Constitutive and Hyperresponsive Signaling by Mutant Forms of *Saccharomyces cerevisiae* Amino Acid Sensor Ssy1†

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Received 4 July 2003/Accepted 8 July 2003

Sensing of extracellular amino acids results in transcriptional induction of amino acid permease genes in yeast. Ssy1, a membrane protein resembling amino acid permeases, is required for signaling but is apparently unable to transport amino acids and is thus believed to be a sensor. By using a novel genetic screen in which potassium uptake was made dependent on amino acid signaling, we obtained gain-of-function mutations in *SSY1*. Some alleles confer inducer-independent signaling; others increase the apparent affinity for inducers. The results reveal that amino acid transport is not required for signaling and support the notion that sensing by Ssy1 occurs via its direct interaction with extracellular amino acids.

Free-living microorganisms cope with changes in the extracellular concentrations of nutrients in part by controlling the rates of uptake of these molecules. A key aspect of this control is the ability of microbes to sense extracellular concentrations of certain nutrients. In *Saccharomyces cerevisiae*, distinct mechanisms have evolved to sense the extracellular presence of carbon sources such as glucose (19, 20, 23, 24) and nitrogen sources including amino acids (6, 13, 16, 17).

Key components of the amino acid sensing signal transduction pathway, including Ssy1, Ptr3, Ssy5, and Stp1, were identified in a genetic screen for mutants that failed to take up branched-chain amino acids (16). Because several genes in *S. cerevisiae* encode perases capable of transporting branched-chain amino acids, the screen revealed mutations that reduced the expression of all of them simultaneously, thereby focusing on components of the signaling pathway itself. Thus, *SSY1* was found to be required for normal amino acid-dependent transcriptional induction of several target permease genes, including *BAP2, BAP3, PTR2*, and *TAT1* (6).

Consistent with their regulation of multiple transporter genes, components of the amino acid signaling pathway were independently discovered through other investigations, including screens for mutations that confer resistance to toxic dipeptides (14), amino acid analogues (4), or high concentrations of histidine (17).

Ssy1 is a plasma membrane protein (17) evolutionarily related to the amino acid permeases in *S. cerevisiae* (22) and thus is positioned at the presumed head of the amino acid signal transduction pathway. Ssy1 interacts at the plasma membrane with Ptr3 and Ssy5, forming a complex designated SPS for Ssy1-Ptr3-Ssy5 (8). Presumably, amino acid binding confers a conformational alteration in Ssy1 producing a signal that is transmitted to various target promoters. Proteolytic activation of the transcription factor Stp1 (or Stp2) is a key event in the signal transduction process (2). The discovery that overexpression of the amino terminus of Ssy1 interferes with amino acid signaling (4, 8) revealed the importance of this part of Ssy1.

Although the structure of Ssy1 is related to that of amino acid permeases, available evidence supports the notion that Ssy1 functions as a sensor rather than as a transporter. First, transport of amino acids by bona fide permeases fails to elicit signaling in *ssy1* mutant cells (6, 13, 16, 17). In addition, Ssy1 itself seems to be unable to mediate amino acid transport. Although citrulline elicits the signaling response in *SSY1 gap1* mutant cells (13), such cells are unable to take up enough citrulline as a sole nitrogen source to confer growth (11). Similarly, although *SSY1* is required for maximal uptake of branched-chain amino acids, *SSY1* overexpression fails to restore their uptake in cells in which the four genes known to encode branched-chain amino acid permeases have been deleted (6). Nor does the expression of *SSY1* restore uptake of tryptophan in cells in which the genes for the authentic tryptophan permeases have been deleted (13). However, such tests cannot rule out the possibility that Ssy1 transports small quantities of the signaling molecule, and if any transport occurs, the sensor that actually recognizes the signaling amino acid would not have to be Ssy1 but could be a closely associated protein. Thus, a key question is: does signaling require the uptake and intracellular appearance of an inducer?

To answer this question, we sought to determine if Ssy1 could be converted genetically into a sensor capable of activating the signaling pathway during growth on medium lacking amino acids. By using a novel genetic screen, we identified gain-of-function mutations in *Ssy1* that conferred constitutive signaling. Some alleles also increased the apparent affinity for inducers, supporting the hypothesis that Ssy1 interacts directly with extracellular amino acids to initiate signaling.

