Hal4 and Hal5 Protein Kinases Are Required for General Control of Carbon and Nitrogen Uptake and Metabolism†‡

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Received 27 July 2010/Accepted 7 October 2010

The yeast protein kinases Sat4/Hal4 and Hal5 are required for the plasma membrane stability of the K+ transporter Trk1 and some amino acid and glucose permeases. The transcriptomic analysis presented here indicates alterations in the general control of the metabolism of both nitrogen and carbon. Accordingly, we observed reduced uptake of methionine and leucine in the hal4 hal5 mutant. This decrease correlates with activation of the Gcn2-Gcn4 pathway, as measured by expression of the lacZ gene under the control of the GCN4 promoter. However, with the exception of methionine biosynthetic genes, few amino acid biosynthetic genes are induced in the hal4 hal5 mutant, whereas several genes involved in amino acid catabolism are repressed. Concerning glucose metabolism, we found that this mutant exhibits derepression of respiratory genes in the presence of glucose, leading to an increased activity of mitochondrial enzymes, as measured by succinate dehydrogenase (SDH) activity. In addition, the reduced glucose consumption in the hal4 hal5 mutant correlates with a more acidic intracellular pH and with low activity of the plasma membrane H+-ATPase. As a compensatory mechanism for the low glycolytic rate, the hal4 hal5 mutant overexpresses the HXT4 high-affinity glucose transporter and the hexokinase genes. These results indicate that the hal4 hal5 mutant presents defects in the general control of nitrogen and carbon metabolism, which correlate with reduced transport of amino acids and glucose, respectively. A more acidic intracellular pH may contribute to some defects of this mutant.

The proper regulation of cellular ion homeostasis is a fundamental requirement for all living organisms. During the last decades, much work has led to the molecular definition of many ion transport proteins. In this respect, the eukaryotic model organism, Saccharomyces cerevisiae, has been instrumental not only in the identification of founding members of many ion transporter and channel families but also in the cloning and/or characterization of heterologous transporters from both mammals and plants (e.g., see references 25 and 34). With the more recent elucidation of the structures of many channels and transporters, our knowledge regarding the molecular mechanisms of how several classes of these transport proteins function has increased considerably (reviewed in reference 3). However, in many cases, the posttranslational mechanisms by which these proteins are regulated in response to external stimuli are largely unknown.

We have studied the regulation of the major potassium transporter in yeast, Trk1, as a model protein. This transporter is largely responsible for maintaining internal potassium at concentrations much higher than that normally found extracellularly (11). The activity of this transporter does not appear to be influenced by transcriptional mechanisms, as the levels of TRK1 mRNA do not change significantly in response to potassium starvation or different ionic stresses (reviewed in reference 2). Therefore, the regulation of Trk1 is thought to be largely posttranslational. Evidence for phosphorylation-dependent regulation of Trk1 has been provided by reports showing that this transporter is phosphorylated in vivo and negatively regulated by the type 1-like protein phosphatases Ppz1 and Ppz2 (36, 37).

The identification of yeast halotolerance genes has defined many important regulators of ion homeostasis (10, 12, 19, 20). These genes were identified in a functional screening for genes that confer tolerance to toxic amounts of LiCl and NaCl upon overexpression in Saccharomyces cerevisiae. Among these genes, the structurally and functionally related Hal4 (also known as Sat4) and Hal5 kinases are proposed to regulate the high-affinity potassium transporter Trk1. This hypothesis is based largely on the growth characteristics of the hal4 hal5 double mutant strain, which requires potassium supplementation for maximal growth and displays a marked defect in rubidium uptake (19). These results suggested that these kinases may regulate Trk1 by direct phosphorylation and thus play an opposing role to that of the Ppz1 and Ppz2 phosphatases. Despite much effort, no experimental evidence demonstrating a direct physical interaction or direct phosphorylation of Trk1 by these kinases has been generated. More recently, we reported that upon removal of potassium supplementation, Trk1
is quickly targeted to the vacuole and degraded in the hal4 hal5 mutant. Moreover, this retargeting is not specific for Trk1; other nutrient transporters, such as the Can1 arginine permease, the Hxt1 glucose transporter, and the Fur4 uracil permease, also accumulate in the vacuole under the same conditions (24). These observations led to the hypothesis that the Hal4 and Hal5 kinases may have an indirect effect on the Trk1 potassium transporter.

