz et al. 1994; van der Rest et al. 1995) and Pma1 is highly phosphorylated on multiple serine and threonine residues in vivo (Kolarov et al. 1988; Bertorello et al. 1991; Chang and Slayman 1991; Serrano et al. 1991). Two-dimensional phosphopeptide gel analysis of Pma1 in temperature-sensitive secretion (sec) mutants has revealed that phosphorylation of plasma membrane-specific sites is associated with increased ATPase activity during growth on glucose (Holcomb et al. 1988; Chang and Slayman 1991). Upon glucose starvation, dephosphorylation occurs concomitantly with a decrease in enzymatic activity and both are rapidly reversed by the addition of glucose (Chang and Slayman 1991; Estrada et al. 1996). These results suggest that reversible, site-specific phosphorylation adjusts the ATPase activity and protein stability (Hasper et al. 1999; Gong and Chang 2001) in response to glucose.

This work was motivated by the observation that glc7-109, one of 20 charged to alanine-scanning alleles, uniquely confers a NaCl-sensitive phenotype (Baker et al. 1997). Defects in ion homeostasis have been previously noted for null mutants in genes encoding the phosphatases calcineurin/PP2B (Nakamura et al. 1993; Breuder et al. 1994; Mendoza et al. 1994; Farcasanu et al. 1995) and Ppz1 (Posas et al. 1995) but not for mutants in GLC7. We show here that glc7-109 mutants are sensitive to cations, alkaline pH, and aminoglycoside antibiotics. These defects can be partially ameliorated by exogenous K+ or mutations in PMA1. The mutant exhibits an increased rate of uptake of the fluorescent dye DiOC6(3) and the amino acid l-leucine. Together, these results suggest that Glc7 plays a role in the cellular response to salt stress, possibly by regulating ion transport or membrane potential.

MATERIALS AND METHODS

Yeast strains and media: The yeast strains used in this study are listed in Table 1. All strains used in this study except K1849 are congenic to strain K1112 (MATa ura3-52 leu2 his3; Stuart et al. 1994). The trk1::KanMX and gip2::KanMX (Research Genetics, Huntsville, AL) and cub1::LEU2 (Cyvert and Thorner 1992) mutations were introduced into the K1112 background by seven serial backcrosses. All growth phenotypes shown in this study required media that contained Difco (Detroit) peptone, yeast extract, and agar. In this study, media made from constituents manufactured by Angus Buffers and Biochemicals impaired the growth of glc7-109 and suppressor mutants on salt and antibiotic media. Yeast strains were routinely incubated at either 24° or 30° in YPD (1% yeast extract, 2% Bacto-peptone, 2% dextrose) or synthetic media (0.67% yeast nitrogen base, 2% glucose, and the appropriate 0.01–0.05% amino acids). Plate media contained 2% agar.

Assays for ion tolerance were performed by liquid growth assays or spot dilution tests on agar media. Liquid growth assays were performed by diluting stationary phase cultures to 4×10⁵ cells/ml with media described below. Cell concentrations were determined by measuring optical density at 600 nm (OD₆₀₀) and/or by microscopic cell counting using a standard hemacytometer. Cultures were incubated in the specified media for 1–3 hr. Spot dilution tests were done by spotting three serial 10-fold dilutions of 1×10⁵ cells/ml of each strain on YPD plates, synthetic media, or YPD supplemented with various concentrations of salts and drugs. Growth of strains at pH 3, 5, and 8.5 was tested in liquid YPD medium that was adjusted with 10 n KOH or 1 m HCl and buffered with 100 mM potassium phosphate or 50 mM sucinate, respectively. Medium containing 0.2 mM KCl consists of a synthetic medium containing 0.15 M ammonium sulfate, 2% glucose, 8 mM ammonium phosphate, 17 mM NaCl, 2 mM MgSO₄, 0.2 mM CaCl₂, 0.2 mM KCl, plus amino acids, vitamins, and trace elements as described (Chester et al. 1968). Quantitative glycogen accumulation measurements were performed as described (Chester 1968). Quantitative glycogen accumulation measurements were performed as described (Wu and Tatchell 2001). Isolation of plasmid yeast DNA and genomic DNA was performed by a variation of the “smash and grab” protocols by Hoffman (Hoffman and Winston 1987) as described by Ausubel et al. (1989). DNA sequence was determined using the T7 Sequenase Version 2.0 kit (Amersham, Life Sciences) according to the manufacturer’s instructions.

Plasmid construction: Plasmids used in this study are listed in Table 2. Plasmid p1407 is a genomic library clone in the cent URA3 vector, YCp50, that contains PMA1 and a 460-bp open reading frame, YGL1007. To analyze the previously described alanine-scanning allele, glc7-109 (Baker et al. 1997), genomic integration of the deletion of glc7-109 (K259A, R260A, and R260P mutations in PMA1) was performed as described (Venturi et al. 2000) using plasmid pTW2. Plasmid pXZ03 (a gift from James Haber) contains genomic integration of the MRR1 or membrane potential. an HA epitope-tagged medium containing 0.2 mM KCl and incubated for 4 hr at 30° prior to flow cytometry analysis.

General methods: Escherichia coli strains DH5α F', XLI-Blue, and HB101 were used for cloning and propagation of plasmids. Diploid cells were induced to sporulate on YPA (2% potassium acetate, 2% peptone, 1% yeast extract) agar media. Yeast transformations were performed by the lithium acetate method as described (Giertz et al. 1992). Qualitative glycogen accumulation measurements were performed as described (Chester 1968). Quantitative glycogen accumulation measurements were performed as described (Wu and Tatchell 2001). Isolation of plasmid yeast DNA and genomic DNA was performed by a variation of the “smash and grab” protocols by Hoffman (Hoffman and Winston 1987) as described by Ausubel et al. (1989). DNA sequence was determined using the T7 Sequenase Version 2.0 kit (Amersham, Life Sciences) according to the manufacturer’s instructions.
**TABLE 1**

**Yeast strains**

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</tr>
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</tr>
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</table>