### MATERIALS AND METHODS

**Media, yeast strains, and plasmids.** Standard SD, SC, MP, and YPD media were prepared as described by Grauslund et al. (10). In some cases, the media were supplemented with 100 mM potassium as KCl. Such high-potassium media are designated SDHP, SCHP, MPHP, and YPDHP, respectively. YPD medium was prepared by replacement of 100 mM KCl with 1% (w/v) glucose.
supplemented with metsulfuron methyl (YPD + MM) was prepared as described by Jørgensen et al. (16).

The strains used are presented in Table 1. From an initial cross between HY438 (H. Liang and R. F. Gaber, unpublished data) and M4871, a recipient strain suitable for the expression of an SPS-dependent AGP1-KAT1 reporter construct was generated by backcrossing appropriate strain YLR011C trk1a trk2a mating progeny with the HY438 parent. The resulting strain, RGY4990, harbors a wild-type GAP1 gene. All strains containing the trkΔ1 trkΔ2 mutations were derived from this strain. Strain M5003, containing the pSPG1-KAT1 reporter construct integrated at the TRP1 locus, was constructed by transformation of strain RGY4990 with XbaI-digested plasmid pOK6 (described below). M5025, containing the pSPG1-KAT1 reporter integrated at the AGP1 locus, was constructed by transformation of strain RGY4990 with BglII-digested plasmid pOK6K. All strains harboring the wild-type TRK1 and TRK2 genes are congenic with S288c.

Strains M5030 and M5031, harboring the Escherichia coli lacZ gene integrated at the AGP1 locus, were constructed from M4054 (gap1 ura3) and M4871 (ssy1 gal1 ura3), respectively. By using plasmid pUG6lacZ (5) as the template, a 4.6-kb fragment containing the downstream of the GAP1 gene, the KanMX4 cassette system (12). Successful integration of the disruption cassettes was verified by diagnostic PCR, and the inserted element was evicted upon transformation of strain RGY4990 to Trp$^+$.

TABLE 1. Yeast strains used in this study

| Strain       | Genotype | Reference |
|--------------|----------|-----------|
| M4054        | MATa suc2 trp1 met5::HIS3 trk2::HIS3 trp1 his3 | This study |
| M4871        | MATa suc2 trp1 met5::HIS3 trk2::HIS3 trp1 his3 | This study |
| M4873        | MATa suc2 trp1 met5::HIS3 trk2::HIS3 trp1 his3 | This study |
| M5030        | gap1::agp1-lacZ::kanMX4 in M4054 | This study |
| M5031        | ssy1::agp1-lacZ::kanMX4 in M4871 | This study |
| RGY4542 trp1 | MATa trk1::HIS3 trk2::HIS3 trp1 his3 | This study |
| RGY4990 TRP1 | MATa trk1::HIS3 trk2::HIS3 trp1 his3 | This study |
| RGY5000      | RGY4990 with pAGP1-KAT1 (TRP1) and pRS316 (URA3) | This study |
| RGY5001      | RGY4990 with pAGP1-KAT1 (TRP1) and pRS316 (URA3) | This study |
| RGY5003      | RGY4990 with pAGP1-KAT1 (TRP1) and pSSY1 (SSY1) | This study |
| RGY5004      | RGY4990 with pAGP1-KAT1 (TRP1) and pSSY1 (SSY1) | This study |
| RGY5012      | RGY4990 with pRS314 (TRP1) | This study |
| M4941        | RGY4990 with pAGP1-KAT1 (TRP1) (1) | This study |
| M5003 trp1   | pSGP1-KAT1-TRP1 in RGY4990 | This study |
| M5025        | AGP1-pSPG1-KAT1-TRP1 in RGY4990 | This study |

and the corresponding fragment from pSSY1-101 was inserted into pRS306 to produce pOK14.

**SSY1 mutagenesis strategies.** To generate random mutations in SSY1 for the mutant screen, a 2.76-kb fragment of DNA encompassing the SSY1 ORF was subcloned into the Mutagenic PCR vector pRS304 [SSY1-101] and the corresponding fragment from pSSY1-101 was inserted into pRS306 to produce pOK14.

