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Identification of a Calcineurin-Independent Pathway Required for Sodium Ion Stress Response in *Saccharomyces cerevisiae*

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ABSTRACT

The calcium-dependent protein phosphatase calcineurin plays an essential role in ion homeostasis in yeast. In this study, we identify a parallel ion stress response pathway that is independent of the calcineurin signaling pathway. Cells with null alleles in both *STD1* and its homologue, *MTH1*, manifest numerous phenotypes observed in calcineurin mutants, including sodium, lithium, manganese, and hydroxyl ion sensitivity, as well as alpha factor toxicity. Furthermore, increased gene dosage of *STD1* suppresses the ion stress phenotypes in calcineurin mutants and confers halotolerance in wild-type cells. However, Std1p functions in a calcineurin-independent ion stress response pathway, since a *std1 mth1* mutant is FK506 sensitive under conditions of ion stress. Mutations in other genes known to regulate gene expression in response to changes in glucose concentration, including *SNF3*, *RGT2*, and *SNF5*, also affect cell growth under ion stress conditions. Gene expression studies indicate that the regulation of *HAL1* and *PMR2* expression is affected by *STD1* gene dosage. Taken together, our data demonstrate that response to ion stress requires the participation of both calcineurin-dependent and -independent pathways.

YEAST cells are able to adapt to many environmental stresses, including changes in the osmolarity of their surroundings. When incubated in hypertonic media, yeast cells adapt by inducing the synthesis of high concentrations of intracellular glycerol (Brewster *et al.* 1993). Indeed, the enzyme responsible for glycerol synthesis, the product of the *GPD1* gene, is essential for growth in hypertonic media (Albertyn *et al.* 1994). Changes in osmolarity are detected by two independent osmosensors that in turn regulate the activity of the *HOG1* mitogen activated protein (MAP) kinase pathway (Posas *et al.* 1996). Activation of the *HOG1* MAP kinase cascade signals the induction of transcription from a number of genes through stress response elements (STREs) (Schuller *et al.* 1994).

Independent of their effects on osmolarity, certain ions are toxic to yeast cells due to their ability to inhibit specific metabolic pathways. Sodium and lithium ions are particularly toxic to yeast cells due to their inhibition of the 3′,5′-bisphosphate nucleotidase enzyme that is required for sulfate assimilation (Murguia *et al.* 1995, 1996). This enzyme is encoded by the *MET22/HAL2* gene and was isolated initially as a methionine auxotroph and subsequently by virtue of its ability to confer sodium tolerance when present on a high-copy plasmid (Gaxiola *et al.* 1992; Glaser *et al.* 1993). The inhibition of the 3′,5′-bisphosphate nucleotidase activity by sodium can be reversed by addition of potassium (Murguia *et al.* 1995), suggesting that the Na^+/K^+ ratio is more important to this enzyme's activity than is the absolute concentration of sodium. This finding may explain why yeast cells undergoing sodium ion stress act both to reduce intracellular sodium ion concentration and to retain intracellular potassium ions. Although the molecular mechanism is unclear, the *HAL3* gene appears to play a critical role in determining the intracellular Na^+/K^+ ratio by regulating cation transport (Ferrando *et al.* 1995).

Yeast cells undergoing sodium ion stress increase both the expression and the activity of the Pmr2p, a P-type ion pump thought to be responsible for Na^+ and Li^+ ion efflux (Weil and *et al.* 1995). Both of these responses to Na^+ ion stress require the activity of the calcium signaling pathway. Ca^{2+}/calmodulin binds to and activates Pmr2p, thereby directly stimulating Na^+ ion efflux (Weiland *et al.* 1995). In addition, Ca^{2+}/calmodulin binds to and activates the protein phosphatase calcineurin. Although the phosphoprotein substrates of calcineurin have not yet been identified, calcineurin activity is required for the transcriptional induction of the *PMR2* gene (Garciadeblas *et al.* 1993; Cunningham and Fink 1996). Recent studies have identified a zinc finger transcription factor that is required for calcineurin-mediated stimulation of transcription of the *PMR2*, *PMC1*, and *FKS2* genes (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). Thus, the calcineurin-dependent signaling pathway leading to increased expression of the *PMR2* gene comprises one essential response to Na^+/Li^+ ion stress.

Calcineurin is a highly conserved (Cyert *et al.* 1991)

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heterodimer composed of one catalytic subunit and one regulatory subunit. The regulatory subunit, a calcium-binding protein containing EF hands related to calmodulin, is required for calcineurin activity (Cyert and Thorner 1992), although the molecular role played by this subunit has not yet been determined. The catalytic subunit contains two distinct domains (Guerini 1997); the N-terminal half comprises the phosphatase active site conserved in numerous protein phosphatases, while the C-terminal domain contains an auto-inhibitory sequence that blocks phosphatase activity. In addition, the regulatory domain contains a calmodulin-binding motif whose presence distinguished calcineurin from other protein phosphatases. Binding of calcium-calmodulin activates calcineurin by relieving the effects of the auto-inhibitory sequence. Indeed, truncation of the C-terminal domain results in a constitutively active calcineurin enzyme (Hubbard and Klee 1989).

In mammals, calcineurin plays an important role in signal transduction in a number of cell types. In activated T-cells, the primary target of calcineurin appears to be members of the NFAT (nuclear factor of activated T-cells) family of transcription factors (Rao *et al.* 1997). In resting T-cells, the NFAT proteins are phosphorylated and thereby trapped in the cytoplasm. Dephosphorylation by calcineurin unmasks the nuclear localization signal, thereby promoting translocation to the nucleus where NFAT cooperates with AP-1 (*fos-jun*) to activate transcription of cytokine genes. In renal tubule cells, calcineurin promotes Na^+ ion retention by dephosphorylating DARP32 (Aperia *et al.* 1992), an inhibitor of protein phosphatase 1. Protein phosphatase 1, in turn, activates the Na^+,K^+-ATPase, which provides the concentration gradient necessary for Na^+ ion readsorption. Thus, in mammals and in yeast, calcineurin plays a crucial role in Na^+ ion homeostasis.

Our studies have focused on the Std1 protein, whose gene was cloned as a high-copy-number suppressor of a mutation in the TATA binding protein (TBP) (Ganster *et al.* 1993). *STD1* (*MSN3*) was also isolated as a high-copy-number suppressor of the growth defect exhibited by *snf4Δ2* mutants on raffinose antimycin media and was shown to be a gene-dosage-dependent modulator of glucose repression (Hubbard *et al.* 1994). Yeast cells contain a single homologue of *STD1* designated *MTH1.* Null alleles in this pair of genes suggested that they acted redundantly because neither single knockout produced a detectable phenotype, whereas the double knockout strain was defective in the derepression of the *SUC2* gene. Std1p has also been shown to interact in the two-hybrid system and biochemically with both the Snf1 kinase (Hubbard *et al.* 1994) and with TBP (Tillman *et al.* 1995). One hypothesis for these data posits that Std1p couples the glucose starvation signal from the Snf1 kinase complex to transcriptional machinery.

In a search for additional phenotypes in cells lacking Std1p function, we discovered that *std1Δ mth1Δ* cells grew poorly on media containing high concentrations of NaCl but not KCl. Because this phenotype is similar to that observed in calcineurin mutants, we tested the *std1Δ mth1Δ* mutant for other calcineurin phenotypes. Our results indicate that the Std1p is required for Na^+ ion stress response in *Saccharomyces cerevisiae* but that it functions in a signaling pathway that is distinct from the calcineurin pathway.

MATERIALS AND METHODS

Yeast strains, media, and genetic techniques: *S. cerevisiae* strains utilized in this study are described in Table 1. Except where indicated, growth of yeast utilized standard media (Rose *et al.* 1990) at 30°. Glucose, sucrose, or raffinose were present at 2% (g/100 ml). Antimycin A was included at 1 μg/ml where indicated. Standard procedures were utilized for genetic crosses, sporulation, and tetrad analysis (Rose *et al.* 1990). Transformations of yeast strains utilized the lithium acetate procedure (Gietz *et al.* 1995). FK506 was provided by Fujisawa USA Inc.