**Isolation and characterization of glc7-109 revertants:** Three independent cultures of glc7-109 strains (KT1596 and TW25) were grown in YPD at 24°C to 3×10⁶ cells/ml, plated at 2×10⁴ cells/ml on YPD plates containing 0.1 M CsCl, and incubated for 2 days at 24°C. This concentration of CsCl completely inhibits the growth of the glc7-109 mutant. Six of 105 Cs⁺-tolerant revertants were characterized in detail. TW30 (pma1-s1 glc7-109), TW31 (pma1-s2 glc7-109), and TW33 (pma1-s3 glc7-109) were isolated from the first culture, TW38 (pma1-s4 glc7-109) was isolated from the second culture, and TW43 (pma1-s5glc7-109) and TW45 (pma1-s6 glc7-109) were isolated from the third culture. To assay for dominance, each revertant was mated to a glc7-109 strain and the resulting diploids were assayed for resistance to CsCl and glycogen accumulation. To determine if the reversion events were extragenic, each revertant was mated to a GLC7 strain, the resultant diploids were sporulated, and tetrad analysis was performed to assay for recovery of the glc7-109 phenotype. Linkage and complementation between the suppressors was determined by performing tetrat analysis on glc7-109 diploid strains heteroallelic for two different suppressors. On the basis of complementation and genetic linkage analysis, the six suppressors were assigned to one complementation group.

**Cloning of the suppressor locus:** A pma1-s2 glc7-109 mutant strain (TW31) was transformed with a yeast genomic library in the CEN URA3 vector Ycp50, and transformants were screened for growth at 37°C and failure to grow on synthetic media containing 0.1 M CsCl. One transformant that was complemented for both traits was obtained from approximately 66,000 transformants. A plasmid (p1407-1) was recovered from this transformant and verified to be responsible for the complementation upon retransformation into TW31. p1407-1 was able to complement the cold sensitivity of TW60 (pma1-s1 glc7-109), TW38 (pma1-s4 glc7-109), and TW78 (pma1-s6 glc7-109). The sequence of p1407-1 was determined at the junctions between the vector and genomic insert using T7 Sequenase Version 2.0 (Amersham, Arlington Heights, IL) and primers that annealed to the Ycp50 vector flanking the insert. The
PMA1 gene in p1407-1 was solely responsible for complementation of the revertant phenotype, as shown by the ability of plasmid pXZ03 (a gift from James Haber), which contains only the PMA1 gene, to complement the conditional phenotypes and restore CsCl sensitivity to TW61 (pma1-s1 glc7-109), TW64 (pma1-s2 glc7-109), and TW71 (pma1-s4 glc7-109).

Genetic analysis of pma1 and calcineurin strains: Each of the suppressor strains TW61 (pma1-s1 glc7-109), TW64 (pma1-s2 glc7-109), TW68 (pma1-s3 glc7-109), TW71 (pma1-s4 glc7-109), TW75 (pma1-s5 glc7-109), and TW79 (pma1-s6 glc7-109) were mated to strain TW82 (cnb1::LEU2) or TW83 (cnb1::LEU2). The resultant diploid strains were sporulated and the haploid segregants were assayed for conditional growth defects relative to growth on leu-/H11002 haploid segregants were assayed for conditional growth defects relative to growth on leu-/H11002. The mutants containing each suppressor (pma1) and calcineurin (cnb1::LEU2) were characterized.

1-Leucine uptake assays: 1-Leucine uptake assays were performed as previously described (Kotliar et al. 1994; Norbeck and Blobel 1998) with some modifications. Briefly, the 1-leucine uptake assays were performed with wild-type and glc7-109 haploid strains grown to an OD 600 of ~0.5 (5 × 10^6 cells/ml) at 30°C in synthetic media supplemented with 0.01% l-leucine, histidine, and uracil. A solution of 14C-radiolabeled mosomal leucine (11.2 GBq/mmol; CEB67, Amersham) at 304 mCi/mmol was added to the cell culture to a final concentration of 0.05 mM. At 1, 2, 3, 4, and 5 min, a 750-μl sample of cells was withdrawn and mixed with 750 μl ice-cold, nonlabeled 0.1 M l-leucine solution to stop uptake. The cells were immediately filtered and washed with ice-cold 0.1 M l-leucine solution as described. The 14C-radiolabeled leucine measurements represent the average of triplicate experiments (Norbeck and Blobel 1998).

Biochemical methods: To assay calcineurin-dependent responsive element (CDRE)-dependent gene expression, strains were transformed with pAMS366 (Stathopoulos-Geronides et al. 1999) and exponentially growing transformants (1–5 × 10^6 cells/ml) were assayed for β-galactosidase activity as described (Miller 1972; Kaiser et al. 1994). Yeast protein extracts were prepared as described (Stuart et al. 1994) from cells at a density of 1 × 10^9 cells/ml and precipitated with 10% trichloroacetic acid as described (Dufour and Goffeau 1978). Pma1p levels were determined from total protein extracts as described (Wittek et al. 1998) using affinity-purified rabbit polyclonal antibody generously provided by Carolyn Goffeau (Nakamoto et al. 1991). The signal was visualized with enhanced chemiluminesence and a horseradish peroxi-

dase-conjugated secondary antibody (Amersham). Pma1p levels were quantitatively measured using NIH Image with phosphoglycerate kinase as a loading control to compare Pma1p in 5, 10, and 15 μg total protein extracts from wild-type and glc7-109 cells. Protein concentrations were determined as described (Bradford 1976) with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard. Membrane fractions were separated on 10% SDS-polyacrylamide gels and immunoblotted as described (Ausubel et al. 1989). Total membrane fractions were isolated and V0,S-sensitive ATPase activity was assayed at pH 5.7 with 5 mM ATP as described (Serrano 1983b). To determine relative membrane potential in our strains, we performed flow cytometry analysis using a FACScan Calibur (Becton Dickinson) as described (Madrid et al. 1998).

**RESULTS**

**glc7-109 mutants have a recessive, salt sensitive and a dominant, hyperglycogen phenotype:** A single GLC7 mutant (glc7-109) from a group of 20 alanine-scanning mutants was uniquely sensitive to high concentrations of NaCl (Baker et al. 1997). It also accumulated higher than normal levels of glycogen, as observed previously for several other alleles (Baker et al. 1997; Ramaswamy et al. 1998). In crosses with strains containing the chromosomal glc7-109, sensitivity to a variety of monovalent and divalent ions and two aminoglycoside antibiotics segregated with the hyperglycogen phenotype, indicating that the single mutation was responsible for both traits. In these crosses we noted that the salt and drug sensitivities were recessive to GLC7 but the glycogen hyperaccumulation trait was clearly dominant. As shown in Figure 1A, a diploid strain heterozygous for glc7-109 is as resistant to CsCl and paromomycin sulfate as the wild-type GLC7 strain but accumulates glycogen levels intermediate between the glc7-109 homozygous strain and the wild type, indicating that the hyperglycogen phenotype of glc7-109 is semidominant.