The Hal4 and Hal5 kinases belong to a family of yeast kinases that includes Ptk2 and Npr1, both implicated in the regulation of plasma membrane transport proteins. Ptk2 has been implicated in the phosphorylation of a plasma membrane proton ATPase, Pma1, although the mechanism by which this phosphorylation event may regulate transporter activity is not yet determined (9). Npr1 has been proposed to be a target of the rapamycin (TOR) kinase pathway and to regulate the trafficking of several amino acid permeases, including Gap1, Bap2, and Tat2 (7, 15, 23, 27). In this case, Npr1-dependent phosphorylation is proposed to affect Rsp5-dependent ubiquitination of these permeases, thus influencing the amount of these proteins present in the plasma membrane.

In order to ascertain the extent of the effect of transporter mislocalization in this mutant, we have analyzed several physiological aspects of the hal4 hal5 mutant, such as the global gene expression pattern under several experimental conditions, amino acid uptake, activation of the general control nondepressing (GCN) pathway, glucose consumption, cytosolic pH, and Pma1 
^+_ATPase activity. These analyses have revealed a general role for the Hal4 and Hal5 kinases in the uptake of both amino acids and sugar, thus influencing the proper control of nitrogen and glucose metabolism.

### MATERIALS AND METHODS

#### Yeast strains and culture conditions.

All strains of *Saccharomyces cerevisiae* used in this work are listed in Table 1. The BY4741 strain lacking *HAL4* and *HAL5* was constructed by disrupting *HAL5* in the *hal4* background obtained from the Euroscarf collection using the LEU2 disruption cassette described previously (19). YPD medium contained 2% glucose, 2% peptone, and 1% yeast extract. In the case of the acidified YPD medium (that with a pH of 4.5 [here designated YPD pH 4.5 medium]), 50 mM succinic acid was also added. Minimal medium (synthetic defined medium) contained 2% glucose, a 0.7% yeast nitrogen base (Difco) without amino acids, 50 mM succinic acid adjusted to pH 5.5 with Tris, and the amino acids and purine and pyrimidine bases required by the strains. Growth assays were performed with solid media by spotting serial dilutions of saturated cultures onto plates with the indicated composition.

| Strain | Relevant genotype | Reference or source |
|--------|------------------|---------------------|

#### Plasmids used in this work.

The *TRK1*-hemagglutinin (HA) (*TRK1::HA*), and the *pYP352-HAL5* plasmids were previously described (24). The *pYEP352-HAL4* plasmid was constructed by inserting the SacI/XbaI fragment containing the promoter and terminator sequences into the same sites in the *pYP352 vector. For the construction of the *pYPEG15-pHluorin* plasmid, the open reading frame (ORF) encoding a modified version of green fluorescent protein (GFP) called pHluorin (18) was amplified by PCR and cloned into the *pYEG15* vector using primer-derived XhoI sites. The correct orientation of the insert was then tested by NdeI digestion. The p180 plasmid, containing the *GCN4* promoter, was used for the β-galactosidase activity assays (35). The *MUP1-GFP* plasmid was used for confocal microscopy and Western blot analysis (29).

#### RNA labeling.

Total RNA, obtained using standard phenol-chloroform extractions, was further purified using the CleanUp protocol of the RNeasy mini kit (Qiagen). Thirty micrograms of RNA was retrotranscribed in the presence of modified nucleotides [5'-3-amino-allyl]-2-deox-UTP by using a oligo(dT) primer in order to obtain mRNAs to single-strand modified cDNAs. cDNA synthesis was performed at 50°C during at least 3 h, using the reverse transcriptase Superscript III (Invitrogen). The cDNA was then purified using Qiagen (Qiagen) and labeled with Cy3 and Cy5 dyes, as recommended by the manufacturer (Amersham Biosciences). cDNAs were then purified again with Qiagen (Qiagen) columns and labeled with Cy3 and Cy5 dyes, as recommended by the manufacturer (Amersham Biosciences). cDNAs were then purified again with Qiagen (Qiagen) columns, and the labeling was confirmed by measuring the absorbance at the appropriate wavelength, using a Nanodrop ND 1000 (Thermo Scientific).

#### Microarray experimental design and hybridization.

The chosen design for all the experiments was a balanced block design with four biological replicates and two dye swaps. The microarrays were generated at the Autonomous University of Barcelona Genomics Facility by spotting approximately 6,200 PCR-amplified yeast ORFs (1). For analysis, they were submersed for 45 min in a prehybridization solution (0.1% SDS, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% bovine serum albumin [BSA]) at 42°C. Microarrays were then hybridized with the labeled probes in hybridization solution (0.1% SDS, 5× SSC, 50% formamide, 0.1 mg/ml salmon sperm DNA) using hybridization chambers (Telechem) overnight at 42°C. After the hybridization, microarrays were washed in different dilutions of SSC and SDS and then scanned to obtain digital images using a GenePix 4000B scanner (Axon Instruments) with a 10-μm resolution.

