Gis4, a New Component of the Ion Homeostasis System in the Yeast Saccharomyces cerevisiae†

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Gis4 is a new component of the system required for acquisition of salt tolerance in Saccharomyces cerevisiae. The gis4Δ mutant is sensitive to Na⁺ and Li⁺ ions but not to osmotic stress. Genetic evidence suggests that Gis4 mediates its function in salt tolerance, at least partly, together with the Snf1 protein kinase and in parallel with the calcineurin protein phosphatase. When exposed to salt stress, mutants lacking gis4Δ display a defect in maintaining low intracellular levels of Na⁺ and Li⁺ ions and exporting those ions from the cell. This defect is due to diminished expression of the ENA1 gene, which encodes the Na⁺ and Li⁺ export pump. The protein sequence of Gis4 is poorly conserved and does not reveal any hints to its molecular function. Gis4 is enriched at the cell surface, probably due to C-terminal farnesylation. The CAAX box at the C terminus is required for cell surface localization but does not seem to be strictly essential for the function of Gis4 in salt tolerance. Gis4 and Snf1 seem to share functions in the control of ion homeostasis and ENA1 expression but not in glucose derepression, the best known role of Snf1. Together with additional evidence that links Gis4 genetically and physically to Snf1, it appears that Gis4 may function in a pathway in which Snf1 plays a specific role in controlling ion homeostasis. Hence, it appears that the conserved Snf1 kinase plays roles in different pathways controlling nutrient as well as stress responses.

Sodium ions are toxic because they take the place of potassium, which is required for the activity of numerous proteins (39, 40). Moreover, sodium specifically inhibits the normal function of certain proteins, as demonstrated for the Hal2 protein phosphatase (41). For these reasons, cells have developed mechanisms to acquire sodium (Na⁺) tolerance. Detailed knowledge about the mechanisms underlying Na⁺ toxicity and the inducible mechanisms to acquire sodium tolerance is important for understanding how eukaryotic cells mediate intracellular ion homeostasis and for the improvement of such tolerance in crop plants. The yeast Saccharomyces cerevisiae has proven to be a useful model organism for the study of ion homeostasis. In this work, we describe a new component of the ion homeostasis system, Gis4, and we provide evidence that Gis4 mediates its effects, at least partly, via the Snf1 protein kinase and regulation of the Na⁺ export pump Ena1.

Ena1 is crucial for yeast sodium and lithium tolerance; mutants lacking the export pump are highly sensitive to these ions (17). Expression of the ENA1 gene is controlled in a complex manner by different regulatory pathways, all of which seem to be activated by sodium indirectly. The osmosensing high-osmolarity glycerol (HOG) pathway controls ENA1 expression via the Sko1 transcriptional regulator (30–32). This mechanism requires high, osmotically relevant levels of NaCl. The calcineurin protein phosphatase, consisting of the Cmp1 and Cmp2 catalytic subunits and the Cnb1 regulatory subunit, controls expression of ENA1 via the Crz1 transcription factor (18, 25, 26, 42). Probably, sodium disturbs cellular calcium homeostasis, thereby activating this pathway. It seems that the calcineurin pathway is also responsible for known effects on sodium tolerance of the Ppz phosphatases (36) and Hal3 (9). In addition, it has been reported that Snf1 and Mig1, which control glucose repression/derepression, control ENA1 expression (1, 31). The reason for this putative link between ion homeostasis and glucose metabolism is still unclear. In addition, salt-sensitive mutants have led to the discovery of secretory pathway components, such as Sro7 and Sro77, which are needed to transport Ena1 to the cell surface (46).

While screening transposon insertion mutants for salt-sensitive clones, we have isolated a mutant with a transposon inserted in the GIS4 gene (8). The GIS4 gene was isolated first in a screen for multicopy suppressors of a snf1Δ mig1Δ srb8 triple mutant on galactose. The incentive of that screen was to identify novel components of the RNA polymerase mediator complex. However, a possible role of Gis4 in transcription regulation has remained unclear (3). Snf1 is a protein kinase needed for glucose derepression, closely related to plant and mammalian AMP-activated protein kinases (5, 6). In yeasts, Snf1 controls, among others, the activity of the Mig1 transcriptional repressor (11, 34, 37, 38). Srb8 is a component of the RNA polymerase mediator complex (4). Overexpression of GIS4 suppressed the growth defect of a snf1Δ mig1Δ pde2Δ (Pde2 is phosphodiesterase) triple mutant as well as a snf1Δ mutant on galactose and raffinose, while it did not suppress the temperature sensitivity of the snf1Δ mutant (3). Studies furthermore suggested some link to the upstream part of the yeast Ras/cyclic AMP (cAMP) pathway, since GIS4 overexpression caused an aggravated growth defect of a temperature-sensitive
cdc25 mutant (Cdc25 is Ras GDP/GTP exchange factor). Furthermore, the gis4Δ and pde2Δ mutations caused similar synthetic effects when combined with snf1Δ and snf1Δ mig1Δ mutations (3). More recently, it has been demonstrated that Gis4 directly interacts with Snf1, and this interaction appears to require Grr1-dependent ubiquitination of Gis4 (22). Despite being ubiquitinated, Gis4 seems to be a stable protein. The same study reported that Gis4 stimulates Snf1 activity and that deletion of Gis4 affects expression of well-known Snf1 target genes, such as SUC2 (22).