To test the possibility that the dominant glycogen

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**TABLE 2**

Plasmids

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**RESULTS**

**glc7-109 mutants have a recessive, salt sensitive and a dominant, hyperglycogen phenotype:** A single GLC7 mutant (glc7-109) from a group of 20 alanine-scanning mutants was uniquely sensitive to high concentrations of NaCl (Baker et al. 1997). It also accumulated higher than normal levels of glycogen, as observed previously for several other alleles (Baker et al. 1997; Ramaswamy et al. 1998). In crosses with strains containing the chromosomal glc7-109, sensitivity to a variety of monovalent and divalent ions and two aminoglycoside antibiotics segregated with the hyperglycogen phenotype, indicating that the single mutation was responsible for both traits. In these crosses we noted that the salt and drug sensitivities were recessive to GLC7 but the glycogen hyperaccumulation trait was clearly dominant. As shown in Figure 1A, a diploid strain heterozygous for glc7-109 is as resistant to CsCl and paromomycin sulfate as the wild-type GLC7 strain but accumulates glycogen levels intermediate between the glc7-109 homozygous strain and the wild type, indicating that the hyperglycogen phenotype of glc7-109 is semidominant.

To test the possibility that the dominant glycogen
Figure 1.—The salt sensitivity and glycogen hyperaccumulation traits of the glc7-109 mutant are separable. (A) Strains were incubated on YPD medium at 24°C for 2 days and then replica plated onto agar containing YPD, YPD supplemented with 1 mg/ml of paromomycin sulfate (Paro), 0.1 M CsCl, and synthetic complete medium stained with iodine (I2) as described in MATERIALS AND METHODS. The glycogen [Gly] accumulation of all strains was quantitated as described in MATERIALS AND METHODS. The strains used are wild-type haploid and diploid, KT1937 and KT1943, respectively; GLC7/glc7-109, KT1944; glc7-109/glc7-109, KT1945; K259A; and glc7-109 (K259A R260A), WU322; glc7-K259A, WU341; glc7-R260A, WU318; and glc7-R260P, WU320.

and recessive salt-sensitive phenotype of glc7-109 are independently caused by the two missense mutations in glc7-109 (K259A and R260A), we constructed mutants that contained the single missense mutations. As shown in Figure 1B, glc7-R260A confers a phenotype similar to that of glc7-109 while glc7-K259A confers a phenotype very similar to that of the wild type. Another missense mutant with proline substituted for Arg260 retains the salt and paromomycin sulfate sensitivities of R260A, but has lost the hyperglycogen phenotype. In fact, glycogen levels are reduced below that of the wild type in the glc7-R260P mutant (Figure 1B). The fact that the glc7-R260P strain retains only the salt-sensitive trait of glc7-109 supports the hypothesis that the salt-sensitive and glycogen traits are physiologically unrelated.

glc7-109 partially bypasses the requirement for the Glc7-regulatory subunits for glycogen accumulation: The Glc7 holoenzyme that dephosphorylates and activates glycogen synthase is thought to contain Gac1, a targeting subunit that binds directly to both Glc7 and glycogen synthase (Wu et al. 2001). Gac1 is rate limiting for glycogen accumulation (François et al. 1992; Stuart et al. 1994) and mutations in GLC7 or GAC1 that prevent the association cause a reduction in glycogen levels (Stuart et al. 1994; Wu et al. 2001). Gac1 most likely makes multiple contacts with Glc7, as judged by the locations of amino acid substitutions in Glc7 that exhibit reduced interactions with Gac1 (Baker et al. 1997; Ramaswamy et al. 1998; Wu et al. 2001). One of these contacts is a hydrophobic channel that is thought to interact with a conserved VXF-motif found in many PP1- and Glc7-targeting subunits (Egloff et al. 1997). Arg260, the residue altered in Glc7-109 that is responsible for the hyperglycogen phenotype, is near F256, a residue in the hydrophobic channel that makes contact with the phenylalanine residue of the VXF-motif (Egloff et al. 1997). A Glc7 F256A mutant has defects in binding to multiple targeting subunits (Wu and Tatchell 2001), supporting the hypothesis that this residue has a role in binding to VXF-containing proteins. The locations of R260 and the hydrophobic channel are highlighted in the crystal structure of PP1 (Figure 2). On the basis of this structure, we propose that R260 might partially occlude the binding of Gac1; a substitution of this residue for alanine may increase the affinity for Gac1, resulting in an increase in the concentration of...
Thus, Gac1 binding is not responsible for the glc7-109 phenotype of the mutant. Contrary to expectations, a Glc7p- and glycogen-targeting subunit. Serial dilution were imaged on YPD and YPD stained with iodine. Only the patches with the lowest serial dilution were imaged on YPD and YPD + CsCl but both the lowest and second lowest dilutions were imaged for the iodine-treated samples. The strains are ascospores from a cross between KT1939 (MATα gac1::LEU2 pig1::URA3) and TW321 (MATα gip2::KanMX glc7-109).

**Figure 3.**—glc7-109 and glycogen-targeting subunits. Serial dilutions of strains with the designated genotypes were incubated for 24 hr at 30°C on YPD, YPD containing 0.1 M CsCl, and YPD stained with iodine. Only the patches with the lowest serial dilution were imaged on YPD and YPD + CsCl but both the lowest and second lowest dilutions were imaged for the iodine-treated samples. The strains are ascospores from a cross between KT1939 (MATα gac1::LEU2 pig1::URA3) and TW321 (MATα gip2::KanMX glc7-109).

the Gac1-Glc7 holoenzyme and increased glycogen levels. Consistent with this hypothesis, we detected an increased interaction between Glc7-109 and Gac1 using the two-hybrid assay (D. L. Frederick and K. Tatchell, unpublished data). If such an occlusion model is responsible for the hyperglycogen phenotype of the glc7-109 mutant, then the gac1 null mutation should be epistatic to glc7-109. Contrary to expectations, glc7-109 gac1::LEU2 mutants accumulate high levels of glycogen (Figure 3). Thus, Gac1 binding is not responsible for the glc7-109 phenotype.