**Screening of the SSY1::HIS3 degenerate library.** Strain M5025, carrying the pSPG1-KAT1 reporter gene integrated at the AGP1 locus, was transformed with plasmid DNA from the SSY1::HIS3 degenerate library with selection on SD containing 100 mM KCi. Randomly picked colonies were suspended in water and diluted to an optical density at 600 nm (OD$_{600}$) of 0.1, and 4-μl droplets of each were applied to SD medium and tested for growth. Colonies that exhibited stronger growth than the wild-type control were selected for rescue of the resident library plasmid into E. coli. All rescued plasmids were reintroduced into strains M5025 and M4941 to confirm the phenotypes.

Sequence analysis was performed with a Megabace1000 (Amersham Life Science). Alleles SSY1-108 through SSY1-120 from the SSY1::HIS3 degenerate library were identified by determination of the sequence of the region immediately surrounding codon 382 of the SSY1 ORF.

**β-Galactosidase assays.** For β-galactosidase assays, a modified protocol based on those of Didion et al. (7) and Ausubel et al. (3) was used. Typically, 1.2 ml of a saturated overnight culture was used to inoculate 60 ml of fresh medium. Fifty milliliters of culture at an OD$_{600}$ between 0.5 and 1.0 was chilled, and cells were harvested for the assay. For the affinity experiment (see Fig. 5), however, cultures were inoculated with exponential-phase precultures and into minimal medium containing L-citrulline at appropriate concentrations; the cells were allowed to grow for an additional 5 h before being harvested at an OD$_{600}$ between 0.5 and 0.7; the data for this experiment were fitted by nonlinear regression with the Sigma2000 software (SPSS Inc., Chicago, IL.).

**RESULTS**

Development of a novel reporter system for amino acid sensing.** To facilitate isolation of mutations in SSY1 that alter amino acid sensing, we developed a system in which cell growth is coupled to amino acid signaling but independent of the uptake of amino acids. This allowed us to exploit amino acids solely as signaling molecules independently of their effects as nutrients. We constructed a strain in which cell growth depended on induction of KAT1, a heterologous potassium channel encoding the Arabidopsis thaliana (1). KAT1 was placed under the control of the amino acid-inducible AGP1 promoter and introduced into cells in which the endogenous potassium transporter genes TRK1 and TRK2 had been deleted. In the trkΔ1 trkΔ2 background, growth is nearly abolished unless the
growth medium is supplemented with high concentrations of potassium (18). Expression of the Kat1 channel restores potassium uptake, permitting growth on normal yeast medium (Fig. 1A). The AGP1 promoter was chosen to drive KAT1 expression because the basal level of AGP1 transcription is low but becomes high in the presence of certain amino acids (13).

Introduction of pAGP1·KAT1 into trk1Δ trk2Δ recipient cells conferred upon them the ability to grow on restrictive (non-potassium-supplemented) media such as SD and MP, but in a manner strictly dependent on the presence of inducers, such as leucine (Fig. 1A). To confirm that expression of AGP1::KAT1 relied on Ssy1-dependent signaling, pAGP1·KAT1 was introduced into ssy1Δ trk1Δ trk2Δ cells. Although the ssy1Δ trk1Δ trk2Δ pAGP1·KAT1 cells grew normally on medium supplemented with 100 mM KCl (SDHP), they were unable to grow on the restrictive medium either in the presence or in the absence of amino acids (Fig. 1A).

The coupling of amino acid signaling to the uptake of potassium resulted in a growth assay that could be used to assess the relative efficacies of various amino acids with regard to their ability to induce the AGP1::KAT1 reporter. In these tests, strain RG5004, which expresses Ssy1 and AGP1::KAT1 from plasmids, was tested for the ability to grow on SD supplemented with different concentrations of various amino acids as inducers. The results of this experiment generated a profile of the relative potencies of the amino acids tested (Fig. 1B). Leucine was most potent, conferring growth at concentrations of less than 5 μM. In contrast, even at concentrations more than 1,000-fold higher, glutamate and aspartate were only marginally effective. Still others, including glutamine, lysine, glycine, proline, arginine, and cysteine, were completely ineffective. Although the potency profile shown in Fig. 1B differs somewhat from a profile reported previously (13) in which the abilities of different amino acids (at 5 mM) to induce an AGP1::lacZ reporter were compared, AGP1::KAT1-dependent growth proved to be an exquisitely sensitive measure of the relative potencies of different inducers and thus served as a convenient assay for the specificity of Ssy1-dependent signaling.