Starting from the observation that deletion of GIS4 causes sensitivity to Na⁺, we analyzed the role of Gis4 in more detail. Here, we report that Gis4 appears to play a specific role in the acquisition of Na⁺ and Li⁺ tolerance by controlling, in conjunction with Snf1 but in parallel with the calcineurin phosphatase, expression of the ENA1 gene. Since, in our hands, deletion of GIS4 only has a minor, if any, effect on glucose derepression, Gis4 may be a new component of the Snf1 pathway linking it specifically to the control of ion homeostasis.

### MATERIALS AND METHODS

#### Yeast strains and plasmids

The S. cerevisiae strains used in this study are isogenic to W303-1A (Table 1). Strains YSH1516, YSH1518, and YSH1622 were generated by crossing and tetrad analysis.

The multicopy vector pH81 has been described previously (28), and the plasmids pH81-GIS4 and pH81-PDE2 were kindly provided by H. Ronne (3). pAN30-ENA1, a centromeric plasmid expressing ENA1 from its own promoter, was a gift of I. Wadskog. pRS415 (44) was used as vector control in some experiments. GIS4 was subcloned into the centromeric vector YCp53 (13) for low-copy-number expression and mutagenesis (see below) following PCR from genomic DNA (Table 2), digestion of vector and PCR product with XhoI, and NotI, ligation, and confirmation by sequencing.

The plasmid pRN295 (29) was used for constructing an expression cassette for N-terminal fusion of green fluorescent protein (GFP) to Gis4. The construct is expressed from the MET25 promoter. Linearized pRN295 was amplified by PCR with the forward and reverse primers indicated in Table 2. The GIS4 gene was amplified from genomic DNA by PCR with primers that contained 20 base pairs complementary to the GIS4 genomic sequence plus flanking regions of about 60 bases complementary to plasmid pRN295. The two fragments were cotransformed into the gis4Δ:TRPI mutant for gap-repair to obtain a circular plasmid. Colonies were picked after 2 to 3 days, DNA was isolated and transformed into Escherichia coli, and plasmids were purified to confirm the construct by DNA sequencing.

#### TABLE 1. Yeast strains used in this study

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| W303-1A | MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 | 45 |
| H174 | W303-1A mig1Δ:LEU2 | 27 |
| YSH372 | W303-1A snf1Δ:LEU2 | S. Hohmann collection |
| H803 | W303-1A gis4Δ:URA3 | 3 |
| H804 | W303-1A gis4Δ:TRPI | 3 |
| YN10 | W303-1A ena1::HIS3 | Ramon Serrano |
| H867 | W303-1A pde2Δ::TRPI | 3 |
| YSH818 | W303-1A hog1Δ:LEU2 | 33 |
| YMR70 | W303-1A pkl1Δ::URA3 pkl2Δ::HIS3 | S. Hohmann collection |
| YMR72 | W303-1A bcy1Δ::LEU2 pkl1Δ::URA3 pkl2Δ::HIS3 | S. Hohmann collection |
| YSH891 | W303-1A cmp1Δ::URA3 cmp2Δ::HIS3 | S. Hohmann collection |
| YSH1516 | W303-1A cmp1Δ::URA3 cmp2Δ::HIS3 gis4Δ:TRPI | This study |
| YSH1518 | W303-1A cmp1Δ::URA3 cmp2Δ::HIS3 snf1Δ:LEU2 | This study |
| H805 | W303-1A snf1Δ::HIS3 gis4Δ::URA3 | 3 |
| H807 | W303-1A snf1Δ::HIS3 mig1Δ::LEU2 gis4Δ::URA3 | 3 |
| YSH1622 | W303-1A cmp1Δ::URA3 cmp2Δ::HIS3 pde2Δ::TRPI | This study |