Plasmid constructions: Increased gene dosage of *STD1* was obtained by transforming cells with p6A5U, which contains the 3.9-kb *Pvu*II to *Sal*I fragment from p6A (Ganster *et al.* 1993) bearing the entire *STD1* gene cloned into the 2μ plasmid YEp352 (Hill *et al.* 1986). The *SNF5* centromeric plasmid was constructed by placing the 4380-bp genomic *Eco*RI fragment (−1159 to +3671, relative to the ATG codon) that contains the entire *SNF5* open reading frame and flanking sequences into the CEN plasmid pUN55 (Elledge and Davis 1988). The *STD1* centromeric plasmid, pRG70U, was constructed by inserting the 3429-bp *Sac*I to *Eag*I fragment from p6A encompassing the entire *STD1* gene and flanking sequences into the *Sac*I and *Eag*I sites of pUN55 (Elledge and Davis 1988). The 2μ *CNB1* plasmid, pAMS283 (kindly provided by Angela Stathopoulos and Martha Cyert), contains a 1.4-kb genomic DNA fragment encompassing the entire *CNB1* gene inserted into YEp352 (Hill *et al.* 1986).

Spot dilution growth assays: Liquid cultures were grown for at least two cell generations to an OD_{600} between 0.2 and 0.4. Cell densities were normalized to an OD_{600} of 0.2, and 5–10 μl of 10-fold serial dilutions of the liquid cultures were spotted onto solid media and incubated at 30° for 2–5 days.

Strain constructions: All *S. cerevisiae* strains used in this study are in the S288c background. Strains with null alleles in *STD1* and *MTH1* were constructed with plasmids pJH104 and pJH124, respectively (Hubbard *et al.* 1994). The *mth1Δ2* allele was constructed using the 3796-bp *Eco*RV genomic DNA fragment encompassing the *MTH1* locus from which the 1791-bp *Eco*RI fragment (−986 to +805, relative to the initiating ATG codon) had been deleted. A strain bearing the *mth1Δ1::URA3* allele was transformed with the modified *Eco*RV fragment, and 5-FOA media was used to select Ura^- transformants. The resulting deletion of the 5′ half of *MTH1* was confirmed by Southern blot. The *mth1Δ2* allele does not confer any *MTH1* function because cells bearing the *mth1-Δ1::URA3* allele or the *mth1Δ2* allele were equally defective for growth on raffinose antimycin media when present in combination with a *std1* null allele. A null allele of the *SNF4* gene was engineered by replacing the entire *SNF4* coding sequence with the loxP-kanMX-loxP cassette from plasmid pUG6 (Guldener *et al.* 1996). The loxP-kanMX-loxP cassette was amplified in a PCR reaction using oligonucleotides with 22 and 19 bases complementary to the kanMX cassette at the 3′ end (lower case) and 40 bases complementary to the *SNF4* locus at the 5′ end (upper case). The primer sequences were as follows: 5′-CTGTGT

TABLE 1

***S. cerevisiae* strains used**

Strain	Genotype	Source or reference
FY14	*MAT***a** *ura3-52 trp1Δ63*	Winston *et al.* (1996)
FY86	*MATα ura3-52 leu2-Δ1 his3-Δ200*	Winston *et al.* (1996)
MCY2099	*MATα ura3-52 his3-Δ200 ade2-101 snf5-Δ2*	Laurent *et al.* (1990)
MSY182	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63*	This study
MSY192	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 std1::HIS3 mth1-Δ2*	This study
MSY314	*MATα ura3-52 leu2-Δ1 his3-Δ200 snf4::KAN*	This study
MSY317	*MATα ura3-52 leu2Δ1 trp1Δ63 snf1::KAN*	This study
MSY401	*MATα ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63*	This study
MSY402	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 snf3::hisG rgt2::HIS3*	This study
MSY403	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 rgt2::HIS3*	This study
MSY404	*MATα ura3-52 leu2 Δ1 his3-Δ200 trp1 Δ63 snf3::hisG*	This study
YPH499	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 lys2-801 ade2-101*	Sikorski and Hieter (1989)
MCY300-1	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 lys2-801 ade2-101 cna1-Δ1::hisG cna2-Δ1::HIS3*	Cyert *et al.* (1991)
DD12	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ63 lys2-801 ade2-101 cnb1-Δ1::hisG*	Cybert and Thorner (1992)

TAGCATTAGGAGGAAGCGAAAAGGAAAATACATAgcataggc cactagtggatctg and 5′-TTTATTTATAGTATGTACACAAAAA TCTCATCGGCTCGTTcagctgaagcttcgtacgc. A diploid strain (FY14 × FY86; Winston *et al.* 1996) was transformed with the PCR product, and G418-resistant clones were analyzed by PCR using one primer complementary to the kanMX gene and one primer upstream of the *SNF4* open reading frame. One strain that was positive by PCR was sporulated and segregants analyzed for growth on G418 and raffinose media containing 1 μg/ml antimycin A. Of the 15 complete tetrads analyzed, all 15 showed 2:2 segregation of G418 resistance and all G418-resistant segregants displayed a *snf*$^-$ phenotype on raffinose-antimycin media. To confirm that the *snf*$^-$ phenotype was due to disruption of the *SNF4* locus, one *snf4::KAN* haploid strain was mated with a wild type, a *snf5* strain, and with a *snf4Δ2* strain. Only the *snf4Δ2* strain failed to complement the *snf4::KAN* allele. A null allele of the *SNF1* gene was constructed by PCR amplification of the kanMX cassette of pUG6 with long flanking homology regions using the procedure described by Wach (1996). The primers used to amplify the 5′ flank of the *SNF1* locus were T-324: 5′-GGCACATCAA CAGGTAGCGTTATAGGGG and SK-B-1: 5′-gcgtacgaagcttcag ctggcggccgcGTTGACTTTATTAAGGGAGTGTAGC. The primers used to amplify the 3′ flank were SK-T1903: 5′-tctgcc ggtctccctatagtgagtcgTGGTGGAACGTAAAAGAATGATATGG and B2260: 5′-GGTTGTATTTTTGTCGCAGACTCCG. Nucleotides written in the upper case are complementary to the *SNF1* locus and those written in lower case are complementary to the kanMX cassette. The 5′ and 3′ flanking sequences were amplified, gel purified, and used in a second PCR reaction using pUG6 as a template and primers T-324 and B2260. The resulting 2.3-kb PCR product was used to transform a diploid (FY14 × FY86; Winston *et al.* 1996) to G418 resistance. Disruption of the *SNF1* locus was confirmed by Southern blot. Tetrad analysis showed 2:2 segregation of the G418 resistance and cosegregation of a *snf*$^-$ phenotype in all tetrads analyzed. A null allele of the *SNF3* gene was constructed using plasmid pBM3103, kindly provided by Sabire Ozcan and Mark Johnston, Washington University (Ozcan *et al.* 1996). A null allele of the *RGT2* gene was created by PCR amplification using the identical primers and strategy described by Ozcan *et al.* (1996). Correct integration for both the *snf3::hisG-URA3-hisG* allele and the *rgt2::GFP-HIS3* allele were confirmed by Southern blot analysis. The pmr2::lacZ plasmid pFR70 (Marquez and Serrano 1996) was kindly provided by A. Rodriguez-Navarro.

Alpha factor toxicity: Yeast cultures that had been grown overnight in low-pH YEPD (Withee *et al.* 1997) were diluted in the same media to an OD_{600} of 0.2. The cultures were grown an additional 2 hr, at which point ($t = 0$) alpha factor [synthesized Millipore (Bedford, MA) model 9050 plus Synthesizer] was added to a final concentration of 50 μm. Aliquots were removed, diluted in water, and plated on solid YEPD media in order to determine viable cells per ml. Experiments with 2μ plasmids were conducted in low-pH SC media lacking uracil.

Northern blot analyses: Liquid cultures (10 ml) were harvested in log phase, and total RNA was prepared by the hot phenol method (Kohrer and Domdey 1991). A total of 10 μg of RNA isolated from each culture was subjected to electrophoresis in formaldehyde-1% agarose gels. The quantity and integrity of the RNA samples were analyzed by ethidium bromide staining in 0.1 m ammonium acetate. The RNA was electrotransferred to nylon membrane and hybridized to ^{32}P-labeled DNA sequences. The DNA probes included gel-purified DNA fragments of the entire *HAL1* open reading frame generated by PCR, the 2084-bp genomic *Hin*dIII fragment containing the *SUC2* open reading frame, and the 240-bp genomic *Bgl*II-*Kpn*I fragment of *TUB2*. All fragments were radiolabeled by the random priming method.