**Identification of a constitutive signaling mutation in Ssy1.**

To identify sites within Ssy1 that may be directly involved with the amino acid sensing or signaling mechanism, the Ssy1 ORF was amplified by PCR under mutagenic conditions and introduced into an ssy1Δ trk1Δ trk2Δ pAGP1·KAT1 recipient by cotransformation with a linearized plasmid containing regions of sequence overlap with the amplified Ssy1 ORF. Transformants were selected on SC medium supplemented with a high potassium concentration but lacking uracil (SCHP-URA). Colonies that developed were tested for their Ssy phenotype by replica plating to non-potassium-supplemented medium either with (SD plus Leu) or without (SD) leucine as an inducer. Ssy transformants were detected by their inability to grow on SD plus Leu. Under the most mutagenic conditions, approximately
25% of the Ura+ transformants were also Ssy−. In contrast, less than 1% of transformants exhibited the Ssy− phenotype in a control experiment in which a high-fidelity polymerase was used. A broad range of Ssy− phenotype severity was observed among the transformants.

Among the approximately 10,000 Ura+ transformants screened, a single colony was identified that grew on both SD plus Leu and SD, suggesting that AGP1::KAT1 expression was no longer dependent on the presence of an inducer. Plasmid pSSY1-101, isolated from this transformant, was reintroduced into the ssy1Δ trk1ΔΔ trk2A pAGP1-KAT1 recipient and found to confer the ability to grow on SD in the absence of amino acids (Fig. 2A).

Suppression of the trk1Δ trk2Δ-encoded phenotype can occur by mutations in transporter genes that produce increased membrane permeability to potassium (21, 26). Thus, to determine whether growth on SD was a direct effect of the mutant Ssy1 protein expressed from pSSY1-101, e.g., because of potassium leakage through the mutant Ssy1 protein, or if it resulted in increased expression of the AGP1::KAT1 reporter, pSSY1-101 was introduced into a trk1Δ trk2A mutant host lacking the pAGP1-KAT1 reporter. All of the transformants grew on SDH but failed to grow on SD, even in the presence of an inducer (data not shown). Thus, pSSY1-101 conferred the ability of ssy1Δ trk1Δ trk2A mutant cells to grow on SD in a manner independent of an inducer but dependent on the pAGP1-KAT1 reporter.

Sequence analysis of pSSY1-101 revealed two mutations compared to the published SSY1 ORF sequence. A transition from C to T at position 704 resulted in a codon change from alanine to threonine (A237T), and a C-to-T transversion at position 1145 resulted in a change from threonine to lysine (T382K). To determine which mutation(s) was responsible for the mutant phenotype, the two single mutations and the double mutant were constructed by site-directed mutagenesis of an otherwise wild-type SSY1 gene expressed from plasmid pSSY1. Plasmids with the SSY1-102 (Ssy1T382K), SSY1-103 (Ssy1A237T), SSY1-104 (Ssy1A237T, T382K), or wild-type alleles were introduced into a ssy1Δ trk1Δ trk2A mutant recipient in which the pAGP1-KAT1 reporter was integrated at the AGP1 locus, and the transformants were tested for the ability to grow on SD (Fig. 2A). Both pSSY1-102 and pSSY1-104 conferred growth on SD lacking an inducer, indicating that the T382K mutation is sufficient to confer the mutant phenotype. The A237T mutant had no discernible phenotype by itself and did not strengthen the phenotype conferred by the T382K mutation.

Suppression of the trk1Δ trk2Δ-encoded phenotype in a manner dependent on the AGP1::KAT1 reporter suggested that SSY1-102 conferred increased expression of the amino acid-responsive AGP1 promoter. To further test this and to quantify their effect(s) on signaling, plasmids encoding the SSY1-102, SSY1-103, SSY1-104, and wild-type SSY1 alleles were introduced into ssy1Δ mutant cells harboring an integrated AGP1::lacZ reporter construct and β-galactosidase activity was measured in the absence or presence of leucine (Fig. 2B). Inducer-independent expression of the integrated reporter in wild-type SSY1 cells was low, but expression increased approximately fivefold upon induction with leucine. As expected from the growth tests, SSY1-103 conferred no discernible effect in the absence of amino acids but responded normally to an inducer. In contrast, cells harboring the SSY1-102 allele produced maximal levels of β-galactosidase activity even in the absence of an inducer, revealing that the T382K mutation confers constitutive activity of the signaling pathway.