#### TABLE 2. Oligonucleotides used in this study

| Primer (direction) | Sequence (5'–3') |
|--------------------|-----------------|
| IPP1 (forward)     | GACACCCCAACCTACTCAA |
| IPP1 (reverse)     | GAACCGGAGATGAGAACCA |
| ACT1 (forward)     | CTGCCGGTATTGACCAAACCT |
| ACT1 (reverse)     | CGTGATTTCTTTCATTGATT |
| SUC2 (forward)     | GTTACGGTGGGAGGAGATAG |
| SUC2 (reverse)     | GTAAGGGCTGGATGAATGAC |
| pRN295-1-line (forward) | CAGAACAATCTAAAACATGACTGCTGGT |
| pRN295-1-line (reverse) | GACGGTATCGATAAGCTTGATATCGAATTC |
| GIS4 Xhol/NotI (forward) | CCCTATCGATGATATACGTTAGTGAAT |
| GIS4 Xhol/NotI (reverse) | CCGGGGGCGCGCCTTTATCATGTGGCACAACG |
| GIS4-N (forward)   | CAGAAAATCTTTAAAACATGAGCTGTTATACCATCATGTTAG |
| GIS4-N (reverse)   | ATGAATGTGAAAAAATGGAAAAATCCGGTAGT |
| Mut CYS GIS4 (forward) | GCCGATATCGATGATATACGTTAGTGAAT |
| Mut CYS GIS4 (reverse) | TCCACTAATCGATGATATACGTTAGTGAAT |
| Cfirm mut GIS4 (forward) | AGCCGATATCGATGATATACGTTAGTGAAT |
| Cfirm mut GIS4 (reverse) | GCATACCCCTTAGCTGTT |

This study
A point mutant in which C771 of Gis4 was replaced by alanine was constructed by PCR using suitable mutagenic primers (Table 2). Amplification of the plasmid YCP33-Gis4 or pHN295-Gis4 was performed by using the accuprime Pfx DNA polymerase kit (Invitrogen). The fragments were digested by DpnI as described in the QuikChange site-directed mutagenesis kit and then transformed into E. coli. Resulting plasmids were checked by DNA sequencing.

Growth conditions and growth assays. Yeast cells were routinely grown in medium containing 2% peptone and 1% yeast extract supplemented with 2% glucose as a carbon source (YPEP). Selection and growth of transformants carrying a replicating plasmid was performed in yeast nitrogen base medium (43). Plate growth assays were performed by pregerminating cells in YEPD medium or uracil-deficient yeast nitrogen base medium. Cells were resuspended in the same medium to an optical density at 600 mn (OD_{600}) of 1.0. Five microliters of a 10-fold serial dilution of this culture was spotted onto agar plates supplemented with 2% glucose and different concentrations of LiCl, NaCl, or KCl as well as 0.1 µg/ml FK506 (calcineurin inhibitor), where indicated. Growth was monitored after 2 to 3 days at 30°C.

Gene expression analysis. To estimate ENA1 promoter activity, the ENA1-lacZ construct pC201 (7) was transformed into relevant yeast strains. Cells were grown to an OD_{600} of 1.0, NaCl was added to a final concentration of 0.6 M, and samples were taken for protein extraction at different time points. The specific activity of β-galactosidase was determined as described previously (35), and the protein concentration was determined using a kit (Bio-Rad). Data represent the averages and standard deviations of results from three biological replicates.

For reverse transcription (RT)-PCR of SUC2 expression, cells were cultivated in media containing 5% glucose and shifted to 0.2% glucose at an OD_{600} of approximately 0.8. mRNA was extracted, DNase treated, and controlled on an agarose gel. Reverse transcription (Superscript II; Invitrogen) using pd(T)_{12–18} as primers to produce the cDNA and quantitative real-time PCR assays using specific SUC2 primers were performed in an iCycler (Bio-Rad). PCR products were checked by agarose gel and melting curve analysis. Expression data were normalized against ACT1.

Western blot analysis. Cells were grown to OD_{600} of 0.5 to 0.8, harvested by centrifugation, and washed with 500 µl of water. Cells were resuspended in 50 µl of 2 M NaOH supplemented with 7% 2-mercaptoethanol and incubated for 2 min at room temperature. Fifty microliters of 50% trichloroacetic acid was added, and samples were vortexed and sedimented. Samples were washed in 250 µl of 1 M Tris (pH 7.5) and resuspended in 50 µl of 2× sample buffer (0.5 M Tris-Cl containing 0.4% sodium dodecyl sulfate [SDS], 20% glycerol, 4% SDS, 0.001% bromophenol blue, 0.01 M dithiothreitol) and incubated for 5 min at 100°C. Ten microliters of the supernatant was separated by SDS-polyacrylamide gel electrophoresis using 5% polyacrylamide gels and analyzed by immunoblotting using anti-hemagglutinin (HA) antibody (Sigma) and secondary anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase. Secondary antibody (donkey anti-goat immunoglobulin G-horseradish peroxidase; Santa Cruz Biotechnology) was applied in Tris-buffered saline–TWEEN 20 in 1:2,000 and 1:1,000 dilutions, respectively. The Lumi-Light Western blotting substrate (Roche) and the FUJIFILM LAS-1000 camera were used for visualization.

Microscopy and staining methods. Cells were grown to exponential phase in selective media and visualized with a Leica DMRXA microscope using bright field, GFP, and 4,6-diamidino-2-phenylindole (DAPI) filters. DAPI staining was visualized by DAPI (1 µg/ml) for 10 min, and cells were quickly washed four times in water (47). Cells exposed to a final concentration of 0.05 M LiCl were observed after 2 to 3 days at 30°C.