**Microarray data analysis.** Digital images from microarrays were analyzed using GenePix 4.0 (Axon Instruments) program. Only those genes whose intensity was 2-fold higher than local background intensity in at least one of the channels used were included in the analysis. The complete data set has been deposited in NCBI’s Gene Expression Omnibus (GEO: 8). The data from the four biological replicates were analyzed and normalized using the Acuity 4.0 program (Axon Instruments), and statistical selection of genes was performed.
with the significance analysis application (SAM) (32). Genes were selected as differentially expressed when the expression in the mutant was at least 1.5-fold higher or lower than the expression in the wild type (WT) and, when applying the significance analysis (SAM), the false discovery rate (FDR) was lower than 5.5% (see Table S1 in the supplemental material). Relevant functional categories were selected using the Gene Ontology Term Finder as described previously (5). The YEASTRACT repository was searched in order to identify regulatory associations between the mRNAs induced in the hal4 hal5 mutant and transcription factors responsible for this regulation (30).

Measurements of amino acid uptake. For methionine uptake measurements, the strains were grown to exponential phase and collected by centrifugation (3,000 rpm for 5 min). After being washed with sterile water, cells were resuspended in 5 ml of buffer (50 mM succinic acid and 2% glucose [wt/vol], either without or with 0.2 M KCl) and incubated for 20 min at 28°C. The number of cells was determined for each culture, and methionine uptake assays were started by adding [35S]methionine (55 mCi/μmol; American Radiolabeled Chemicals, St. Louis, MO) to a final concentration of 10 μM. Samples were collected at the indicated time points (Fig. 1A) by filtration on glass microfiber filters and dried overnight. Filters were then immersed in scintillation fluid, and the radioactivity was measured in counts per minute and disintegrations per minute by using the Pharmacia Wallac 1410 liquid scintillation counter as described previously (33). Methionine uptake is expressed as nmol of methionine per mg of cells (wet weight).

Leucine uptake measurements were performed as described for the methionine uptake protocols, but using [3H]leucine (315.5 mCi/mmol; Amersham Biosciences).

Protein extraction and fractionation. Protein extracts, fractionation procedures, and Western blot analyses were performed as described previously (24).

Confocal microscopy. Fluorescence images were obtained as described previously (36).

β-Galactosidase assays. Yeast cells transformed with the indicated reporter plasmid (p180) were grown selectively in SD medium and then diluted in YPD pH 4.5 medium. In this case, the BY4741 strain was transformed with the YCplac111 (13) plasmid to match the strains for auxotrophies. Cells were grown to exponential phase and then harvested by centrifugation (3,000 rpm for 5 min) and resuspended in M9 minimal medium. β-Galactosidase activity was determined as described elsewhere (12) and represented as β-galactosidase activity units. Data are from the means of three independent transformants, each measured in triplicate.

Glucose consumption. Strains were grown in YPD pH 4.5 medium until mid-log phase. Cells were collected and resuspended in fresh media to an optical density at 600 nm (OD600) of 0.2. At the indicated time points (see Fig. 4B), aliquots were removed to determine cell number and glucose concentration in the media using standard protocols employing coupled reactions with glucose oxidase (Roche) and horseradish peroxidase (Roche), with the diammonium salt of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Merck) as the chromophore. Error bars represent the standard deviations of triplicate determinations (see Fig. 4B). Identical results were observed in two independent experiments.

Ethanol production. Strains were grown in YPD pH 4.5 medium until mid-log phase (7 × 106 cells/ml). Cells were removed by centrifugation, and the amount of ethanol present in the media was determined by standard procedures for quantifying the production of NADH during the conversion of ethanol to acetaldehyde by alcohol dehydrogenase (Sigma) at 340 nm. The error bars represent the standard deviations of triplicate determinations (see Fig. 4C, right panel).

SDH assay. Mitochondrial-enriched extracts were prepared in TSB buffer (10 mM Tris-HCl [pH 7.5], 0.6 M sorbitol), as described previously (21). Succinate dehydrogenase (SDH) assays were performed with p-iodonitrotetrazolium violet as an artificial electron acceptor for the SDH complex. Extracts were incubated in 300 μl of succinate buffer (10 mM succinic acid in 50 mM phosphate buffer, pH 7.4) with 100 μl of p-iodonitrotetrazolium violet solution (2.5 mg of p-iodonitrotetrazolium violet in 50 mM phosphate buffer, pH 7.4). The reactions were stopped with 1 ml of stop solution (10 g of trichloroacetic acid in 100 ml of 2% ethyl acetate/ethanol; 1:1 [vol/vol]), and the absorbance of the supernatant was measured at 490 nm. Error bars represent the standard deviations of triplicate determinations (see Fig. 4C, left panel). Similar results were observed in two independent experiments.