RESULTS

***STD1* gene function is required for ion stress response:** Cells lacking *STD1* gene function (*std1Δ mth1Δ*) appear indistinguishable from wild-type cells on standard rich (YEPD) and minimal (synthetic complete) media at all temperatures tested (data not shown). However, *std1Δ mth1Δ* strains display a defect in invertase derepression (Hubbard *et al.* 1994) and consequently show impaired fermentative growth on media with sucrose or raffinose as the carbon source (Figure 1A). To identify new functions of the Std1 protein, a set of strains

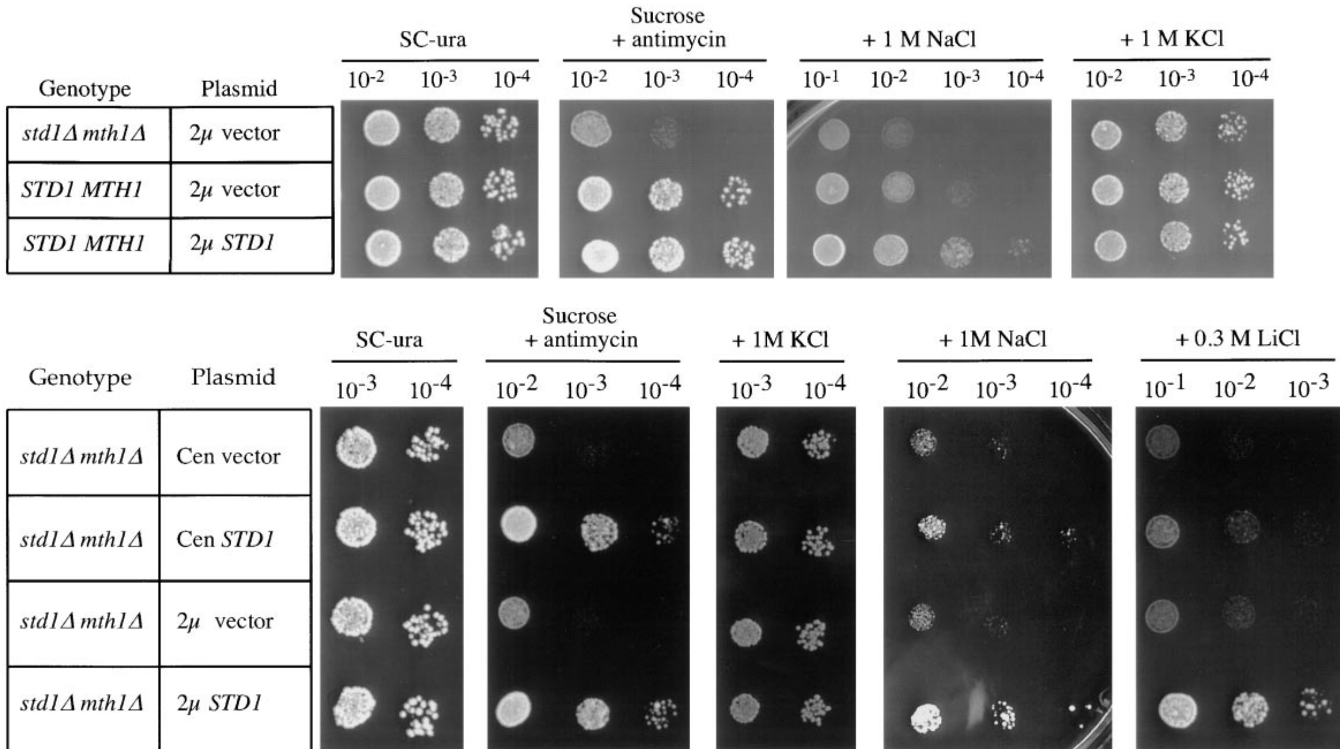

Figure 1.—Effect of *STD1* gene dosage on cell growth under ion stress conditions. Serial dilutions of yeast cultures were spotted onto standard synthetic complete media lacking uracil with 2% glucose, 2% sucrose plus 1 μg/ml antimycin A, or 2% glucose supplemented with either 1 m NaCl, 1 m KCl, or 0.3 m LiCl, as indicated. (A) The strains used in this experiment are MSY192 (*std1*Δ *mth1*Δ) transformed with YEp352 (2μ vector). MSY182 (*STD1 MTH1*) transformed with either YEp352 or p6A5U (2μ *STD1*), as indicated. (B) MSY192 (*std1*Δ *mth1*Δ) was transformed with either pUN55 (CEN vector), pRG70U (CEN *STD1*), YEP352, or p6A5U, as indicated.

that differ in *STD1* gene dosage was analyzed for growth on a wide variety of different media. We have found that *std1*Δ *mth1*Δ strains display impaired growth on media containing 1 m NaCl (Figure 1A). This effect is not due to a defect in the *HOG1*-osmotic stress pathway because no growth defect is observed when this strain is grown in the presence of 1 m KCl (Figure 1A) or 1 m sorbitol (not shown). Furthermore, increased *STD1* gene dosage on high copy number plasmids provides wild-type cells with a growth advantage on media containing 1 m NaCl. This Std1-mediated growth advantage was specific to sodium and was not observed on media containing 1 m KCl (Figure 1A) or 1 m sorbitol (not shown).

To rule out any effect of strain variation on these results, a single *std1*Δ *mth1*Δ strain transformed with low and high copy plasmids containing the *STD1* gene or no insert was analyzed for growth in various media (Figure 1B). Cells lacking *STD1* gene function show no growth defect on synthetic complete media alone or supplemented with 1 m KCl. However, when cell growth is dependent on the fermentation of sucrose, cells lacking *STD1* gene function display a distinct growth defect that is equally well suppressed by low and high copy *STD1* plasmids (Figure 1B). Growth in media supplemented with either 1 m NaCl or 0.3 m LiCl is greatly affected by *STD1* gene dosage. Cells lacking any *STD1* gene function grow very poorly in media containing these concentrations of Na^+ and Li^+. Low copy plasmids containing *STD1* provide a limited growth advantage in the presence of 1 m NaCl, and the high copy plasmid encoding *STD1* is required to promote a growth advantage in the presence of 0.3 m LiCl. The fact that these strains are isogenic except at the *STD1* locus confirms that the *STD1* gene plays an essential role in ion stress response.

Loss of Std1p function confers additional phenotypes found in calcineurin mutants: The PP2B protein phosphatase calcineurin is an important regulator of ion homeostasis in yeast (Nakamura *et al.* 1993). In particular, calcineurin mutants exhibit growth defects in the presence of specific ions, including sodium, lithium, manganese, and hydroxyl ions, but not when challenged by potassium or magnesium. Since *std1 mth1* mutants also exhibited sodium and lithium sensitivity but not potassium sensitivity, it was possible that other calcineurin phenotypes would be shared by *std1 mth1* mutants. Three yeast strains that differ in *STD1* gene dosage were compared for growth in the presence of 10 mm manganese chloride and in media adjusted to pH 8.3 (Figure 2). Cells lacking *STD1* gene function clearly showed growth defects relative to the wild-type cells

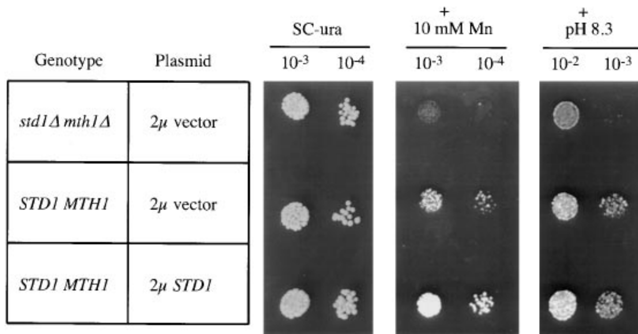

Figure 2.—Loss of *STD1* gene function causes additional phenotypes observed in calcineurin mutants. Serial dilutions of yeast cultures were spotted onto synthetic complete media lacking uracil or the same media supplemented with 10 mM $MnCl_2$ or adjusted to pH 8.3, as indicated.

under both of these conditions. Mutations in either the catalytic subunits (*CNA1* and *CNA2*) or the regulatory subunit (*CNB1*) of calcineurin also cause growth defects under these same ion stresses (Nakamura *et al.* 1993; Pozos *et al.* 1996) (Figure 3). Another phenotype of calcineurin mutants is loss of viability in the presence of the alpha factor mating pheromone (Withee *et al.* 1997). Pairs of isogenic strains lacking either calcineurin (Sikorski and Hieter 1989; Cyert *et al.* 1991; Cyert and Thorner 1992) or *STD1* gene function were incubated with 50 μM alpha factor, and then cell viability was determined over a 6-hr time course. Cells lacking calcineurin (*cnb1*) lose viability in alpha factor with <10% of the cells being viable after 6 hr (Figure 4A). Likewise, the *std1*Δ *mth1*Δ strain also showed a similar loss of viability over the same time course and in contrast to the isogenic wild-type strains. Thus cells lacking either calcineurin function or *std1 mth1* function share an identical set of phenotypes with respect to ion stress and alpha-factor toxicity.