Invertase assays. Cells were harvested by centrifugation 5 h after a shift to 0.2% glucose. Protein extracts and measurements of invertase activity were performed as described previously (14, 20). The protein concentration was determined by using the DC protein assay kit (Bio-Rad).

Determination of Na⁺ or Li⁺ content and efflux. For determination of the cellular Na⁺ or Li⁺ content, cells were grown overnight in YEPD medium and incubated in the same medium plus 0.1 M NaCl or LiCl for 1.5 h. Cells were harvested, washed with cold water, and resuspended in assay buffer (10 mM MES [2-bis(2-hydroxyethyl)aminomethane] acid) adjusted to pH 5.5 with Ca(OH)₂ and supplemented with 0.1 mM MgCl₂. The cells were then extracted with acid and analyzed by atomic emission spectrophotometry (2, 15).

For determination of Na⁺ or Li⁺ efflux, cells were grown overnight in YEPD medium and incubated in the same medium plus 0.2 or 0.5 M NaCl or 0.03 or 0.06 M LiCl. At an OD_{600} of 0.3 to 0.35, cells were collected on Millipore filters and rapidly washed with 20 mM MgCl₂. The cells were then extracted with acid and analyzed by atomic emission spectrophotometry (2, 15).

RESULTS

Gis4 genetically interacts with the calcineurin and Snf1 pathways. We have previously identified Gis4 when screening transposon insertion libraries for mutants unable to grow in the presence of high NaCl concentrations (8). The gis4 mutant was not osmosensitive, since it could grow in the presence of 1.5 M sorbitol. Sensitivity to Na⁺ was confirmed in this study. In addition, the gis4Δ mutant is sensitive to Li⁺ (Fig. 1A) but not to 1 M KCl (not shown). While sensitivity was already apparent with 0.4 M NaCl and 0.05 M LiCl, the phenotype was more pronounced at 1 M NaCl (Fig. 1A). Na⁺ and Li⁺ sensitivity was neither influenced by the marker used to delete Gis4 (gis4Δ::TRPI and gis4Δ::URA3 mutants displayed identical phenotypes; not shown) nor the genetic background (tested in BY4741 and W303-1A; not shown).

Sensitivity to Na⁺ and Li⁺ is typical for mutants lacking the export pump Ena1; ena1Δ mutants are exclusively sensitive to these ions (17) (Fig. 1A). The phenotype of the gis4Δ mutant was less severe than that of the ena1Δ mutant. The sensitivity of the gis4Δ mutant was comparable to that of the cmp1Δ cmp2Δ mutant, which lacks the known ENA1 regulator calcineurin. Taken together, these observations indicated that the gis4Δ mutant is specifically defective in acquiring tolerance to toxic Na⁺ and Li⁺ ions, as is common for mutants defective in Ena1.

We therefore tried to link Gis4 to any of the known pathways that control ENA1 expression. The HOG pathway mediates osmotic responses in yeast. Since a gis4Δ mutant is salt sensitive but not osmosensitive and since the hog1Δ mutant is osmosensitive but not Li⁺ sensitive (Fig. 1A), it was regarded unlikely that Gis4 functions in the HOG pathway.

The initial report proposed Gis4 to be involved in the Ras-cAMP-protein kinase A (PKA) pathway (3). It has previously been reported that PKA activity affects ENA1 expression (23). We tested whether mutants with diminished (tpk1Δ tpk2Δ) or enhanced (tpk1Δ tpk2Δ bcy1Δ and pde2Δ) activity of protein kinase A showed a salt-sensitive phenotype similar to a gis4Δ mutant. We chose these particular PKA pathway mutants, since strains with extremely high or absent PKA activity are either not viable, display numerous indirect effects and/or accumulate suppressor mutations. The mutants we tested were not Na⁺ or Li⁺ sensitive, even at higher NaCl concentrations (Fig. 1A). Hence, the Na⁺ and Li⁺ sensitivity of the gis4Δ mutant was unlikely to be linked to activity of the Ras-cAMP-PKA pathway.

Gis4 was isolated in a genetic screen involving the SNF1 gene. Mutants lacking Snf1 are sensitive to Na⁺ and Li⁺, although only at higher concentrations (Fig. 1A). Quite surprisingly, a mutant lacking Mig1, which is a repressor controlled by Snf1 (and hence Snf1 and Mig1 have opposite effects in glucose repression), also conferred weak salt sensitivity (Fig. 1A). Taken together, the phenotypes of the snf1Δ and gis4Δ mutants are similar, although of different severity.