The yeast genome contains three additional genes whose products are similar to Gac1. **PIG1**, **PIG2**, and **GIP2** were identified in a two-hybrid screen for Gsy2-interaction proteins (Cheng et al. 1997; Wu et al. 2001) while Gip2 was also identified independently as a Glc7p-interacting protein (Tu et al. 1996). Although null mutations in these genes were reported to have little effect on glycogen levels, we tested whether the products of several of these might be necessary for the hyperglycogen phenotype of the glc7-109 mutant. Glycogen levels were elevated in glc7-109 strains containing deletions of **GAC1**, **PIG1**, and **GIP2** until stationary phase, when glycogen levels dropped below that of the wild type. As shown in Figure 3, the higher serial dilution of the gac1 pig1 gip2 glc7-109 strain stained more with iodine vapor than did the lower serial dilution, presumably reflecting a later stage in the growth phase. These results indicate that glc7-109 partially bypasses the need for Gac1, Pig1, and Gip2 for glycogen accumulation, which may have important implications for the role of Glc7 in glycogen metabolism (see Discussion).

**Glc7 and calcineurin act through different pathways to affect ion homeostasis:** Yeast strains lacking the ser/thr protein phosphatase calcineurin/PP2B are sensitive to high concentrations of Na+, Li+, and Mn2+ (Nakamura et al. 1993; Breuder et al. 1994; Mendoza et al. 1994; Farcasanu et al. 1995), due to their failure to induce expression of a number of membrane ATPases. The sensitivity of calcineurin mutants to Mn2+ is at least partly caused by reduced transcription of PMR1 (Cunningham and Fink 1996), encoding a Golgi P-type ATPase (Antebi and Fink 1992); sensitivity to Na+ and Li+ is caused by reduced transcription of PMR2/ENA1 (Mendoza et al. 1994), the gene encoding the major plasma membrane Na+ATPase (Rudolph et al. 1989). Calcineurin mutants are also defective in induction of Pmc1, a Ca2+ATPase, which causes them to be resistant to media containing 200 mM CaCl2 (Cunningham and Fink 1994). These transcriptional defects are due to the failure of calcineurin mutants to activate the Crz1/Tcn1 transcription factor (Mathews et al. 1997; Stathopoulos and Cyert 1997).

The apparent similarity between the calcineurin and glc7-109 phenotypes prompted us to directly compare the two mutants. We used a cnb1::LEU2 null mutant for the comparison. CNB1 encodes a conserved regulatory subunit of calcineurin (Kuno et al. 1991; Cyert and Thorner 1992) that is necessary for phosphatase activity. The cnb1::LEU2 and glc7-109 mutants have qualitative and quantitative differences in ion sensitivity. The glc7-109 mutant is generally more sensitive to mono- and divalent ions. As shown in Figure 4, the glc7-109 mutant fails to grow on YPD medium containing 25 mM NaCl, whereas the cnb1::LEU2 strain exhibits no defect at this or higher concentrations tested (50 and 100 mM). glc7-109 and cnb1::LEU2 mutants are equally sensitive to 5 mM MnCl2, but the glc7-109 mutant is more sensitive to Li+ and Na+ (data not shown). Furthermore, the glc7-109 strain does not exhibit the calcium resistance observed for calcineurin mutants (Figure 4). Differences were also noted in the temperature dependence of Li+ sensitivity of glc7-109 and cnb1::LEU2 strains. The glc7-109 mutant exhibits greater sensitivity to LiCl at 24°C than at 30°C, but no such temperature differential was noted for the cnb1::LEU2 strain. An additional difference was the sensitivity to aminoglycoside antibiotics. The cnb1::LEU2 mutant does not exhibit growth defects on media containing 50 μg/ml hygromycin B (Figure 4) and 1 mg/ml paromomycin sulfate (data not shown) in contrast to the hypersensitivity of glc7-109 mutants. An indication that Glc7 and calcineurin act in separate pathways is the finding that the glc7-109 cnb1::LEU2 dou-
The glc7-109 strain exhibits increased rates of l-leucine uptake: The glc7-109 mutant is hypersensitive to the aminoglycoside antibiotics paromomycin sulfate and hygromycin B. Since the mechanisms of action of these two antibiotics are different, it is likely that the hypersensitivity of glc7-109 strains to these drugs is caused by defects in uptake or export. Although we have not been able to distinguish between these two possibilities, we note that glc7-109 strains grow more rapidly than the wild type on synthetic media containing low concentrations of amino acids (data not shown). To confirm that this difference is due to a change in amino acid uptake we measured l-leucine uptake in glc7-109 mutants. The rate of l-leucine uptake in the glc7-109 mutant was approximately twofold higher than that in the wild type (Table 3).

Many defects due to glc7-109 are K⁺ remedial: To rule out the possibility that the sensitivity of glc7-109 mutants to high concentrations of ions was due to osmotic effects, we tested the growth properties of glc7-109 strains

### Table 3

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CDRE activation a</th>
<th>t-Leucine uptake b</th>
<th>Pma1 amount c</th>
<th>Pma1 activity d</th>
</tr>
</thead>
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<tr>
<td>GLC7 CNB1</td>
<td>88 ± 7.0</td>
<td>2212 ± 56</td>
<td>2.10 ± 0.26</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>glc7-109 CNB1</td>
<td>73 ± 9.1</td>
<td>770 ± 153</td>
<td>5.62 ± 1.2</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>GLC7 cnb1</td>
<td>3 ± 0.6</td>
<td>2 ± 0.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>glc7-109 cnb1</td>
<td>3 ± 0.6</td>
<td>2 ± 0.6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

a Wild-type (KT1112), glc7-109 (KT1596), and calcineurin-deficient (TW82) strains were transformed with pAMS366, which contains calcineurin-dependent responsive elements (4XCDREs) and a LacZ reporter gene (Stathopoulos and Cyert 1997). CDRE activation is quantitated in Miller units of β-galactosidase activity (Miller 1972). β-Galactosidase activity was measured as described in MATERIALS AND METHODS after growth in selective media or media supplemented with 200 mM CaCl₂ or 10 mM MnCl₂ as indicated. Values represent the average of three separate experiments.

b Micromoles of [¹⁴C]leucine per gram dry weight per minute; data represent the average of three separate experiments.