Constitutive induction by SSY1-102 was not limited to the AGP1 promoter. Quantification of β-galactosidase produced in cells expressing the amino acid-responsive BAP2::lacZ reporter revealed that SSY1-102 conferred inducer-independent expression from the BAP2 promoter as well (data not shown). Ssy1T382K confers a dominant gain of function. As a test for dominance, wild-type SSY1 cells expressing a second wild-type or mutant copy of SSY1 integrated at the UR3 locus were generated with plasmids pOK13 and pOK14, respectively, and both inducer-dependent and inducer-independent signaling was quantified with the AGP1::lacZ reporter. Compared to the cells harboring the single SSY1 gene, cells expressing an additional wild-type gene produced a modest increase in both
inducer-independent (from 0.006 to 0.012 Miller units) and inducer-dependent (from 0.025 to 0.065 Miller units) β-galactosidase activities. In contrast, when the second copy of SSY1 contained the T382K mutation, full activity of the AGP1::lacZ reporter (0.12 Miller units) occurred even in the absence of an inducer (in the presence of an inducer, 0.11 Miller units). Thus, T382K confers a dominant gain of function with regard to signal transduction.

**Constitutive signaling by Ssy1**

Amino acid signaling requires the participation of other proteins, including Ptr3 and Ssy5, both of which exist in a complex with Ssy1 (8). Two tests were performed to determine if the requirement for Ptr3 or Ssy5 was bypassed by the constitutive SSY1-102 mutation. In one, SSY1-102 cells were tested for the ability to grow on YPD + MM (15). Branched-chain amino acid biosynthesis is inhibited by MM, so growth depends on SPS induction of BAP2 and other permeases. Wild-type SSY1 and mutant SSY1-102 cells grew normally on YPD + MM, but growth was impaired upon deletion of either PTR3 or SSY5 (Fig. 3A).

In a second test, induction of the dipeptide transporter gene, PTR2, was assessed by sensitivity to the toxic dipeptide L-leucyl L-ethionine. Consistent with its constitutive effect on signaling, cells expressing Ssy1**382K** exhibited somewhat greater sensitivity to L-leucyl L-ethionine than did wild-type cells on SD medium lacking an inducer, whereas both were equally sensitive in the presence of leucine (Fig. 3B). In contrast, both SSY1 and SSY1-102 cells harboring a ptr3Δ or ssy5Δ mutation were fully resistant. Thus, the SSY1-102 allele is unable to induce PTR2 in cells with either Ptr3 or Ssy5 deleted. We conclude from these experiments that constitutive signaling via Ssy1**382K** does not bypass the requirement for the other components of the SPS complex.

Ssy1**382K**, Ssy1**382R**, Ssy1**382H**, and Ssy1**382L** confer amino acid-independent signaling. On the basis of the topology of the related permease Gap1p (9) and the online prediction facility www.cbs.dtu.dk/services/TMHMM, the amino acid altered by SSY1-102 is predicted to lie on the extracellular face of the plasma membrane. Since SSY1-102 resulted in constitutive signaling, we reasoned that other mutations at this site might produce interesting phenotypes. Additional codons at position 382 were therefore generated and assessed for their effects on signaling. Most of these mutations were obtained by screening of two independently generated libraries of mutations at codon 382 made by degenerate primer-mediated PCR. Each Ssy1 allele was introduced into an sylΔ trk1Δ trk2Δ mutant recipient in which the AGP1::KAT1 reporter construct had been integrated at the AGP1 locus, and the transformants were tested for the ability to grow on SD in the absence of an inducer.

A screen of 90 yeast transformants from the library pools identified 17 that were capable of growth on SD without an inducer. Plasmids from these transformants were retested for the ability to confer the constitutive phenotype, and the SSY1 alleles were sequenced. The gain-of-function alleles that were found encode Lys, Arg, His, or Leu at codon 382. Sequence analysis of 96 randomly selected library plasmids that were recovered in *E. coli* identified 11 additional SSY1 alleles, encoding Ala, Cys, Gly, Ile, Met, Phe, Pro, Ser, Trp, Tyr, or Val at codon 382. A mutant allele containing an Asp codon at this site was independently generated by site-directed mutagenesis. Plasmids encoding each of these 12 new SSY1 alleles were introduced into an sylΔ trk1Δ trk2Δ mutant recipient in which the AGP1::KAT1 reporter construct had been integrated at the AGP1 locus, and the transformants were tested for the ability to confer growth on non-potassium-supplemented medium in the presence or absence of an inducer. On SD, the growth of these transformants was not greater than the growth of wild-type SSY1 cells. On the other hand, all of the transformants grew well on SD plus leucine, indicating that these mutations did not impair the ability of Ssy1 to initiate signaling in response to amino acid (not shown).