FK506 is an inhibitor of the calcineurin phosphatase. For unknown reasons, FK506 somewhat improved growth of the cmp1Δ cmp2Δ mutant, which is defective in calcineurin (Fig. 1A). FK506 conferred salt sensitivity to the wild type, although this effect was less strong than that of the cmp1Δ cmp2Δ mutant, suggesting that the block of calcineurin is only partial. In
FK506 strongly aggravated the Na\(^+\) and Li\(^+\) sensitivity of the gis4\(\Delta\) mutant (Fig. 1). Also the snf1\(\Delta\) and mig1\(\Delta\) mutants, which showed weak sensitivity to Na\(^+\) and Li\(^+\), became strongly salt sensitive in the presence of FK506. The hog1\(\Delta\) mutant or mutants with altered PKA activity did not show such an effect beyond that apparent in the wild type (Fig. 1). Taken together, these observations indicated that Gis4 as well as Snf1 (and Mig1) mediate salt tolerance.

**FIG. 1. Phenotypic analysis.** Yeast strains were grown to an OD\(_{600}\) of about 1.0 and adjusted to an OD\(_{600}\) of exactly 1.0, and 5 \(\mu\)l was spotted in 1:10 dilution steps onto the indicated media. **SNF1** encodes the Snf1 protein kinase in glucose derepression, which inactivates the transcriptional repressor Mig1. Hog1 is the mitogen-activated protein kinase in the HOG osmosensing pathway. **CMP1** and **CMP2** encode the catalytic subunits of the protein phosphatase calcineurin. Tpk1 and Tpk2 are catalytic subunits, Bcy1 is the regulatory (inhibitory) subunit of protein kinase A, and Pde2 encodes a cAMP phosphodiesterase. Hence, the *tpk1Δ tpk2Δ* mutant has reduced and the *tpk1Δ tpk2Δ bcy1Δ* and *pde2Δ* mutants have moderate, constitutive, and increased protein kinase A activity, respectively. *Ena1* is the Na\(^+\)/Li\(^+\) export ATPase. (A) The gis4\(\Delta\) mutant is sensitive to Na\(^+\) and Li\(^+\), and this phenotype is aggravated by the calcineurin inhibitor FK506. (B and C) **GIS4** genetically interacts with **SNF1** and **CMP1/CMP2**. (D) Expression of **ENA1** on a centromeric plasmid (i.e., one or few additional copies per cell) partially suppresses the growth defects of the gis4\(\Delta\) and snf1\(\Delta\) mutants on Na\(^+\) and Li\(^+\) media.
together with the calcineurin phosphatase via a common regulatory target.

To address genetic interactions of Gis4 with the calcineurin pathway as well as the Snf1 pathway in further detail, Na\textsuperscript{+}/H\textsuperscript{+} and Li\textsuperscript{+}/H\textsuperscript{+} sensitivity of mutants affected in both GIS4 and SNF1 as well as CMP1 and CMP2 was tested. While deletion of either GIS4, SNF1, or CMP1 plus CMP2 caused no or only very moderate sensitivity to 0.2 M NaCl or 0.02 M LiCl, gis4Δ cmp1Δ cmp2Δ and snf1Δ cmp1Δ cmp2Δ triple mutants were highly sensitive (Fig. 1B). No such effect was observed when the cmp1Δ cmp2Δ mutations were combined with deletion of PDE2. At the same time, the double mutant gis4Δ snf1Δ showed only moderately or no enhanced sensitivity to Li\textsuperscript{+} and Na\textsuperscript{+} compared to the single gis4Δ mutant, even at a higher salt concentration (Fig. 1C). Additional deletion of MIG1 did not enhance salt sensitivity further (Fig. 1C). These observations corroborate the idea that Gis4 and the calcineurin pathway control a common target in Li\textsuperscript{+} and Na\textsuperscript{+} tolerance through parallel pathways. Also Snf1 (and Mig1) and calcineurin appeared to operate in parallel. On the other hand, Gis4 and Snf1 might operate in a common pathway. To dissect a possible Snf1-Gis4 pathway, we tried to suppress the snf1Δ mutant by overexpression of GIS4. The overexpression plasmid used in this experiment could complement the salt sensitivity of the

![Graphs of Na\textsuperscript{+} and Li\textsuperscript{+} content and efflux](image-url)