Measurements of cytosolic pH. For the determination of the cytosolic pH, a modified version of GFP referred to as ratiometric pHluorin was employed (18). The strains of interest were transformed with pYPGE15-pHluorin and grown to mid-log phase in SD medium with or without potassium supplementation (0.2 M KCl). Cells were then harvested, and cytosolic pH estimations and calibration curves were performed as previously described (6) using a PerkinElmer LS50B luminescence spectrometer equipped with FLWinLab software.

Measurement of H+-ATPase activity. The in vitro H+-ATPase activity was measured as described previously (14, 28).

Microarray data accession number. The complete data set has been deposited in NCBI’s GEO database and is accessible through GEO series accession number GSE22976 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22976).

RESULTS

Transcriptomic analysis of the hal4 hal5 mutant. As an approach to further investigate the biological roles of the functionally and structurally related Hal4 and Hal5 protein kinases, we compared the transcription profiles of the hal4 hal5 double mutant and the wild-type strains in two different genetic backgrounds: W303-1A and BY4741. Both strains were grown to mid-log phase in YPD pH 4.5 medium. We initially chose this acidic rich medium based on the more severe growth defect displayed by the hal4 hal5 mutant. Moreover, we wanted to compare the phenotypic differences between the wild-type and mutant strains under steady-state conditions.

Using the experimental design described in Materials and Methods, 709 genes were differentially expressed in the W303-1A genetic background (see Table S1 in the supplemental material). Of these genes, 457 had higher mRNA levels in the hal4 hal5 mutant than in the wild type, whereas 252 transcripts accumulated to lower levels in the hal4 hal5 mutant than in the wild type. In the BY4741 genetic background, the number of differentially expressed genes was smaller: 260. There were 194 genes whose expression was higher in the hal4 hal5 mutant than in the wild type and 66 genes whose mRNA accumulated to lower levels in the hal4 hal5 mutant than in the wild type (see Table S1 in the supplemental material).

Although the number of differentially expressed genes is considerably larger in the W303-1A genetic background than in the BY4741 genetic background, many of the same genes were identified in both genetic backgrounds. Approximately 70% (139 of 194) of the induced genes and 60% (40 of 66) of the repressed genes identified in the hal4 hal5 mutant in the BY4741 genetic background were also identified in the W303-1A genetic background.

To characterize the biological processes represented by these genes, we employed the GO Term Finder tool (5). As shown in Table 2, among the genes induced in the hal4 hal5 mutant in the BY4741 genetic background, two functional categories, related to energy metabolism and methionine biosynthesis, are significantly overrepresented (GO Term Finder P value, <0.01). In the W303-1A genetic background, where the number of induced genes was almost doubled, these two functional categories are also overrepresented among the induced genes, as were additional functional categories related to carbohydrate metabolism and stress response.

Among the genes repressed in the hal4 hal5 mutant in the BY4741 genetic background, functional categories related to nucleotide metabolism (adenine biosynthesis), amino acid metabolism, and iron assimilation (three genes) are overrepresented. In the W303-1A genetic background, we also observed an overrepresentation of functional categories related to adenine biosynthesis and amino acid metabolism, but not those related to iron assimilation. On the other hand, in addition to the categories mentioned above, there is also an overrepresentation of genes related to the functional categories of ribosome biogenesis and ergosterol biosynthesis.
It is worth noting that these experiments have been performed for genetic backgrounds with different genotypes in terms of adenine and methionine auxotrophies. The W303-1A genetic background is ade2 MET15, whereas the BY4741 genetic background is ADE2 met15. Therefore, the identification of differentially expressed genes related to methionine and adenine biosynthesis is not due to the genetic background of the strain employed. In addition, we performed Northern blot analyses to confirm the induction and repression of selected genes by comparing the W303-1A hal4 hal5 mutant to a control strain in which we introduced the wild-type versions of both LEU2 and HIS3 by homologous recombination to match the auxotrophies (data not shown).

Moreover, we performed the same experiment using cells from the W303-1A genetic background grown in YPD pH 6.0 medium (see Table S1 in the supplemental material). Under these conditions, the functional categories that were overrepresented among the induced and the repressed genes were exactly the same as those that were identified in both genetic backgrounds when cells were grown at pH 4.5 (for induced genes, energy metabolism and methionine biosynthesis; for repressed genes, adenine biosynthesis and amino acid metabolism).

Thus, these results suggest that strains lacking the HAL4 and HAL5 genes display alterations in the general control of both nitrogen and carbon uptake and/or metabolism.