Increased gene dosage of *STD1* suppresses calcineurin mutations: Since *std1 mth1* mutants shared several phenotypes with calcineurin mutants, it seemed possible that the Std1p (and Mth1p) might act in the same or in a parallel ion response pathway to calcineurin. If true, then increased gene dosage of *STD1* might be able to compensate for the loss of calcineurin function. To test this hypothesis, we transformed a set of isogenic strains that differ only at the *CNA1*, *CNA2*, and *CNB1* loci with 2μ plasmids containing either the *STD1* gene or no insert. These strains were then tested for growth under a variety of ion stresses. Cells lacking either both copies of the calcineurin catalytic subunits or the single regulatory subunit all display a marked growth defect when grown on media containing 0.9 M NaCl, 0.3 M LiCl, or 10 mM $MnCl_2$ or in media adjusted to pH 8.3 (Figure 3). The growth defects observed in the calcineurin mutants, however, were in all cases suppressed by increased gene dosage of *STD1.* The suppression by 2μ *STD1* is observed in both calcineurin mutant strains as well as in wild-type cells whose calcineurin activity was inhibited by the immunosuppressant drug FK506 (data not shown). Increased gene dosage of *MTH1*, the *STD1* gene homologue, was also able to suppress calcineurin ion stress phenotypes (data not shown). However, a second calcineurin phenotype, loss of viability in alpha factor, was not suppressed by increased gene dosage of *STD1* (Figure 4B). In this experiment, a *cnb1*$^-$ strain was transformed with high copy plasmids containing either the *STD1* gene, the *CNB1* gene, or no insert. The *CNB1* plasmid restored viability to the *cnb1* mutant, indicating that plasmid-based suppression in this media was possible. Increased gene dosage of *MTH1* was also unable to suppress this *cnb1* defect (data not shown). The ability of the Std1p and the Cna1p and Cnb1p to interact directly was tested in the two-hybrid system. Two-hybrid constructs containing

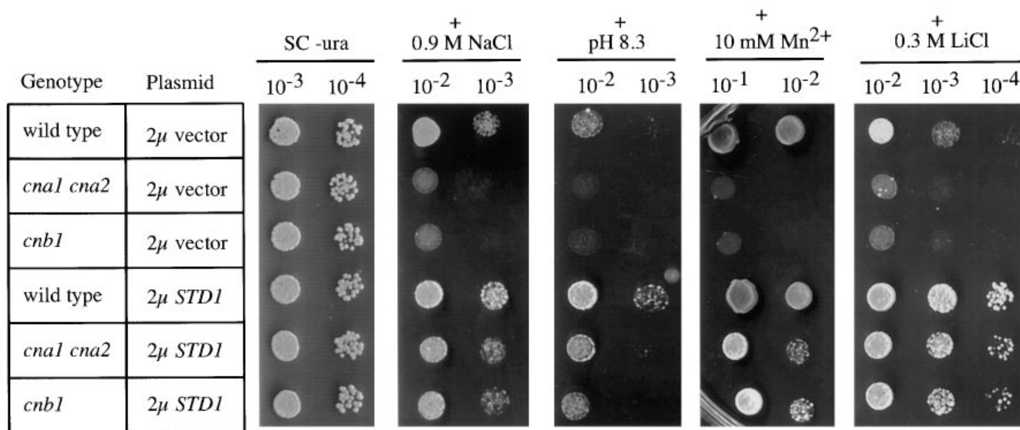

Figure 3.—Increased gene dosage of *STD1* suppresses ion stress-induced growth defects in calcineurin mutants. Serial dilutions of yeast cultures were spotted onto synthetic complete media lacking uracil with 2% glucose or 2% glucose supplemented with 0.9 M NaCl, 10 mM $MnCl_2$, pH 8.3, or 0.3 M LiCl, as indicated. The set of isogenic strains used in this experiment, YPH499 (*CNA1 CNA2 CNB1*), MCY300 (*cna1*Δ *cna2*Δ), and DD12 (*cnb1*Δ), were transformed with either YEp352 (2μ vector) or p6A5U (2μ *STD1*), as indicated.

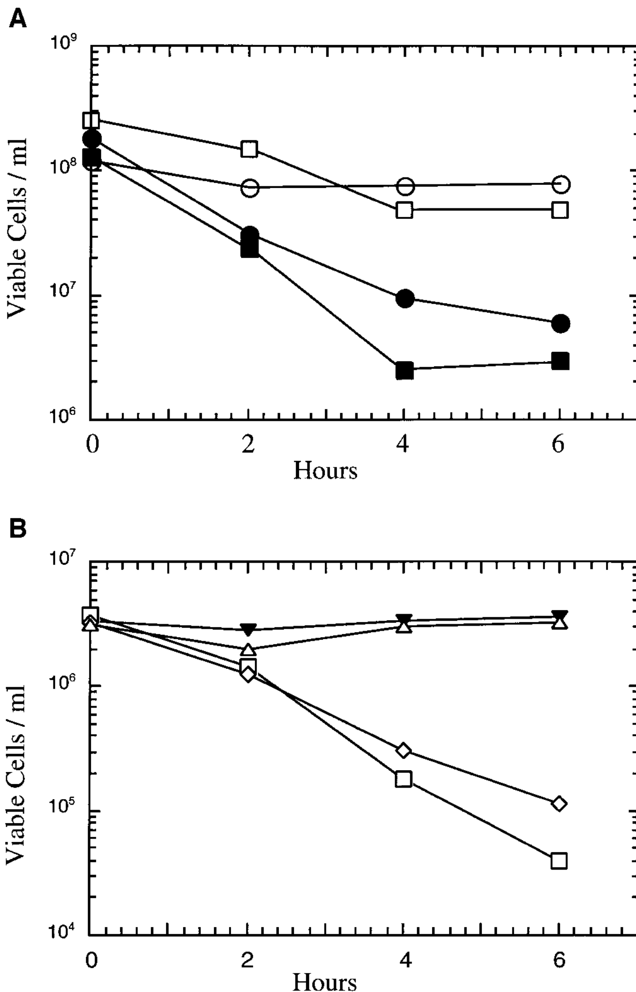

Figure 4.—Alpha factor toxicity shared by both calcineurin and *std1Δ mth1Δ* strains. (A) Cells were grown overnight in low-pH YEPD and diluted to an OD_{600} of 0.2 in the same media. Cells were grown an additional 2 hr, at which point (time 0) alpha factor was added to a final concentration of 50 μm. Aliquots were removed at 2, 4, and 6 hr after addition of alpha factor and the number of viable cells per ml determined by plating dilutions onto YEPD. The strains used were YPH499 (wild type, □), MCY300 (*cnb1*, ■), MSY182 (wild type, ○), and MSY192 (*std1Δ mth1Δ*, ●). (B) Increased gene dosage of *STD1* fails to suppress alpha factor toxicity in calcineurin mutants. Cells were grown overnight in low-pH SC-ura and then diluted in fresh media for an additional 2 hr of growth, at which point (time 0) alpha-factor was added to a final concentration of 50 μm. Aliquots were removed and the number of viable cells per ml determined by plating dilutions on SC-ura. The strains used were YPH499 (wild type) transformed with YEp352 (▼), DD12 (*cnb1*) transformed with either YEp352 (◇), p6A5U (2μ *STD1*, △), or pAMS283 (2μ *CNB1*, △).