FIG. 2. Mutants lacking Gis4 or Snf1 display increased content of Na\textsuperscript{+} and Li\textsuperscript{+} and a defect in exporting these ions from the cells. (A) Cells were incubated overnight at the indicated ion concentration, and then the cellular content of Na\textsuperscript{+} and Li\textsuperscript{+} was determined. (B) Efficiency of Na\textsuperscript{+} and Li\textsuperscript{+} efflux. Cells were incubated in the presence of 0.1 M NaCl or LiCl for 1.5 h. Then cells were washed and resuspended in assay buffer adjusted to pH 5.5 and supplemented with 50 mM KCl to trigger the efflux process.
The observed effects on the intracellular Na⁺ levels correlated very well with the rate of Na⁺ extrusion observed when the cells were initially loaded with Na⁺ (Fig. 2B). While the wild type rapidly exported most of the Na⁺, the ena1Δ was unable to export any Na⁺. The gis4Δ mutant clearly showed a diminished ability to export Na⁺, while the snf1Δ mutant and, in particular, the mig1Δ mutant were less affected. In this test, the snf1Δ gis4Δ double mutant and the gis4Δ snf1Δ mig1Δ triple mutant were somewhat more affected than the gis4Δ single mutant. The data on Li⁺ export were very similar to those observed for Na⁺ export (Fig. 2B). Taken together, the Na⁺ and Li⁺ sensitivity of all the mutants tested here seems to be due to diminished export of these ions. Since double snf1Δ gis4Δ mutants show a somewhat more severe defect than the single mutants, both proteins may cooperate but probably, in addition, have separate roles in controlling ion homeostasis.

**Gis4 is involved in controlling expression of ENA1.** The phenotype of the gis4Δ mutant suggested that it was defective in the expression of the ENA1 gene or the Ena1 protein following the exposure to Na⁺ and Li⁺ ions. To test this directly, we performed analysis of mRNA expression by Northern blot analysis, RT-PCR (data not shown), and β-galactosidase activity employing a plasmid carrying ENA1-lacZ (Fig. 3A). In wild-type cells, ENA1-lacZ transcriptional activity rapidly increased following a shift to 0.6 M NaCl. Deletion of GIS4 as well as SNF1 caused a drastic reduction in transcriptional activity. While the basal Ena1 protein level was similar in the wild type and the gis4Δ mutant, only in the wild type was that level strongly increased following salt treatment (Fig. 3B). These data are in accordance with the mutant phenotypes. The gis4Δ snf1Δ mutant also displayed very low ENA1 transcriptional activity, which appeared to be in about the same range as both single mutants. The mig1Δ mutant appears to express ENA1 normally, and hence, its salt sensitivity is probably unrelated to ENA1 expression (data not shown).

**Gis4 is a poorly conserved protein.** Gis4 is a predicted protein of 774 amino acids. There are no apparent paralogs in the yeast genome. The protein is rich in acidic (14.8%) and polar amino acids (asparagine plus serine alone, 25.2%), which together make up 35% of the protein sequence. Consistently, several stretches of Gis4 classify as low complexity domains. There are no homologs to Gis4 outside the fungal kingdom and, in fact, sequence similarity outside yeasts is doubtful. There are no apparent protein motifs in Gis4 that could hint at its molecular function.

We used predicted protein sequences from nine yeasts (Fig. 4; the complete alignment is shown in Fig. S1 in the supplemental material) in multiple alignments. The first 120 amino acids contain several conserved segments. Another conserved stretch is located between positions 435 and 520 (Gis4 from S. cerevisiae). In several instances these regions contain alternating short stretches of hydrophobic and charged residues, which
may play a role in protein-protein interaction. Putative PEST regions (22) do not seem to be conserved and, hence, have doubtful functional significance. Using conserved elements individually in BLAST searches did not reveal any other proteins than those already depicted (Fig. 4; Fig. S1 in the supplemental material). Low sequence conservation and complexity suggest that Gis4 does not perform an enzymatic function but rather may have a structural role, for instance, in a protein complex.

Gis4 is located at the plasma membrane. To determine the subcellular localization of Gis4, we first used a chromosomal C-terminal Gis4-GFP fusion from the global GFP fusion collection (12, 21). The strain carrying this fusion displayed an intermediate degree of salt tolerance, indicating that this fusion protein is only partially functional (not shown). Consistent with the data reported on the website http://yeastgfp.ucsf.edu/ (12, 21), this fusion protein appeared to be evenly distributed over cytosol and nucleus (whose position was deter-
The C terminus of Gis4 contains a CAAX sequence, with the cysteine and a terminal methionine being perfectly conserved in homologs from other yeasts (Fig. 4). Such a sequence could be target for prenylation, a modification that attaches proteins to cell membranes. Since the C-terminal Gis4-GFP fusion was not fully functional and the C-terminal GFP fusion likely affected a potential prenylation, we constructed an N-terminal GFP-Gis4 fusion. Expression of this construct was driven by the \textit{MET25} promoter and kept at a low level by supplementing the medium with methionine. The GFP-Gis4 fusion is functional, since the plasmid could complement the growth defect of the \textit{gis4}/H9004 mutant on salt medium (Fig. 5A). Quite remarkably, this construct showed an entirely different localization pattern than the C-terminal fusion: there was clear enrichment of the protein at the cell surface. At the same time, it appeared that a fraction was present in the cytosol and vacuolar staining could be observed in many cells. This vacuolar staining is probably an artifact, since it appears with different unrelated proteins expressed at low levels. The GFP-Gis4 subcellular distribution pattern did not change when cells were exposed to 0.05 M LiCl (not shown).