**Nitrogen uptake/metabolism.** (i) Methionine and leucine uptake are decreased in the hal4 hal5 mutant. The functional category of methionine biosynthesis was overrepresented among the genes induced in the hal4 hal5 mutant under all conditions tested. Virtually all the genes involved in the biosynthesis of methionine are induced in the hal4 hal5 mutant in at least one of the experiments described (see Fig. S1 in the supplemental material). We reasoned that a defect in methionine uptake in this mutant could explain the observed induction of these genes. In order to test this hypothesis, methionine uptake assays were performed for both the hal4 hal5 mutant and wild-type strains. Cells were grown to exponential phase in YPD pH 4.5 medium in order to maintain the same conditions as those in the transcriptomic analysis. As shown in Fig. 1A, the hal4 hal5 mutant displays a marked decrease in the maximum methionine uptake after 10 min (around 50% of the maximum methionine uptake observed for the wild type) and also a lower initial uptake velocity.

In order to determine if the defect in methionine uptake was due to mislocalization of the Mup1 high-affinity methionine permease, we analyzed the localization of a Mup1-GFP fusion protein in wild-type and hal4 hal5 strains. As shown in Fig. 1B, reduced amounts of Mup1-GFP are observed in the plasma membrane of the hal4 hal5 mutant, whereas GFP accumulates in the vacuole of these strains in both the presence and the absence of K+ supplementation of the growth media. We confirmed these results by anti-GFP Western blot analysis of fractionated protein extracts, observing a reduction in the Mup1-GFP signal in the plasma membrane-containing insoluble fraction and an increase in free GFP in the soluble fraction.

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**TABLE 2. Summary of functional categories of genes differentially regulated in the hal4 hal5 mutant**

| Gene category       | Functional categories for the indicated strain (growth medium) |
|---------------------|---------------------------------------------------------------|
|                     | W303-1A (YPD pH 4.5)       | BY4741 (YPD pH 4.5)       | W303-1A (YPD pH 6.0)       |
| **Induced genes**   |                                                                 |
| Energy metabolism   | Energy metabolism       | Energy metabolism       |
| ATP biosynthesis    | ATP biosynthesis        |
| Aerobic respiration | Aerobic respiration    |
| Electron transport  | Electron transport      |
| Sulfate assimilation| Sulfate assimilation   |
| Methionine biosynthesis| Methionine biosynthesis |
| Carbohydrate metabolism |                                     |
| Trehalose biosynthesis |                                |
| Glycogen biosynthesis |                                |
| Alcohol catabolism  |                                |
| Monosaccharide catabolism |                             |
| Stress response     |                                |
| **Repressed genes** |                                                                 |
| Nucleotide metabolism |                                   |
| Adenine biosynthesis | Adenine biosynthesis       |
| Amino acid metabolism | Amino acid metabolism     |
| Ribosome biogenesis  | Iron assimilation        |
| Ergosterol biosynthesis |                                 |

*Functional categories were identified using the Gene Ontology Term Finder available through the Saccharomyces Genome Database (http://www.yeastgenome.org/). Categories with a P value of <0.01 were considered significant. Relevant subcategories are shown in italics.*
which contains the contents of vacuolar lumen) in the hal4 hal5 mutant (Fig. 1C).

In order to test whether this defect in amino acid uptake is specific to methionine or a more general effect, we also performed leucine uptake assays. We observed a similar decrease in leucine uptake in the hal4 hal5 mutant compared to that in the wild-type control (Fig. 2).
in order to distinguish between those effects that are caused by the lack of high-affinity potassium transport from those that are directly caused by the absence of the Hal4 and Hal5 kinases. In the trk1 trk2 strain, β-galactosidase activity levels were intermediate between those observed for the hal4 hal5 mutant and those observed for the wild type (Fig. 3).

**Carbon metabolism.** (i) **Mitochondrial activity is increased in the hal4 hal5 mutant.** Analysis of the transcriptomic data of the hal4 hal5 mutant reveals the overaccumulation of mRNAs corresponding to groups of genes related to energy metabolism. For example, genes specifically related to the respiratory chain accumulated to higher levels in the hal4 hal5 mutant than in the wild type (Fig. 4A). As reported above, this mutant displays a defect in both methionine and leucine uptake, which may reflect a more general starvation, one not only of amino acids but also of other nutrients, such as glucose. We previously reported a defect in the plasma membrane localization of the Hxt1 glucose transporter in the hal4 hal5 mutant (24). Moreover, the high-affinity hexose transporter HXT4 and three hexokinase genes, HXK1, HXK2, and GLK1, are induced in the hal4 hal5 mutant. These results are consistent with a relative reduction in glucose uptake. Therefore, we measured glucose consumption in the wild type and the hal4 hal5 mutant. As shown in Fig. 4B, the mutant strain consumes less glucose than the wild-type control. This reduction in glycolytic flux may explain the altered metabolic state of this mutant toward respiration in order to optimize energy production. Consistent with this hypothesis, we observed dramatically higher succinate dehydrogenase (SDH) activity and a reduction in ethanol production in the hal4 hal5 mutant (Fig. 4C).