c The abundance of Pma1 protein in whole cell extracts was determined as described in MATERIALS AND METHODS. Values represented are the median percentage of Pma1 relative to 100% in the wild type (KT1112) from four separate determinations.

d Inorganic phosphate (micromoles per milligram per minute) liberated; values presented represent the average of three separate experiments.
on YPD media containing 1 m KCl and 1 m sorbitol. While no growth defects were observed on media with only KCl or sorbitol, KCl partially relieved the salt and drug sensitivities of glc7-109 strains. The presence of 200 mM KCl in the medium reduces the sensitivity of glc7-109 to Cs\(^+\), Li\(^+\), hygromycin B, and paromomycin sulfate (Figure 5), and to Mn\(^{2+}\) and Na\(^+\) (data not shown). The K\(^+\) remedial phenotype caused by glc7-109 led us to examine its effect on growth on low concentrations of KCl, because mutants defective in the potassium transporters Trk1 and Trk2 are hypersensitive to hygromycin B and toxic cations (Mulet et al. 1999). Growth of yeast cells on media containing low concentrations of KCl (0.2 mM) requires the activity of the high-affinity K\(^+\) transporter encoded by TRK1 (Gaber et al. 1988). We found that a trk1 strain (TW272) failed to grow on synthetic medium containing 0.2 mM KCl whereas glc7-109 and wild-type strains grew equally well on this medium. Therefore, the ion sensitivities of the glc7-109 mutant are unlikely to be caused by a defect in high-affinity K\(^+\) transport.

**Suppressors of the Cs\(^+\)-sensitive phenotype of glc7-109:** We characterized six spontaneous glc7-109 revertants that grew on YPD medium containing 0.1 m CsCl, as described in MATERIALS AND METHODS. The mutations responsible for the reversion events were recessive, as shown by CsCl sensitivity of diploid strains heterozygous for suppressor mutations and homozygous for glc7-109. To determine if the mutations responsible for suppression were extragenic to GLC7, tetrad analysis was performed on diploid strains created by mating each of the six revertants to a wild-type strain. The salt-sensitive phenotype of glc7-109 was observed in approximately one-fourth of the spore clones from each cross, indicating that the mutation responsible for the reversion was unlinked to GLC7. To determine if the suppressors were in the same complementation group, diploid strains were constructed that were homozygous for glc7-109 and heterozygous for two different suppressors. All diploids were resistant to 0.1 m CsCl, indicating that all suppressors were in the same complementation group. Furthermore, tetrad analysis of meiotic progeny of these diploid strains revealed that all spore clones retained the Cs\(^+\)-resistant phenotype of the parents, indicating that the suppressor loci are tightly linked.

Although these suppressors are genetically linked, they confer diverse phenotypes. While all six revertants are able to grow on media containing 0.1 m CsCl or 0.9 m NaCl, only pma1-s1, pma1-s3, and pma1-s4 suppress the Li\(^+\) defect of glc7-109 (Figure 6). In contrast, pma1-s1 and pma1-s4 strains actually grow better than the wild-type strain on 0.1 m LiCl (Figure 6). No revertant strains except those containing pma1-s2 exhibit the glc7-109 growth defect on 10 mM MnCl\(_2\) (Figure 6). Some of these revertants also have conditional growth defects in the glc7-109 genetic background. pma1-s2 and pma1-s5 strains grow more slowly than the wild-type strain at 37\(^\circ\)C while pma1-s1, pma1-s4, and pma1-s6 strains grow more slowly than the wild type at 15\(^\circ\)C (Figure 6). These six suppressor mutants were also characterized in a wild-type GLC7 background. pma1-s6 strains grow poorly on 0.1 m CsCl and 0.1 m LiCl, whereas pma1-s2 grows poorly on LiCl (Figure 7). In contrast, the pma1-s1, pma1-s3, pma1-s4, and pma1-s5 strains grow faster than the wild-type strain on LiCl (Figure 7). Most of the conditional growth defects observed in a glc7-109 background were also apparent in a GLC7\(^+\) background. For example, pma1-s1 and pma1-s4 strains grow more slowly than the wild type at 15\(^\circ\)C (Figure 7) and pma1-s2 strains grow more slowly than the wild type at 37\(^\circ\)C (data not shown).

**The glc7-109 suppressors are allelic to PMA1:** The suppressor locus was cloned by complementation of the temperature sensitivity of strain TW64 (pma1-s2 glc7-109) as described in MATERIALS AND METHODS. The complementing plasmid clone (p1407-1) also complemented the cold-sensitive defects of strains TW30 (pma1-s1 glc7-109) and TW38 (pma1-s4 glc7-109). These transformants grew well at 37\(^\circ\)C and 15\(^\circ\)C, but failed to grow on 0.1 m CsCl media. Sequence analysis revealed that the genomic insert in p1407-1 contained PMA1 and an adjacent 378-bp open reading frame, YGL007. A plasmid (pXZ03) containing PMA1 but lacking YGL007 was also

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**Figure 5:** KCl suppresses the salt and drug sensitivities of the glc7-109 mutant. Serial dilutions of wild type (KT1112) and glc7-109 (TW25) strains were incubated for 2–4 days at 24\(^\circ\)C on media containing YPD, YPD containing 1 m KCl, 0.05 m CsCl, 0.05 m CsCl + 0.2 m KCl, 0.1 m LiCl, 0.1 m LiCl + 0.2 m KCl, 50 \(\mu\)g/ml hygromycin B, 50 \(\mu\)g/ml hygromycin B + 0.2 m KCl, 1 mg/ml paromomycin sulfate, and 1 mg/ml paromomycin sulfate + 0.2 M KCl.
able to suppress the temperature sensitivity and Cs⁺ resistance of TW64 (pma1-s2 glc7-109), indicating that PMA1 is responsible for the complementation. To confirm that the suppressor mutations are alleles of PMA1, we mapped the distance between pma1-s4 and leu1, which lies ~1 cM centromere proximal to PMA1. Tetrad analysis of a cross between KT1850 (MATa ura3-52 his3 glc7-109 pma1-s4) and KT1849 (MATa ura3-52 his3 leu1) revealed that the distance between leu1 and pma1-s4 is 1.1 cM (P:NP:T = 43:0:1), identical to the previously determined map distance (P:NP:T = 869:0:22; McCusker and Haber 1988). Together, our linkage and complementation data indicate that all six glc7-109 suppressors are alleles of PMA1.