To further analyze the relevance of the CAAX element at the Gis4 C terminus for localization and function, we generated a C771A mutation in the N-terminal GFP-Gis4 fusion. The resulting GFP-Gis4C771A did not fully complement the \textit{gis4}/H9004 mutant, and the fusion protein was not enriched at the plasma membrane (Fig. 5B). We also introduced the C771A mutation into \textit{GIS4} expressed from a centromeric plasmid and its own promoter (but lacking GFP). This \textit{GIS4C771A} allele only partially complemented the salt sensitivity of the \textit{gis4}/H9004 mutant (Fig. 5C). In conclusion, it appears that at least a significant fraction of Gis4 is attached to the plasma membrane and such localization seems to be important, but not absolutely essential, for its function in acquisition of salt tolerance.

Gis4 does not play a major role in glucose derepression. \textit{GIS4} was initially isolated as a suppressor of the growth defect of the \textit{snf1}/H9004 mutant (in fact a \textit{snf1 mig1 srb8} mutant) on galactose and raffinose, suggesting that overexpression of \textit{GIS4} could cause stimulated expression of the \textit{GAL} and \textit{SUC} genes independently of Snf1 activity. Since our analysis suggested

\textbf{FIG. 5.} Localization of Gis4. (A) Cells were transformed with a centromeric plasmid expressing a fusion of GFP to the N terminus of Gis4 (GFP-Gis4). Expression is mediated by the \textit{MET25} promoter and kept low by supplementing the growth medium with methionine. The plasmid complements the Li\textsuperscript{+} sensitivity of the \textit{gis4} mutant and, hence, the fusion is functional. The fusion protein is mainly localized at the cell surface. (B) The putative farnesylation sequence at the Gis4 C terminus was mutated (C771A) in the GFP-Gis4 fusion plasmid. The resulting plasmid complements the \textit{gis4} mutant, although less well than wild-type \textit{GIS4}. The GFP-Gis4C771A fusion protein is localized throughout the cytosol. (C) The C771A mutation was introduced into \textit{GIS4} (without GFP) expressed from its own promoter on a centromeric plasmid. Also, this plasmid partly complements the Li\textsuperscript{+} sensitivity of the \textit{gis4} mutant.
that Gis4 functions in the same pathway as Snf1 for salt tolerance and since glucose derepression is the best-known function of Snf1, we wondered if Gis4 played a role in this response pathway. A role in glucose derepression of Gis4 was inferred by La Rue et al. (22).

While the snf1Δ mutant was unable to grow on medium with ethanol or raffinose as the sole carbon source, the gis4Δ mutant grew like the wild type (Fig. 6A for the W303-1A background; the BY4741 background showed the same phenotype). The snf1Δ gis4Δ double mutant showed the same phenotype as the snf1Δ single mutant. While the snf1Δ mutant and the snf1Δ gis4Δ double mutant did not increase their specific invertase activity (the product of the glucose-repressible SUC2 gene) when incubated in raffinose medium, the gis4Δ mutant produced as much invertase as the wild type. These data indicated that Gis4 does not play a significant role in glucose derepression (Fig. 6B).

To test derepression of the SUC2 gene more accurately, we performed time course gene expression analysis after a shift from 8% to 0.2% glucose by RT-PCR. While the snf1Δ and snf1Δ gis4Δ mutants did not show any expression of SUC2 in this experiment, it appeared that the gis4Δ mutant derepressed SUC2 expression, although we observed consistently that this occurred somewhat slower than in wild-type cells (Fig. 6C).

**DISCUSSION**

We describe, in this study, Gis4 as a new component of the system that establishes tolerance to Na⁺ and Li⁺ ions in the yeast S. cerevisiae. Based on the phenotype of the gis4Δ mutant, Gis4 performs its function in the acquisition of salt tolerance by participating in the transcriptional regulation of the ENA1 gene, which encodes the critical Na⁺/Li⁺ export pump. We provide evidence that Gis4 collaborates with the Snf1 kinase and that both Snf1 and Gis4 function in a pathway that operates in parallel to the calcineurin pathway.

Several pieces of evidence link Gis4 to the Snf1 pathway. (i) The GIs4 gene was initially isolated as a multicopy suppressor of the snf1Δ mig1Δ srb8Δ mutant and then shown to suppress also the single snf1Δ mutant for growth on galactose and raffinose. These data appear to place Gis4 down-stream (or in parallel) of Snf1 in the glucose derepression pathway (3). However, as discussed below, it appears that Gis4 does not normally have an important function in glu-
cose derepression. (ii) Gis4 physically interacts with Snf1. The protein was identified in a complex purified by immuno-precipitation with FLAG-tagged Snf1, which in addition, contained the known Snf1 interaction partners Snf4 and Gal83 (19). A direct interaction between Snf1 and Gis4, which seems to require Grr1-dependent ubiquitination of Gis4, has recently been demonstrated (22). (iii) While deletion of *SNF1* and of *GIS4* causes strong synthetic salt-sensitive phenotypes in combination with *CMP1* and *CMP2*, it appears that the *snf1Δ gis4Δ* double mutant has a phenotype that is very similar to that of the single *gis4Δ* mutant. This genetic evidence places Gis4 and Snf1 into the same pathway. At the same time, we note that in some assays, such as the Na\(^+\) and Li\(^+\) content and export experiments, the double *snf1Δ gis4Δ* mutant performs somewhat more poorly than the *gis4Δ* single mutant. This might be due to the unique role of Snf1 in cellular energy homeostasis and the requirement for ATP for Ena1-mediated ion export.