(ii) **The intracellular pH and Pma1 activity of the hal4 hal5 mutant are decreased.** It has been proposed that alterations in potassium transport could affect intracellular pH due to the strict requirement of maintaining electrical neutrality in cells. We reported experimental evidence supporting this hypothesis by showing that the internal pH is increased in yeast mutants that aberrantly accumulate potassium (37). We hypothesized that the decrease in the intracellular potassium concentration observed for the hal4 hal5 mutant (24) would lead to proton accumulation and consequent cytosolic acidification.

In order to analyze the cytosolic pH of the hal4 hal5 mutant and the wild-type strain, both strains were transformed with a plasmid containing a modified version of GFP, referred to as ratiometric pHluorin, which changes its emission spectrum depending on the cytosolic pH of the cells (18).

As expected, we found that the hal4 hal5 mutant (BY4741 genetic background) has a lower cytosolic pH than the control strain 2 h after transfer to minimal medium without potassium supplementation (6.47 ± 0.08 versus 6.91 ± 0.10, respectively). Under these conditions, the hal4 hal5 mutant displays a marked decrease in the internal potassium concentration (136 ± 7 mM for the WT versus 92 ± 8 mM for the hal4 hal5 mutant) (24). Strikingly, when we performed the same analysis with these strains grown in potassium supplemented medium, with the hal4 hal5 mutant and the wild-type strains having similar internal potassium concentrations (161 ± 11 mM versus 162 ± 18 mM), we observed that the hal4 hal5 mutant retained a significantly lower cytosolic pH (6.79 ± 0.17 for the WT versus 6.46 ± 0.06 for the hal4 hal5 mutant). These results were confirmed with an alternative genetic background (W303-1A and B, in all cases, the BY4741 strain transformed with a centromeric plasmid carrying the p180 plasmid that contains a fusion of the hal4 hal5 promoter with the lacZ gene. Increased production of β-galactosidase is regulated by a translational control mechanism that increases the cellular concentration of this transcriptional activator is regulated by a translational control mechanism that increases the cellular concentration of this transcription factor in amino acid-starved cells (reviewed in reference 17).

In order to analyze the GCN pathway, wild-type and hal4 hal5 mutant strains were transformed with the p180 plasmid that contains a fusion of the GCN4 promoter with the lacZ gene. Increased production of β-galactosidase is correlated with increased Gcn4 activity and is observed upon amino acid starvation (16). When cells were grown in rich medium (YPD), β-galactosidase activity was higher in the hal4 hal5 mutant than in the wild type for strains of two different genetic backgrounds (Fig. 3 and data not shown). In the case of the BY4741 background, the wild-type control was cotransformed with a centromeric plasmid carrying the LEU2 gene (data not shown).

(ii) **GCN activity is increased in the hal4 hal5 mutant.** The decrease in methionine and leucine uptake described for the hal4 hal5 mutant may lead to a general state of amino acid starvation. In order to investigate this possibility, we measured the activity of the GCN pathway in this mutant. The Gcn4 transcriptional activator is regulated by a translational control mechanism that increases the cellular concentration of this transcription factor in amino acid-starved cells (reviewed in reference 17).

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A) and are in agreement with those recently reported for the

These observations suggest that the lower intracellular po-
tassium concentration of the hal4 hal5 mutant is not the only
cause of the cytosolic acidification of this mutant. In order to
add experimental support to this tentative conclusion, we an-
alyzed the cytosolic pH of the hal4 hal5 mutant and the wild-
type strains transformed with a centromeric plasmid carrying
either the wild-type version of TRK1 or the truncated version
lacking the last 35 amino acids of the potassium transporter
(TRK1/H900435). We previously reported that when the hal4 hal5
mutant expresses the highly stable and active Trk1/H900435, growth
in medium without potassium supplementation is improved
and the mutant is no longer sensitive to salt stress (24). As
expected from the results described above, we did not observe
a statistically significant difference in the cytosolic pH in the
hal4 hal5 mutant expressing the truncated version of TRK1,
TRK1/H900435, compared to that of the hal4 hal5 mutant expressing
the vector containing full-length TRK1.

In order to experimentally corroborate the relative decrease
in the cytosolic pH of the hal4 hal5 mutant, we measured the
activity of the Pma1 H+/H11001-ATPase. As shown in Fig. 5,
we observed a significant decrease in Pma1 activity. This result is
consistent with the observed acidification of the hal4 hal5 cy-
tosol and correlates with the decreased uptake of nutrients,
such as amino acids and glucose, which are symported with
protons.