PMA1 mutants have been isolated that suppress the salt sensitivities of calcineurin mutants (Nass et al. 1997; Withee et al. 1998). To determine if our glc7-109 suppressors also suppress these defects, we crossed strains containing each of our suppressors to a cnb1 strain and assayed the meiotic products for salt sensitivity. Three of our suppressors fail to suppress the ion sensitivities of cnb1, but the other three partially suppress the Na⁺ and/or Li⁺ defects of the cnb1 strain (Table 4). The two cold-sensitive mutations (pma1-s1 and pma1-s4) that most strongly suppress glc7-109 also showed the greatest suppression of cnb1::LEU2.

Many pma1 mutants are sensitive to low pH (McCusker et al. 1987; Withee et al. 1998). We compared the growth of the glc7-109 mutant and a cold-sensitive pma1 mutant strain, TW71 (pma1-s4 glc7-109), in liquid YPD media at pH 3, 5, and 8.5. At pH 5, the growth of the wild-type, glc7-109, and pma1-s4 glc7-109 strains were all comparable (data not shown). As expected, the suppressor strain TW71 (pma1-s4 glc7-109) grows slowly at acidic pH but unexpectedly, the glc7-109 strain grows slowly at alkaline pH (Figure 8).

The ability of pma1 mutations to suppress the salt-sensitive phenotype of glc7-109 led us to assay Pma1 levels and activity in glc7-109 and pma1-s4 mutants. Pma1 levels in the glc7-109 strain were ~90% that of the wild type, as assayed by immunoblot analysis. Vanadate-sensitive ATPase activities, a measure of Pma1 activity, were comparable in the wild-type and glc7-109 strains (Table 3), but that of the pma1-s4 mutant is 61% that of the wild type (data not shown). Indirect immunofluorescence revealed no obvious differences between glc7-109 and wild-type strains in Pma1 localization (data not shown). Thus, we find no compelling evidence that glc7-109 di-

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**Figure 6.**—pma1 mutations suppress the Cs⁺ sensitivity conferred by glc7-109. Serial dilutions of pma1 mutants in a glc7-109 background were plated onto YPD medium, YPD media supplemented with the designated salts, and synthetic complete (SC) medium. The plates were incubated from 2 to 4 days. The strains that were used are wild type (KT1112), glc7-109 (TW25), pma1-s1 glc7-109 (TW30), pma1-s2 glc7-109 (TW31), pma1-s3 glc7-109 (TW33), pma1-s4 glc7-109 (TW38), pma1-s5 glc7-109 (TW43), and pma1-s6 glc7-109 (TW45).

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**Figure 7.**—The phenotype of pma1 mutants in a GLC7 background. Serial dilutions of pma1 mutants in a GLC7 background were plated onto YPD medium, YPD media supplemented with the designated salts, and synthetic complete (SC) medium. The plates were incubated for 2–4 days. The strains that were used are wild type (KT11112), glc7-109 (TW25), pma1-s1 (TW62), pma1-s2 (TW65), pma1-s3 (TW69), pma1-s4 (TW72), pma1-s5 (TW76), and pma1-s6 (TW80).
The potassium-remedial sensitivity of *glc7-109* mutants to toxic cations, high pH, and aminoglycoside antibiotics and the suppression of these defects by mutations in *PMA1* are consistent with the hypothesis that the plasma membrane potential of the *glc7-109* mutant is hyperpolarized. A conventional determination of membrane potential using electrophysiological methods is not possible in yeast, but relative membrane potentials can be assessed using fluorescent dyes. Although such methods suffer from complications caused by the contribution of the mitochondria to the uptake process, *Madrid et al.* (1998) have shown that cellular fluorescence of the cyanine dye DiOC<sub>6</sub>(3) can be determined under conditions in which mitochondria do not interfere. We have followed their procedure closely by growing strains to low density in glucose-containing medium and assaying DiOC<sub>6</sub>(3) fluorescence levels by flow cytometry. As shown in Table 5, DiOC<sub>6</sub>(3) fluorescence in the *glc7-109* strain (KT1596) was ~150–200% that of the wild-type strain. In contrast, DiOC<sub>6</sub>(3) fluorescence in a *pma1-s4* strain, TW72, was only 30–40% that of wild type and that of the *pma1-s4 glc7-109* strain (TW71) was only 50–60% of wild type. As a control, we assayed DiOC<sub>6</sub>(3) fluorescence in a wild-type strain grown in synthetic media containing a low concentration of K<sup>+</sup>, which is known to hyperpolarize the plasma membrane (Madrid et al. 1998). DiOC<sub>6</sub>(3) fluorescence in wild-type cells under these conditions was 450–650% of the wild-type cells grown in YPD medium (data not shown). The results of these assays suggest that many of the defects of *glc7-109* strains are caused either by a hyperpolarized plasma membrane or by the activation of a nonspecific ion conductance.

**DISCUSSION**

Like many *glc7* mutations, *glc7-109* is very pleiotropic. The *glc7-109* mutant hyperaccumulates glycogen and is sensitive to a wide range of cations, high pH, and aminoglycoside antibiotics. The analysis of the single missense mutations that make up *glc7-109* indicated that the R260A substitution is responsible for all the traits conferred by *glc7-109*. It is likely that the hyperglycogen trait and the ion and drug hypersensitivity traits reflect the influence of Glc7 on at least two separate physiological processes. This is because the glycogen phenotype of *glc7-109* is dominant, whereas the other traits are recessive, and because *glc7-R260P* confers the ion-related defects but not the hyperglycogen phenotype. Yeast PP1 has been associated with glycogen metabolism for years; its acronym was derived from the glycogen synthase. PP1 activity toward glycogen synthase is thought to dephosphorylate and activate glycogen synthase. Abundant evidence indicates that Gacl plays this role in yeast. *gac1* null mutants accumulate low levels of glycogen and glycogen synthase in these strains remains in a phosphorylated, inactive form (François et al. 1992). The product of *glc7-1* is the original glycogen-deficient allele of *GLC7*, fails to interact with Gac1 (Stuart et al. 1994). Gac1 contains separate domains required for binding Glc7 and glycogen synthase, both of which are required for functional activity (Wu et al. 2001). Mutations in the glycogen synthase kinase, Pho85/Pcl10, suppress the glycogen deficiency of *glc7-1* and *gac1* strains (Huang et al. 1996, 1998; Timblin et al. 1996). Furthermore, mutations in the major