Std1p and Cna1p or Cnb1p failed to activate reporter gene expression (data not shown), suggesting that these proteins may not stably interact *in vivo.*

The Std1p and calcineurin function in distinct ion stress response pathways: The findings that *std1 mth1* mutants exhibit the same ion stress and alpha factor toxicity phenotypes as calcineurin mutants and that the 2μ *STD1* suppresses the ion stress phenotypes of calcineurin mutants are consistent with a model in which Std1p acts in the same pathway but downstream of calcineurin. Alternatively, Std1p may function in a parallel pathway independent of calcineurin or downstream of calcineurin in a branched pathway. To distinguish these possibilities, yeast strains with null alleles in either calcineurin or in *STD1* and *MTH1* were exposed to LiCl ion stress in the presence and absence of the immunosuppressant drug FK506 that specifically blocks the function of calcineurin (Foor *et al.* 1992; Nakamura *et al.* 1993). FK506 has no effect on the cell growth on YEPD plates (not shown), consistent with the observation that calcineurin is not required for growth on YEPD. Under conditions of ion stress, however, calcineurin function is required for optimal growth. Strains lacking calcineurin function (*cnb1*) or *STD1* gene function (*std1 mth1*) grow poorly in the presence of 0.3 m LiCl (Figures 1B and 3). To distinguish between the different pathway models, these strains were exposed to mild ion stress conditions (0.1 m LiCl) in the presence and absence of FK506 (Figure 5). In the absence of FK506, the wild-type strain has a distinct growth advantage over the calcineurin mutant on media containing 0.1 m LiCl. However, in the presence of FK506, the wild type and calcineurin mutant display very similar growth kinetics, consistent with the earlier data showing that FK506 specifically inhibits calcineurin. In the absence of FK506, the *std1 mth1* cells exhibit a growth defect relative to wild-type cells; however, it is not as severe as the calcineurin mutants. When calcineurin function is blocked by FK506, the *std1 mth1* mutants exhibit a more severe defect than the calcineurin mutant. These data demonstrate that the ion stress responses by calcineurin and Std1p are additive. If Std1p functioned downstream in the same pathway as calcineurin, then the *std1Δ mth1Δ* strain should be unaffected by FK506. However, the opposite result is observed. Furthermore, if Std1p acted downstream of calcineurin in a branched pathway, then one would expect that mutation or biochemical inhibition of calcineurin would produce an ion stress defect that

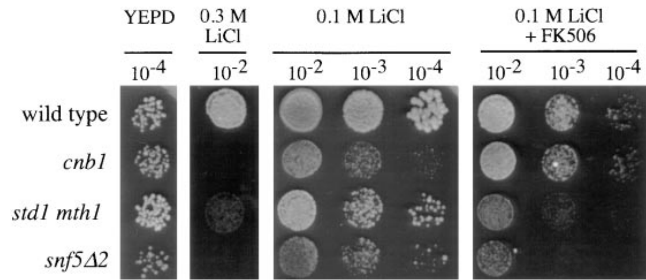

Figure 5.—Calcineurin and Std1p function in distinct ion stress response pathways. Serial dilutions of yeast cultures were spotted onto YEPD media supplemented with 0.3 m LiCl or 0.1 m LiCl in the presence or absence of 1 μg/ml FK506, as indicated. Strains used were YPH499 (wild type), DD12 (*cnb1*), MSY192 (*std1 mth1*), and MCY2099 (*snf5Δ2*).

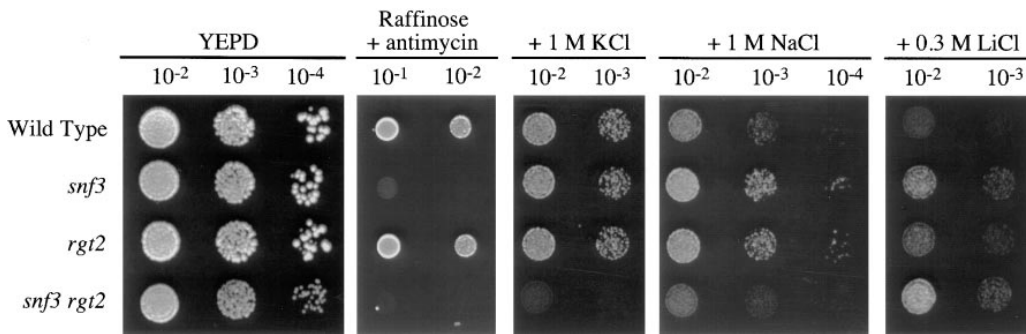

Figure 6.—Growth properties of strains lacking the Snf3 and Rgt2 glucose sensors. Serial dilutions of yeast cultures were spotted onto synthetic complete media lacking uracil with 2% glucose, 2% raffinose containing 1 μg/ml antimycin A, or 2% glucose supplemented with either 1 m KCl, 1 m NaCl, or 0.3 m LiCl, as indicated. The strains used in this experiment were MSY401 (wild type), MSY-404 (*snf3*), MSY403 (*rgt2*), and MSY402 (*snf3 rgt2*).

would not be affected by additional downstream mutations in either branch. However, loss of calcineurin function causes a further reduction of growth in a *std1 mth1* mutant. Therefore, Std1p cannot act exclusively in the same ion stress response pathway as calcineurin, nor can it act downstream of calcineurin in a branched pathway. We conclude that Std1p must function in a parallel ion stress response pathway.

Loss of the glucose sensors Snf3p and Rgt2p confers ion stress phenotypes: Earlier studies have shown that Std1p functions in the glucose derepression pathway. Therefore, we tested whether other components known to function in glucose signaling were also involved in the ion stress response pathway defined by Std1p. We tested the effect of null mutations in the genes encoding the yeast glucose sensors, Snf3p and Rgt2p (Ozcan *et al.* 1996), on growth under various ion stresses. Null alleles of *SNF3* and *RGT2* were constructed in a diploid strain, which was then subjected to tetrad analysis. Multiple tetrads were analyzed, and four representative haploid strains from a single tetratype tetrad are shown in this experiment. The single *snf3* and *rgt2* strains display normal growth on glucose media, whereas the double *snf3 rgt2* mutant displays a slow growth phenotype that is specific for glucose media (Figure 6) and is not observed on media containing glycerol as the carbon source (to be described in detail elsewhere). Both the *snf3* and *snf3 rgt2* strains are defective for growth on raffinose antimycin media, while the *rgt2* strain grows comparably to the wild-type strain. The growth defect of *snf3* strain on raffinose antimycin is the phenotype by which the *SNF3* locus was originally identified (Neigeborn and Carlson 1984). When challenged with 1 m KCl in the media, the single mutants grow with rates similar to the wild-type strain, while the *snf3 rgt2* strain has a distinct growth defect. One surprise from this experiment is the finding that loss-of-function mutations in either *SNF3* or *RGT2* confer a growth advantage on Na^+ and Li^+ containing media. Interestingly, the double *snf3 rgt2* mutant grows more slowly than wild type on 1 m NaCl but has a growth advantage in media containing 0.3 m LiCl. Thus, we conclude that the glucose sensors *SNF3* and *RGT2* affect cell growth rates during ion stress. Loss-of-function alleles in these genes cause a growth defect on K^+ but a growth advantage on Na^+ media.

The Snf1 kinase complex is not required for the response to sodium ion stress: The *SNF1* gene encodes a serine-threonine protein kinase that is required for glucose derepression of many genes (Johnston and Carlson 1992). The Snf4 protein is a subunit of the kinase complex that is required for activation of the protein kinase activity. We constructed *snf1::KAN* and *snf4::KAN* null alleles individually in a diploid strain and tested the growth properties of meiotic segregants. Haploid strains carrying the *snf1::KAN* or the *snf4::KAN* allele displayed distinct growth phenotypes. The *snf1* null strain grows poorly on all media tested and is severely defective on low glucose media (0.1%) and on raffinose antimycin media (Figure 7A), the phenotype that defines the *snf* genes (Carlson *et al.* 1981). In contrast, the *snf4* null strain grows comparably to wild-type cells on rich media but is defective on raffinose antimycin media. The *snf1* and *snf4* null strains were tested for growth under conditions of Na^+, K^+, and Li^+ ion stress in the presence of 2% glucose. None of these ions produced a noticeable growth defect in these strains, indicating that the Snf1 protein kinase complex is not required for response to ion stress under high glucose conditions.