DISCUSSION

As mentioned above, despite much effort, no experimental
evidence has been obtained to demonstrate that the Hal4 and
Hal5 kinases directly regulate the Trk1 potassium transporter.
As we recently demonstrated an alteration in the normal traff-
cicking of this and other nutrient permeases in mutants lacking
HAL4 and HAL5 (24), we hypothesized that these kinases
could be regulating some step of vesicle trafficking and/or
endocytosis. Therefore, we analyzed several other aspects of
the phenotypes of hal4 hal5 strains in order to determine the
extent of the effect of the lack of these kinases. In our previous
studies, we compared the behavior of the hal4 hal5 mutant
strain with that of the wild type during a shift from potassium-
supplemented minimal medium to nonsupplemented medium,
in which the mutant strain cannot grow (24). In the present
study, we have chosen to compare the strains under steady-
state conditions in rich medium, which does not require po-
tassium supplementation for growth of the hal4 hal5 mutant.
Using this approach, we present evidence for a more general
defect in nitrogen and carbon availability. Interestingly, we also observe alterations in the internal pH of this mutant which are independent of the internal potassium concentration. These data suggest that there is no simple direct correlation between fluxes of potassium and protons, at least under the experimental conditions tested.

Based on the previously reported trafficking defects of the Can1 and Tat2 amino acid permeases and the relative decrease in internal pH observed here, we tested whether the combined effect of the decreased plasma membrane permease presence and the unfavorable proton gradient driving amino acid uptake would lead to defects in other aspects of nutrient availability. We observed marked defects in both leucine and methionine uptake, as well as an increase in the activation of the GCN pathway, as measured using a β-galactosidase reporter construct. However, supplementing the growth media with excess amino acids is only marginally effective in ameliorating the growth defects of this mutant (24; data not shown).

The phenotypes described above are consistent with a more general defect in nutrient uptake and/or utilization. This conclusion is reinforced by the gene expression analysis, in which we observed the accumulation of mRNAs corresponding to genes implicated in energy metabolism and amino acid biosynthesis. A more detailed analysis of the genes induced in the hal4 hal5 strain revealed a possible alteration in the metabolic state of this mutant, as reflected by the induction of the nuclear genes encoding virtually all the components of the respiratory chain (Fig. 4A). This altered expression pattern may indicate increased mitochondrial function, presumably to optimize the metabolism of the more limited supply of nutrients. Accordingly, we demonstrate a marked increase in the activity of the mitochondrial enzyme succinate dehydrogenase, although we did not observe any differences in the overall morphology of the mitochondria (data not shown). Interestingly, we also observed decreased glucose consumption and ethanol production in the hal4 hal5 strain compared to the wild type (Fig. 4B and C and data not shown).

Genes involved in the biosynthesis of methionine were also overrepresented in the list of genes induced in the hal4 hal5 mutant. We initially hypothesized that this result could be due to oxidative stress, as many genes controlled by the Yap1 transcription factor also accumulate to higher levels in this strain. However, we were unable to detect any significant difference in reactive oxygen species (ROS) levels using 1,2,3-dihydroxydihydrogenase as a probe (data not shown). Therefore, based on the marked defect in methionine uptake, the instability of the Mup1 high-affinity permease, and the fact that essentially all genes involved in the central pathway for methionine biosynthesis are induced, we propose defects in the transport of this amino acid as the most likely explanation for this phenotype.

It is worth pointing out the remarkable reproducibility among the various gene expression analysis data sets. We performed microarray experiments comparing the wild type and the hal4 hal5 mutants of two different genetic backgrounds (W303-1A and BY4741) and grown in two different media (YPD pH 4.5 and standard YPD [pH 6] media). For example, all but one of the functional categories identified, for both induced and repressed genes, in the BY4741 background were also identified in the W303-1A background, and the same functional categories were identified regardless of the growth conditions. However, we did observe a larger number of genes which were differentially regulated in the W303-1A strain than that for the BY4741 strain. Interestingly, the majority of these additional genes were related to either carbohydrate metabolism or the general stress response pathway, both principally controlled by the Msn2/Msn4 transcription factors. The identification of genes related to carbohydrate metabolism is in agreement with a general defect in nutrient uptake, which in this case would also involve glucose. As mentioned above, we previously observed a defect in the stability of the Hxt1 glucose permease, which would be consistent with this hypothesis and the observed decrease in glucose consumption. In terms of the general stress response genes, this effect is likely to be explained by the fact that, under all stress conditions we have tested, the W303-1A strains are much more sensitive. Therefore, we postulate that the same environmental conditions provoke a more robust stress response in the more sensitive W303-1A strain.