### Table 4

<table>
<thead>
<tr>
<th>Suppressor</th>
<th>Phenotype of <em>pma1</em> mutant in a <em>GLC7</em> background</th>
<th><em>glc7-109</em> mutant defects suppressed by <em>pma1</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>cub1::LEU2</em> mutant defects suppressed by <em>pma1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pma1-s1</em></td>
<td>cs</td>
<td>Cs&lt;sup&gt;c&lt;/sup&gt;, Na&lt;sup&gt;c&lt;/sup&gt;, Li&lt;sup&gt;c&lt;/sup&gt;, Mn&lt;sup&gt;c&lt;/sup&gt;, paro., hygro.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Na&lt;sup&gt;c&lt;/sup&gt;, Li&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><em>pma1-s2</em></td>
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<td>Cs&lt;sup&gt;c&lt;/sup&gt;, Mn&lt;sup&gt;c&lt;/sup&gt;, paro., hygro.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Li&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>—</td>
<td>Cs&lt;sup&gt;c&lt;/sup&gt;, Na&lt;sup&gt;c&lt;/sup&gt;, Li&lt;sup&gt;c&lt;/sup&gt;, paro., hygro.&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Na&lt;sup&gt;c&lt;/sup&gt;, Li&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Li&lt;sup&gt;c&lt;/sup&gt;</td>
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The growth of *pma1* mutants in three genetic backgrounds was tested on various media as described in materials and methods. cs, reduced growth rate at 15<sup>°</sup>C; ts, reduced growth rate at 37<sup>°</sup>C; Li<sup>c</sup>, unable to grow on 0.1 M LiCl; Cs<sup>c</sup>, unable to grow on 0.1 M CsCl; Na<sup>c</sup>, unable to grow on 0.9 M NaCl media; Mn<sup>c</sup>, unable to grow on 0.01 M MnCl<sub>2</sub> media; paro., unable to grow on 1 mg/ml paromomycin sulfate media; hygro., unable to grow on 50 µg/ml hygromycin B media; and OH<sup>−</sup>, reduced growth at alkaline pH.

<sup>a</sup>The *pma1-s4* *glc7-109* mutant (TW75) is the only *glc7-109* suppressor that was tested for suppression of *glc7-109* growth at acidic and alkaline pH.
isoform of glycogen synthase, Gsy2, that cannot be phosphorylated also suppress the glycogen deficiencies of glc7-1 and gac1 strains (Hardy and Roach 1993; Anderson and Tatchell 2001). Together, these data suggest that Gac1 acts as a molecular scaffold to tether Gc7 and Gsy2. We were therefore surprised to discover that the hyperglycogen trait of glc7-109 is epistatic to a gac1 null mutation. Glycogen levels remain high in strains disrupted for GAC1 and two GAC1-related genes, PIG1 and GIP2.

One explanation for these results is that additional glycogen-targeting subunits can substitute for the loss of Gac1, Pig1, and Gip2. Pig2 is a candidate for such a redundant subunit. It is most similar in sequence to Gip2 and although loss-of-function mutations in PIG2 have not been found to alter glycogen levels, it is possible that Gc7-109 could recruit Pig2 in the absence of other targeting subunits. Another possibility is that the high levels of glycogen in glc7-109 strains are partially due to changes in the activity of enzymes other than glycogen synthase. We have not assayed activity levels of glycogen synthase in our strains and we cannot rule out the possibility that the degradative pathway is altered in glc7-109 strains. It is worth noting that Pho85, the kinase that phosphorylates glycogen synthase, also regulates the activity of glycogen phosphorylase through cyclins Pcl6 and Pcl7 (Wang et al. 2001). Furthermore, a large-scale analysis of protein complexes has found that Gip2 and Gc7 associate with glycogen phosphorylase (Ho et al. 2002), suggesting that Gc7, possibly through one or more of its targeting subunits, may regulate glycogen phosphorylase as well as glycogen synthase. A third possibility is that the Gc7-109 mutant protein no longer requires glycogen-targeting subunits to increase its V_{max} toward glycogen synthase, assuming that this is a role of glycogen-targeting subunits in yeast. There is direct biochemical evidence for this effect with a PPI-targeting subunit in smooth muscle (Tanaka et al. 1998) and some genetic evidence for such a role in yeast. Small fragments of Gac1 that contain only the Gc7-binding domain partially complement the glycogen deficiency of a gac1 null mutant when expressed at high levels (Wu et al. 2001). Clearly, further genetic and biochemical studies are necessary to sort out the complex roles of Gc7 in glycogen metabolism.

**Glc7 and ion homeostasis:** The pleiotropic salt-and-drug-sensitive phenotype conferred by glc7-109 can be accounted for by at least two broad mechanisms. One possible explanation is that the glc7-109 mutant has a hyperpolarized plasma membrane potential. In Neurospora crassa, in which membrane voltage measurements can be performed (Slayman 1965a,b), the plasma membrane H\(^+\)ATPase is largely responsible for maintaining the membrane voltage (Slayman et al. 1973; Gradmann et al. 1978). In S. cerevisiae, mutants in the gene encoding the plasma membrane H\(^+\)ATPase, *PMA1*, which are inferred to have a reduced membrane potential (Perlin et al. 1988; Seto-Young and Perlin 1991), have defects that are in general opposite to that of glc7-109 mutants, exhibiting increased resistance to drugs and toxic cations (McCusker et al. 1987). In contrast, yeast cells with defects in the plasma membrane K\(^+\) transporters

**TABLE 5**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>400 mm KCl</th>
<th>Fluorescence of DiOC6(3)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT1112</td>
<td>GLC7 PMA1</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>KT1596</td>
<td>glc7-109 PMA1</td>
<td>+</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>TW72</td>
<td>GLC7 pma1-s4</td>
<td>–</td>
<td>177 ± 18</td>
</tr>
<tr>
<td>TW71</td>
<td>glc7-109 pma1-s4</td>
<td>+</td>
<td>100 ± 10</td>
</tr>
</tbody>
</table>

* Fluorescence intensity of DiOC6(3) in glc7-109 (KT1596), pma1-s4 (TW72), and pma1-s4 glc7-109 (TW71) strains is expressed as the percentage of wild-type levels (see MATERIALS AND METHODS). Values represent the average of three separate experiments performed on three separate days.