A functional Swi/Snf complex is required for halotolerance: The chromatin remodeling Swi/Snf complex is required for the transcriptional induction of a diverse set of yeast genes. To determine whether the Swi/Snf complex was required for ion stress response, a strain carrying the *snf5Δ2* allele (Laurent *et al.* 1990) was tested for growth relative to an unrelated wild-type strain. The *snf5Δ2* strain displayed a severe growth defect when grown in the presence of 1 m NaCl and 0.3 m LiCl but not in the presence of 1 m KCl, indicating that the growth defect is Na^+- and Li^+-specific and not osmotic (Figure 7A). Since strain differences could af-

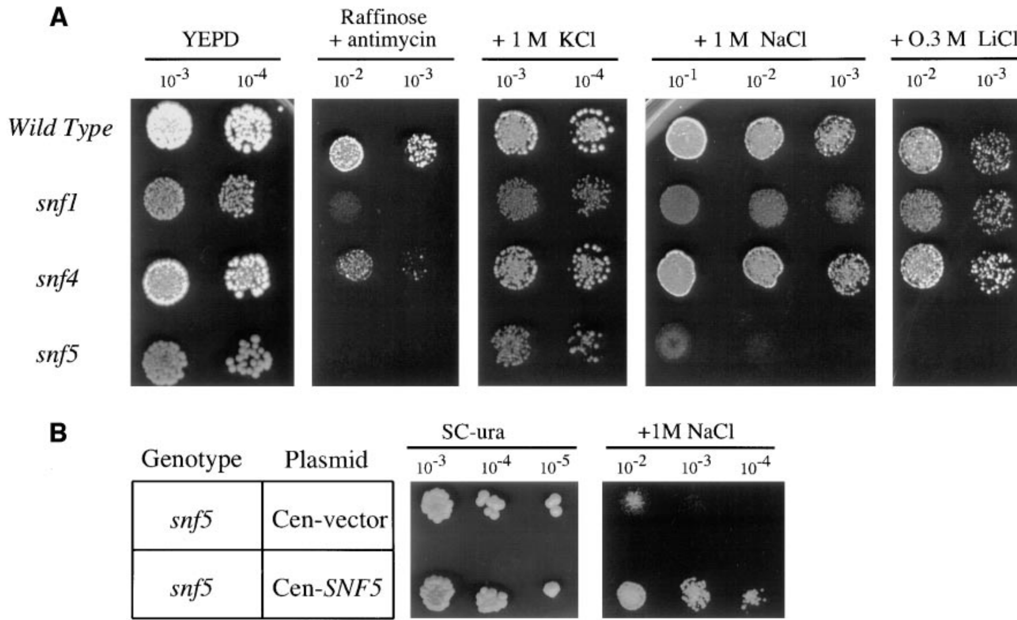

Figure 7.—Ion stress response in strains lacking Snf1, Snf4, or Snf5 proteins. Serial dilutions of yeast cultures were spotted onto synthetic complete media with 2% glucose, 2% raffinose containing 1 μg/ml antimycin A, or 2% glucose supplemented with 1 m NaCl, 1 m KCl, or 0.3 m LiCl, as indicated. (A) The strains used in this experiment were MSY182 (wild type), MSY-317 (*snf1::KAN*), MSY314 (*snf4::KAN*), and MCY2099 (*snf5Δ2*). (B) Serialdilutions of MCY2099 (*snf5Δ2*) transformed with either pUN55 (CEN-vector) or pSNF5 (CEN *SNF5*) were spotted onto synthetic complete media lacking uracil with and without 1 m NaCl.

fect this result, we also analyzed the growth properties of the *snf5Δ2* strain that was transformed with a single copy plasmid containing either the wild-type *SNF5* gene or no insert. These two strains were then isogenic at all loci except *SNF5.* This pair of strains shows no growth difference on glucose media (Figure 7B). The *snf5* strain did have a severe growth defect on raffinose media (Figure 7A) that is complemented by the *SNF5* plasmid (not shown), indicating that the episomal copy of *SNF5* was functional. Similarly, the growth defect observed in the *snf5Δ2* strain in the presence of 1 m NaCl is suppressed by the *SNF5* plasmid, indicating that the defect in the *snf5Δ2* strain observed in ion stress response is due to the *snf5Δ2* mutation. Therefore, we conclude that a functional Swi/Snf complex is required for ion stress response. We also tested whether Snf5p acted in the same ion stress response pathway as calcineurin using the same strategy as was used for the Std1p (Figure 5). The *snf5Δ2* strain grew poorly in the presence of 0.1 m LiCl, and this growth was inhibited by the presence of FK506. The finding that the effect of the *snf5* mutation and loss of calcineurin activity are additive indicates that Snf5p and calcineurin function in distinct ion stress response pathways.

The halotolerance gene *HAL1* is regulated by *STD1* gene dosage, glucose, and Na^+ ion stress: Halotolerance in *S. cerevisiae* can be conferred by increased gene dosage of the *HAL* genes (Gaxiola *et al.* 1992; Ferrando *et al.* 1995; Murguia *et al.* 1996). The *HAL1* gene encodes a protein that affects the intracellular K^+/Na^+ ratio in response to Na^+ ion stress (Rios *et al.* 1997). *HAL1* mRNA accumulation increases as media glucose concentration falls and is also repressed by the Tup1p (DeRisi *et al.* 1997). Since these properties are also shared by *SUC2*, a gene induced by 2μ *STD1*, the *HAL1* gene appeared to be a potential target of *STD1*-mediated regulation and a potential connection between *STD1* and halotolerance. In this experiment, the expression of the *HAL1* gene in response to changes in extracellular Na^+ ion concentration, glucose concentration, and *STD1* gene dosage was analyzed by Northern blot hybridization. Total cellular RNA was prepared from wild-type cells, cells lacking *STD1* gene function (*std1Δ mth1Δ*), and wild-type cells carrying a high copy number *STD1* plasmid. Cells grown in synthetic complete (lacking uracil) 2% glucose media were shifted to media containing 2% glucose plus 1 m NaCl or to 0.05% glucose for 3 hr prior to RNA preparation. Equivalent RNA samples were loaded in each lane as judged by ethidium bromide staining of the ribosomal RNAs (Figure 8). *HAL1* mRNA is detected in all samples, and its accumulation is moderately induced by exposure to 1 m NaCl for 3 hr. Interestingly, the accumulation of the *HAL1* mRNA was greatly affected by both glucose limitation and *STD1* gene dosage. In this respect, *HAL1* mRNA showed similar regulation to that of the *SUC2* gene, a well-characterized glucose-repressed, *STD1*-induced gene. Not all genes are responsive to increased *STD1* gene dosage or glucose limitation, as evidenced by the constitutive expression of the *TUB2* mRNA. *HAL1* and *SUC2* regulation were not identical because the low-glucose induction of the *HAL1* mRNA was not diminished by loss of *STD1* function, whereas the *std1Δ mth1Δ* strain shows a few-fold reduction in *SUC2* expression. However, full induction of *HAL1* mRNA by NaCl did require Std1 protein function because the *std1Δ mth1Δ* strain accumulated less *HAL1* mRNA than wild type in media containing 1 m NaCl. Also of note is that increased gene dosage of *STD1* further induces *HAL1* mRNA, but not *SUC2* mRNA, accumulation during glucose withdrawal, suggesting that the glucose-mediated and Std1-mediated regulation of these two genes is similar but distinct.