Here, we have further investigated the functional categories that correspond to the induced genes: energy metabolism and methionine biosynthesis. We provide experimental evidence which both supports the appearance of these categories and provides a possible explanation in terms of the physiology of the hal4 hal5 mutant, as discussed above. In addition, we also identify two functional categories of repressed genes which were overrepresented in all the transcriptomic analyses of the hal4 hal5 mutant: amino acid metabolism and adenine biosynthesis. The first functional category contains genes several genes involved in amino acid catabolism (CHA1, ASP1, ARO9, ARO10, BAT1, GCV2, GCV3) and amino acid excretion (AQR1). Therefore, if the hal4 hal5 mutant is defective in amino acid uptake, it makes sense that genes involved in excretion and/or catabolism of these nutrients would be downregulated.

However, the interpretation of the appearance of genes involved in the biosynthesis of adenine is not so straightforward. It is known that the adenine biosynthesis route is controlled by feedback inhibition on the enzymatic level and by derepression on the transcriptional level (reference 26 and the references therein). The product of the ADE4 gene, which catalyzes the first step of this pathway, is negatively regulated by ATP and ADP. Therefore, it is unlikely that the nutrient-deprived hal4 hal5 strain accumulates excess ATP to inhibit this pathway. In terms of the derepression, two transcription factors have been implicated: Pho2 and Bas1. Neither of these genes have an altered expression pattern in the hal4 hal5 mutant. The most likely explanation resides in the activation of Pho2 and Bas1 by the pathway intermediates SAICAR (5-amino-4-imidazole-N-succinocarboxamide ribonucleoside) and AICAR (5-amino-4-imidazolecarboxamide ribonucleoside). Although the molecular mechanism of this regulation has not yet been elucidated, it is clear that the relative concentration of these two compounds affects the ability of these two transcription factors to derepress the pathway. Therefore, it is conceivable that differences in the relative concentrations of these intermediates in the hal4 hal5 mutant explain the lack of derepression observed.

Further analysis of the transcriptome of the hal4 hal5 mutant also reveals a significant enrichment in genes known to be transcriptionally regulated by a subset of transcription
factors. For example, two factors that are activated by the Hog1 pathway are overrepresented in our analysis (Hot1 and Sko1, with P values of $1.5 \times 10^{-12}$ and $2.5 \times 10^{-10}$, respectively). This result is not surprising, since the decrease in internal potassium concentrations observed in this strain would be expected to lead to a decrease in turgor and thus activation of the Hog1 pathway through its upstream sensors. Accordingly, the hal4 hal5 mutant presents a decreased cell size and a relative increase in the amount of phosphorylated Hog1 present under standard growth conditions (data not shown).

It is known that the proton gradient generated by the proton ATPase, Pma1, is the driving force for nutrient uptake in yeast (reviewed in reference 4). We show here that the hal4 hal5 mutant presents phenotypes consistent with a general defect in nutrient uptake and a lower internal pH. These data may suggest a role for these kinases as positive regulators of the Pma1 proton ATPase. Preliminary experiments suggest that the in vivo phosphorylation of Pma1 is decreased in the hal4 hal5 mutant, whereas the amount of Pma1 found in plasma membrane-enriched fractions is not significantly altered (L. Yenush, unpublished observations). The related Ptk2 kinase has been proposed to regulate Pma1 by directly phosphorylating it on serine 899 (9). However, it is unlikely that Hal4 and Hal5 act through this mechanism, as the phenotypes of the ptk2 mutant and the hal4 hal5 mutant are considerably different: the ptk2 mutant is tolerant to toxic cation concentrations, whereas the hal4 hal5 mutant is sensitive compared to the wild-type strain, and the ptk2 mutant does not require potassium supplementation for maximal growth on minimal medium. Other reports demonstrate multiple phosphorylation sites for Pma1; therefore, it is likely that multiple kinases regulate this complex ATPase. However, as the function of Trk1 is dependent on the proton gradient generated by Pma1, this is an attractive hypothesis to explain current data regarding Hal4 and Hal5 functions. Experiments are under way to further investigate this possibility.

ACKNOWLEDGMENTS

This work was supported by grants BFU2005-06388-C04-01/BMC from the Spanish Ministry of Education and Science (Madrid), BFU2008-04188-C03-02 and BFU2008-04188-C03-01 from the Ministry of Science and Innovation (Madrid), and Prometeo/2010/038 (Generalitat Valenciana). J. Pérez-Valle was supported by a predoctoral fellowship dedicated to activation of plasma membrane transporters. Mol. Cell. Biol. 26:7654–7661.

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