According to the data presented here, Snf1 and Gis4 share roles in controlling the acquisition of salt tolerance and expression of ENA1, while Gis4 does not seem to function together with Snf1 in glucose derepression. This observation is in clear contrast to the report by La Rue et al. (22), who observed that Gis4 stimulates Snf1 activity and that deletion of *GIS4* largely prevented derepression of *SUC2* expression. Our data are internally consistent in that the *gisΔ* mutant grows normally on carbon sources whose utilization requires Snf1, produces normal levels of invertase, and shows robust stimulation of gene expression following the shift to 0.2% glucose. At the same time, we reproducibly observed that derepression of *SUC2* expression was slightly delayed in the *gisΔ* mutant, suggesting that Gis4 may have a minor contribution to Snf1 activity under these conditions. A possible explanation for these observations is that other yeast proteins share redundant functions with Gis4 in glucose derepression but not salt tolerance; there are, however, no apparent paralogs to *GIS4* in the yeast genome. Taken together, we believe that the difference between our data and the La Rue et al. data may be due to the specific time points when analyses were performed (22) or to particular strain properties (note that we obtained consistent results in two strain backgrounds).

Taken together, it appears that Gis4 could be a component of the Snf1 system that directs pathway activity specifically toward acquisition of salt tolerance and expression of the *ENA1* gene. It has previously been reported that Snf1 plays a role not only in carbon metabolism but also in acquisition of heat tolerance and control of the heat shock transcription factor Hsf1 (16). More recently, it has been shown that Snf1 phosphorylates the general stress transcription factor Msn2 and thereby contributes to long-term adaptation of the acute stress response to glucose depletion (10). In addition, the role of Snf1 in salt tolerance and the control of expression of *ENA1* has previously been established (1, 31). However, these effects could somehow be explained by the higher energy demand of the cell under stress conditions and the consumption of ATP for export of Na\(^+\) in particular. Interestingly, it has been reported that phosphorylation of Thr210 on Snf1, which is required for activation of the kinase, can be specifically stimulated by NaCl addition (24).

What could be the molecular function of Gis4? The ob-

servation that overexpression of *GIS4* suppresses glucose derepression effects of the *snf1Δ* mutant (3) would be consistent with a function downstream of (or in parallel with) Snf1 by stimulating transcriptional activity independent of Snf1-mediated deactivation of the Mig1 repressor (note that *GIS4* was initially identified as a suppressor of the *snf1Δ mig1Δ srb8* mutant, which hence, lacks both Snf1 and Mig1). However, in the acquisition of salt tolerance, the epistatic relationship of Snf1 and Gis4 seems to be more complex, since overexpression of *GIS4* did not suppress the salt sensitivity of the *snf1Δ* mutant (and vice versa). The genetic data are consistent with the two proteins functioning in concert, i.e., in a complex. Whether the direct physical interaction between Snf1 and Gis4 plays a role in salt tolerance remains to be established. It has also been shown that Gis4 can stimulate Snf1 activity (22), although this cannot be its only function, since overexpression of *GIS4* can suppress the lack of Snf1 in glucose derepression (3). The predicted amino acid sequence of Gis4 does not provide any hints toward its biochemical function. Together with poor conservation, this suggests that it does not have a catalytic role but rather may function as an adaptor or scaffold protein. Gis4 seems to preferentially localize to the cell surface, where it may be anchored to the plasma membrane by means of C-terminal farnesylation. However, it appears that the C-terminal CAAX box is important but not essential for Gis4 function in the acquisition of salt tolerance. Moreover, putative homologues from filamentous fungi do not seem to have the C-terminal CAAX box, although identification of likely orthologues is doubtful beyond yeasts due to low sequence conservation.

In conclusion, it appears that Gis4 plays a role in the acquisition of salt tolerance, probably in conjunction with Snf1. Gis4 may therefore control a function of Snf1 that is independent of its role in glucose derepression and by directing Snf1 to a specific location at the cell surface, where it could be activated for its role in acquisition of salt tolerance. Since, however, the salt-sensitive phenotype of the *gisΔ* mutant is stronger than that of the *snf1Δ* mutant; Gis4 may collaborate with additional proteins that function in parallel with Snf1.

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