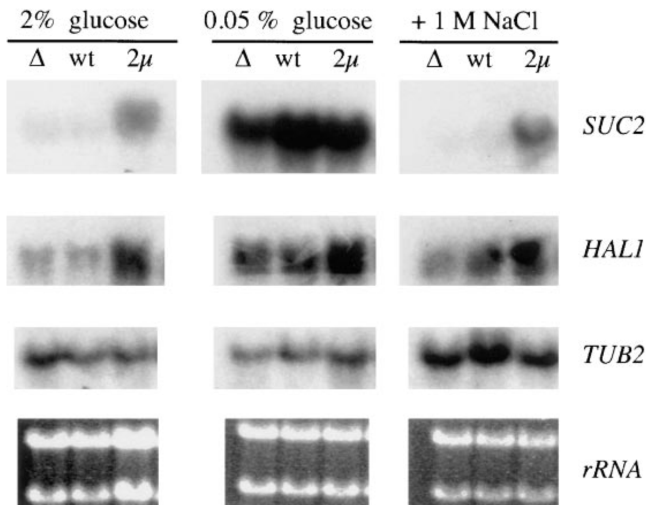

Figure 8.—*HAL1* mRNA is regulated by sodium, glucose, and *STD1* gene dosage. Total RNA was resolved on a 1% agarose-formaldehyde gel and analyzed by hybridization to ^{32}P-labeled DNA. Each row represents a single exposure of the same blot that was hybridized sequentially with sequences complementary to the *SUC2*, *HAL1*, and *TUB2* mRNAs. As a control for equal loading of RNA, the ethidium-bromide-stained rRNAs for this gel are shown. RNA was prepared from cultures grown in synthetic complete media lacking uracil and containing 2% glucose and shifted to the same media containing 0.05% glucose or 2% glucose supplemented with 1 m NaCl for 3 hr prior to RNA extraction. The strains used were MSY192 (*std1*Δ *mth1*Δ) transformed with YEP252 (Δ), MSY182 (*STD1 MTH1*) transformed with either YEP352 (wt) or p6A5U (2μ), as indicated.

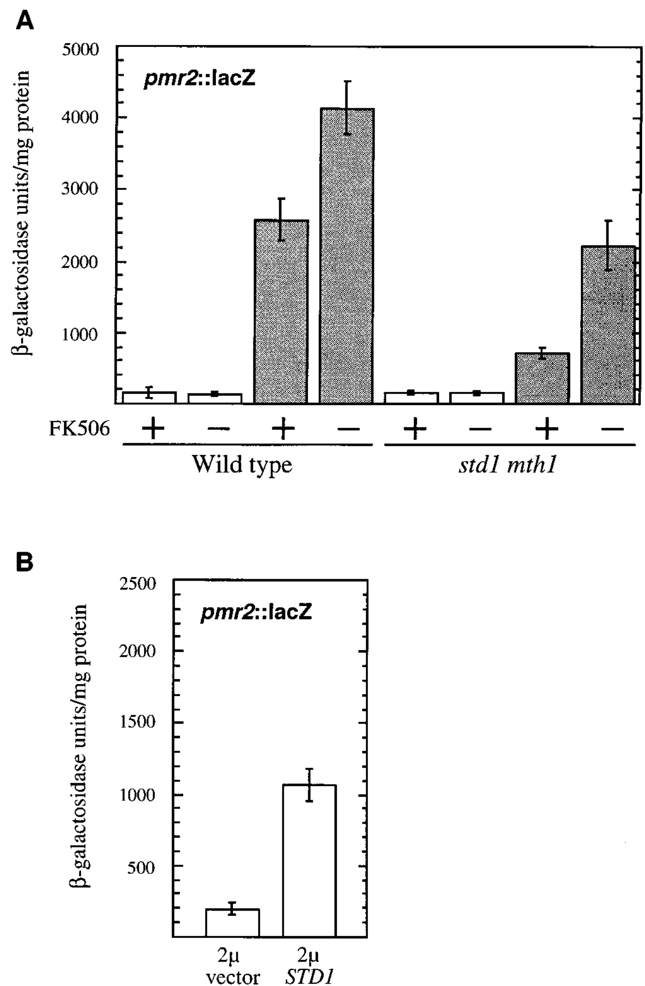

Figure 9.—*PMR2* expression is regulated by Std1p and calcineurin. (A) Cells transformed with plasmid pFR70 (Marquez and Serrano 1996) were grown in synthetic complete media lacking uracil and then diluted into YEPD media for 4 hr with (solid bars) or without (open bars) 1 m NaCl in the presence (+) or absence (−) of 1 μg/ml FK506. The strains used in this experiment were MSY182 (wild type) and MSY192 (*std1 mth1*). (B) Wild-type cells (MSY401) transformed with pFR70 and either YEp351 (2μ vector) or p6A5 (2μ *STD1*) were grown in synthetic complete media lacking uracil and leucine. Cultures were diluted in YEPD for 4 hr prior to harvest. Protein extracts from two independent colonies of each strain were assayed at two different protein concentrations. The mean value is plotted, and the error bars represent one standard deviation.

The *PMR2* gene is regulated by *STD1* gene dosage: The *PMR2* gene encodes a plasma membrane P-type ion pump thought to be responsible for Na^+ and Li^+ ion efflux (Wieland *et al.* 1995). *PMR2* gene expression is induced by high concentrations of Na, calcium (Stathopoulos and Cyert 1997), and low glucose (Alepuz *et al.* 1997). We suspected that the *PMR2* gene might be a target of *STD1* regulation since only a portion of its Na response is calcineurin dependent (Stathopoulos and Cyert 1997). We tested the effect of changes in *STD1* gene dosage on *PMR2* expression using a *PMR2* promoter-lacZ reporter plasmid (Marquez and Serrano 1996). Expression of β-galactosidase can be efficiently induced by incubation in 1 m NaCl for 4 hr (Figure 9A). Consistent with earlier studies using this reporter (Stathopoulos and Cyert 1997), only a fraction of the Na induction of the *PMR2* reporter is sensitive to FK506, suggesting that other Na stress response pathways contribute to *PMR2* induction. One of those additional pathways is Std1p-dependent because mutation of *STD1* and *MTH1* reduces the Na induction of *PMR2.* Addition of FK506 to the *std1 mth1* mutant further reduces but does not eliminate Na induction of the *PMR2* reporter gene, indicating that a calcineurin-, Std1-independent pathway exists. The presence of increased gene dosage of *STD1* induces *PMR2* expression in the absence of Na stress (Figure 9B). Our finding that increased gene dosage of *STD1* induces the expression of both *HAL1* and *PMR2* provides a likely explanation for the halotolerance conferred by 2μ *STD1* plasmids.

DISCUSSION

In this study we report the finding that *std1*Δ *mth1*Δ cells manifest numerous phenotypes that are shared with calcineurin mutants. Both *std1*Δ *mth1*Δ mutants and calcineurin mutants display greatly impaired growth in the presence of Na^+ and Li^+ ions but not in

the presence of K^+ ions. Both calcineurin mutants and *std1*Δ *mth1*Δ mutants show a reduced tolerance to Mn^+ ions and alkaline pH, and both mutants lose viability upon prolonged exposure to the mating pheromone, alpha factor. An additional genetic link between *STD1* and calcineurin is the finding that increased gene dosage of *STD1* can suppress the ion-mediated growth defects observed in calcineurin mutants. The cellular response to Na^+ ion stress endeavors to reduce intracellular Na^+ concentrations by both limiting influx and increasing efflux. Both of these responses utilize Ca^{2+} signals and activated calcineurin. The Trk1p forms an ion channel through which Na^+ ions enter cells, and it has been suggested that calcineurin-mediated dephosphorylation of Trk1p may increase its specificity for K^+ ions, thereby limiting Na^+ ion influx (Mendoza *et al.* 1994). Second, calcineurin is required for the Na^+-induced transcription of the PMR2/ENA1 gene that encodes the P-type ATPase that is responsible for Na^+ and Li^+ efflux (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). Std1p has previously been shown to interact with the TATA binding protein (Tillman *et al.* 1995) and has been implicated in the regulation of gene expression in response to changes in extracellular glucose concentrations (Hubbard *et al.* 1994). Thus, it is possible that the Std1p may also act to mediate changes in gene expression in response to ion stress.

The ability of increased gene dosage of *STD1* to suppress calcineurin growth defects under ion stress conditions suggested that Std1p might act downstream of calcineurin in the same stress response pathway. However, our data did not exclude the possibility that Std1p and calcineurin functioned in parallel pathways. To distinguish between these possibilities, we used the drug FK506, which specifically inhibits calcineurin activity in both mammalian and yeast cells (Foor *et al.* 1992). If Std1p acted downstream of calcineurin in the same stress response pathway, then inhibition of calcineurin by FK506 should not have any effect in a *std1*Δ *mth1*Δ mutant. However, the opposite result was observed. Therefore, calcineurin and Std1p must be acting in parallel ion stress response pathways. A similar result was also observed in a *snf5* mutant, indicating that *snf5* and calcineurin also function in distinct pathways. We propose a model (Figure 10) in which calcineurin and Std1p act in parallel but synergistic ions stress response pathways.