**Figure 8.** The growth of the glc7-109 mutant and the *pma1-s4* glc7-109 mutant in YPD medium adjusted to pH 3 and 8.5. Log-phase cultures of each strain were incubated in liquid YPD media at pH 3 and 8.5 as described in MATERIALS AND METHODS. The growth of each strain was monitored by optical density at 660 nm. The strains that were used are wild type (KT1112), glc7-109 (TW25), and pma1-s4 glc7-109 (TW71).
encoded by TRK1 and TRK2 have been inferred to have an increased membrane potential (MADRÍD et al. 1998) and have a phenotype that is similar, but not identical, to that of the glc7-109 mutant (VIDAL et al. 1990; MADRÍD et al. 1998; MULET et al. 1999). Direct measurements of membrane potential in S. cerevisiae are not possible for technical reasons and indirect determinations using fluorescent dyes are subject to artifacts (BALLARÍN-DENTI et al. 1994). Nevertheless, our analysis of DiOC₆(3) fluorescence is consistent with hyperpolarization of the plasma membrane in glc7-109 cells. The isolation of mutations in PMA1 as suppressors of the CsCl sensitivity of glc7-109 mutants further supports this hypothesis.

How could Glc7 act to influence membrane potential? The two most obvious possibilities are via Pma1 and the potassium transporters, Trk1 and Trk2. Pma1 is essential for maintenance of the plasma membrane potential and its activity is regulated in response to a range of external factors. Pma1 is phosphorylated at multiple sites and at least two classes of protein kinases may regulate its activity (KOLAROV et al. 1988; BERTORELLO et al. 1991; SERRANO et al. 1991). The protein kinase Ptk2 is a positive regulator of Pma1 and appears to act through the activating phosphorylation site in the Ser-899 (GOOSENS et al. 2000). In contrast, the Yck1 and Yck2 kinases may act as negative regulators of Pma1 activity (ESTRADA et al. 1996). Thus, Pma1 is a likely candidate to be regulated by Glc7. However, we have not found a direct involvement of Glc7 in Pma1 regulation. Pma1 levels and Pma1-dependent H⁺-ATPase activities are similar in glc7-109 and GLC7 strains, but we cannot exclude the possibility that glc7-109 exerts a subtle effect on Pma1 activity or that Glc7 regulates Pma1 in response to specific environmental stresses.

The potassium transporters Trk1 and Trk2 are also candidates for Glc7 regulation. Like glc7-109, trk1 and trk1 trk2 double mutants exhibit high levels of DiOC₆(3) fluorescence (MADRÍD et al. 1998) and share traits in common with glc7-109, including sensitivity to toxic cations and hygromycin B (MULET et al. 1999). However, unlike mutants lacking the potassium transporters, the glc7-109 mutant exhibits no obvious growth defect on media containing low concentrations of potassium. glc7-109 also confers sensitivity to alkaline pH as opposed to the acidic pH sensitivity of trk1 mutants. Surprisingly, K⁺ transport and regulation of membrane potential may be independent properties of Trk1 and Trk2. MADRÍD et al. (1998) have found that trk1 trk2 mutants expressing a K⁺ transporter (HAK1) from Schwannomyces occidentalis that is unrelated in structure to Trk1 or Trk2 exhibit normal levels of K⁺ transport but retain the hyperpolarization of the trk1 trk2 mutant. In contrast, expression of a plant K⁺ transporter related to Trk1 suppresses both the K⁺ transport and membrane potential defects of trk1 trk2 mutants. Using patch clamping on yeast spheroplasts, BÍLHER et al. (1999) have observed an inward, H⁺-dependent current that is dependent upon Trk2. Surprisingly, this current is independent of K⁺ and is also independent of Trk1. A possible scenario arising from these studies is that the K⁺ transporters may have an auxiliary function unrelated to K⁺ transport, which strongly regulates membrane potential. Two related protein kinases, Hal4 and Hal5, may modulate Trk1 and Trk2 activity (MULET et al. 1999). Yeast cells lacking Hal4 and Hal5 have a phenotype similar to that of trk1 trk2 strains, including hypersensitivity to toxic cations and hygromycin B and a deficiency in K⁺ uptake. In contrast, increased expression of Hal4 or Hal5 results in resistance to Li⁺ and Na⁺ (MULET et al. 1999). These results suggest that Hal4 and Hal5 are positive regulators of Trk1 and Trk2. However, if Glc7 regulates K⁺ transport, it probably does not do so by simply opposing the kinase activity of Hal4 and Hal5 since glc7-109 confers a similar phenotype to that of a hal4 hal5 null mutant.

An alternative explanation to hyperpolarization for the pleiotropic drug- and ion-hypersensitive phenotype of the glc7-109 mutant is that glc7-109 results in the activation of a nonspecific uptake mechanism or conductance. In this scenario, a reduction of Pma1 activity or the application of exogenous K⁺ could suppress the drug and ion toxicity of the glc7-109 mutation through indirect effects. In this regard, we note that BÍLHER et al. (1998) have identified a nonspecific cation channel in S. cerevisiae by patch-clamping yeast spheroplasts. This conductance, termed nonselective cation channel 1 (NSC1), carries a large inward current and has low cation specificity. NSC1 is independent of Trk1, Trk2, and the potassium channel Duk1/Tok1, but the gene or genes encoding this channel have not been identified. It is possible that NSC1 remains open in glc7-109 mutants, thus causing an increased toxicity to cations. Of course, we cannot exclude the possibility that Glc7 has multiple targets that influence ion homeostasis. Many GLC7 mutants are pleiotropic and it is not unreasonable to propose that multiple substrates are affected in glc7-109 strains. Further genetic analysis may allow us to identify these targets.

We thank Carolyn Slayman and Ken Allen for providing antibody to Pma1 and for helpful advice concerning Pma1 activity assays. We acknowledge Martha Cyert, James Haber, and Peter Roach for kindly providing strains and plasmids. We thank Andrew Bloecher, Lucy Robinson, Heather Panek, Clifford Slayman, and Guglielmo M. Venuti for helpful discussions. We gratefully acknowledge Clifford Slayman and Lucy Robinson for critically reading this manuscript. This work was funded by National Institutes of Health research grant GM-47789.

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