Mutants in calcineurin lose viability when incubated with the mating pheromone alpha factor (Withee *et al.* 1997). Exposure to alpha factor causes an increase in intracellular calcium concentration that serves to activate both calcineurin and calcium-calmodulin dependent kinase (Moser *et al.* 1996). Our data show that *std1*Δ *mth1*Δ cells also lose viability in the presence of alpha factor. It is not known whether calcineurin- or calcium-calmodulin-dependent kinase-mediated responses are defective in *std1*Δ *mth1*Δ cells. Increased

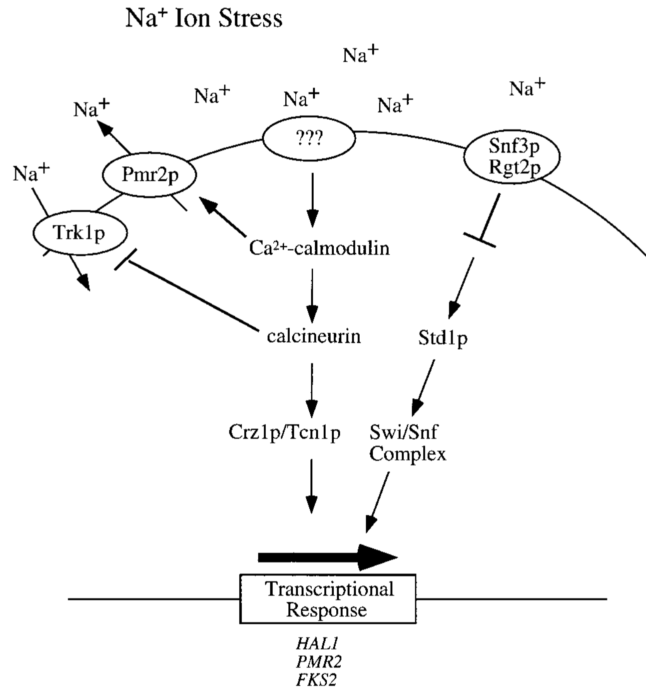

Figure 10.—Model for parallel ion stress response pathways. Cellular response to sodium ion stress is shown in two parallel pathways. Both pathways converge in the nucleus where transcriptional induction of genes such as *HAL1*, *PMR2*, and *FKS2* occur. In addition, cation homeostasis may be directly regulated by both calmodulin and calcineurin acting on the Pmr2p and Trk1p, respectively (Mendoza *et al.* 1994; Weiland *et al.* 1995).

gene dosage of *STD1* is able to suppress calcineurin mutant defects under ion stress conditions but is not able to suppress calcineurin mutant loss of viability in alpha factor. This observation suggests that Std1p and calcineurin may have distinct functions in response to alpha factor adaptation.

Since Std1p has been shown to modulate expression of glucose-regulated genes, we tested other genes whose products are involved in glucose-mediated regulation of transcription for any effects on the Na^+ ion stress response pathway. We found that cells lacking a functional Swi/Snf complex have a greatly reduced tolerance to Na^+ ion stress. This result suggests that the transcriptional response to Na^+ ion stress is likely to involve chromatin remodeling. We also tested the Na^+ stress response in cells lacking a functional Snf1/Snf4 protein kinase complex and found no decreased tolerance to Na^+ ion stress in either *snf1* or *snf4* mutants. A similar study by Alepuz *et al.* (1997) has recently reported the opposite result. In that study, a *snf4*Δ*2* mutant and a *snf1-K84R* mutant showed a severe growth defect in the presence of 1.2 m NaCl and 0.3 m LiCl. In light of their report, we tested additional *snf1* and *snf4* alleles, and in all cases, we have found that these mutants have seemingly normal growth rates under both Na^+ and Li^+ ion stress (data not shown). Indeed,

we have tested the *snf4Δ2* allele used by Alepuz *et al.* (1997) and find no defect in Na^+ ions stress response (data not shown). It is difficult to reconcile these contradictory findings other than to attribute them to strain differences. For instance, one important difference between our strains may be in the copy number or expression of the *PMR2/ENA1* gene since this gene is found in a repeated gene cluster (Weiland *et al.* 1995). Also, it is worth noting that neither strain used by Alepuz *et al.* (1997) contained null alleles of the *snf1* or *snf4* genes, and perhaps some form of dominant negative inhibition could account for the Na^+ ion stress defect observed in their study.

Yeast encode 20 genes whose products are members of the 12-transmembrane hexose transporter superfamily (Kruckeberg 1996). Two of these, the products of the *SNF3* and *RGT2* genes, are structurally distinct due to the presence of a C-terminal hydrophilic domain. Snf3p and Rgt2p are thought to function not as hexose transporters but as glucose sensors (Ozcan *et al.* 1996). We show here that mutations in either of these genes actually enhance the Na^+ stress response. We interpret these results to mean that the Snf3p and Rgt2p are negative regulators of the Na^+ stress response and their loss of function serves to increase expression of Na^+ stress response genes. Additional studies not shown here indicate that the glucose-repressed gene *SUC2* is derepressed in the absence of *SNF3* and *RGT2* (X. Zhang and M. C. Schmidt, unpublished results). We hypothesize that loss of a glucose sensor likewise causes a derepression of the Na^+ stress response genes that are under glucose control and thereby enhances the Na^+ stress response of the cells. In addition, we found that the *snf3 rgt2* mutant is sensitive to high concentrations of K^+. This result may stem from the fact that one of the cell's Na^+ stress responses, to increase intracellular K^+ (Gaxiola *et al.* 1992), may be deregulated in the *snf3 rgt2* mutant. Cells that constitutively act to increase intracellular K^+ may find this maladaptive when presented with high concentrations of extracellular K^+.

Certain members of this 12-transmembrane hexose transporter superfamily are ion/sugar symporters (Baldwin 1990). Although there is no evidence that the yeast hexose transporters couple ion and sugar transport, single amino acid mutations in Hxt1p and Hxt3p can convert these proteins to cation transporters (Liang *et al.* 1998). We show here that mutations in the glucose sensors confer a growth advantage under conditions of Na^+ ions stress and a growth disadvantage under conditions of K^+ ion stress. These effects are completely independent of glucose concentration, which was kept at 2% in these studies. One possible explanation for these data would be a model in which Snf3p and Rgt2p play dual roles as both glucose and cation sensors. The mechanism by which Na^+ ion stress induces a calcium signal in yeast cells is not known. Nor is it known by what mechanism Std1p-mediated gene regulation is induced under conditions of ion stress. Further analysis of Snf3p and Rgt2p will be needed to determine if they play a direct role as sensors of ion stress.

Increased gene dosage of *STD1* confers a growth advantage under Na^+ ion stress conditions. Since increased levels of Std1p have been shown to induce expression of the *SUC2* gene (Hubbard *et al.* 1994; Tillman *et al.* 1995), we looked for halotolerance genes that might be subject to Std1p regulation. Analysis of global patterns of gene expression found that the *HAL1* gene was the only known halotolerance gene whose expression was increased more than twofold by the deletion of *TUP1* (DeRisi *et al.* 1997), a known repressor of *SUC2* expression. We examined the expression of *HAL1* mRNA by Northern blot and found that its accumulation was regulated strongly by glucose concentration and by *STD1* gene dosage. Increased gene dosage of *HAL1* has been shown to induce expression of *PMR2/ENA1*, the Na^+ efflux pump, and also to suppress the ion stress growth defects observed in calcineurin mutants (Rios *et al.* 1997). We also tested the effect of *STD1* gene dosage on *PMR2* expression and found that Std1p is required for the full induction of *PMR2* in response to Na stress. The Na response of the *PMR2* gene is the result of the additive effects of the calcineurin pathway, the Std1 pathway, and at least one additional pathway. The high osmotic glycerol (HOG) MAP kinase pathway is also known to regulate expression of the *PMR2* gene and most likely accounts for the calcineurin- and Std1-independent Na response of the *PMR2* gene (Marquez and Serrano 1996). We also show here that increased gene dosage of *STD1* induces both *HAL1* and *PMR2* expression, suggesting a possible mechanism for the halotolerance and the suppression of calcineurin mutants that is conferred by 2μ *STD1.* Our studies indicate that yeast cells have developed overlapping but distinct stress response pathways for coping with changes in both cation and carbon source concentrations.

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