**Ssy1**382H** and Ssy1**382L** respond to subthreshold concentrations of inducers. Growth tests in which trk1Δ trk2Δ pAGP1::KAT1 cells expressing the gain-of-function SSY1 alleles were compared revealed two phenotypic classes. Cells expressing either the T382K or the T382R allele grew strongly even in the absence of an inducer. In fact, growth of either mutant on SD in the absence of an inducer was nearly indistinguishable from...
Residue at position 382 in Ssy1

$$\begin{array}{cccc}
\text{T (WT)} & K & R & H & \text{ssy1Δ} \\
\hline
\text{No amino acids} & & & & \\
1 \mu \text{M Leu} & & & & \\
10 \mu \text{M Ile} & & & & \\
10 \mu \text{M Phe} & & & & \\
0.5 \text{mM Cit} & & & & \\
0.5 \text{mM Ser} & & & & \\
2.5 \text{mM Glu} & & & & \\
\text{Complete} & & & & \\
100 \text{mM KCl} & & & & \\
\end{array}$$

FIG. 4. Constitutive and supersensitive phenotypes of SSY1 mutants affected at position 382. Droplets of strain M4941 (ssy1 trk1 trk2 [PAGP1-KAT1]) transformed with the SSY1 alleles on a centromere-based vector were spotted onto minimal media supplemented as indicated. Plates were incubated at 30°C for 4 days. Cit, L-citrulline; WT, wild type.

its growth in the presence of amino acids (Fig. 4), suggesting that Ssy1$^{382H}$, like Ssy1$^{382K}$, is fully constitutive. In contrast, cells expressing the T382H or T382L mutant form exhibited growth that was only marginally stronger than that conferred by wild-type SSY1 cells in the absence of amino acids. Both of these mutant forms retained the ability to respond to the presence of amino acids (Fig. 4). Interestingly, however, compared to cells expressing the wild-type sensor, Ssy1$^{382H}$- and Ssy1$^{382L}$-expressing cells were capable of responding to concentrations of amino acids that are normally too low to produce a strong signaling response by this assay (compare relevant inducer concentrations in Fig. 4 with those in Fig. 1B). This suggested the possibility that the T382H and T382L mutations increased the sensitivity of Ssy1 to inducers.

To determine if the increased sensitivity to an inducer was accompanied by altered selectivity, the minimum concentrations of various inducers required to elicit increased growth on SD were determined (Fig. 4). The results of these tests indicated that the relative efficacies of various amino acids did not differ from that established with wild-type SSY1 cells (Fig. 1B). Thus, while the Ssy1$^{382H}$ and Ssy1$^{382L}$ sensors exhibited an increased ability to sense the presence of amino acids, their specificity seemed to be unaltered.

Ssy1$^{382H}$ exhibits greater apparent affinity for an inducer.

To better understand the basis of this phenotype, the ability of cells expressing either the wild-type or Ssy1$^{382H}$ sensor to respond to an inducer was quantified by measuring $\beta$-galactosidase produced from AGP1 lacZ during growth in the presence of different concentrations of citrulline. Citrulline was chosen because it is not taken up by cells with a deletion of the general amino acid permease, Gap1 (11). The results of these experiments were striking (Fig. 5). First, at the lower inducer concentrations (basal level), signaling was increased in the T382H mutant compared with that in the wild type. Second, citrulline was capable of increasing signaling to levels equivalent to that of the wild type at the higher concentrations, confirming that the mutant Ssy1$^{382H}$ sensor did indeed retain the ability to fully respond to an inducer. Third, Ssy1$^{382H}$-expressing cells responded to significantly lower levels of an inducer than did wild-type cells. Whereas cells expressing wild-type Ssy1 exhibited an apparent $K_d$ of approximately 800 $\mu$M citrulline, cells expressing Ssy1$^{382H}$ exhibited an apparent $K_d$ of only ~80 $\mu$M citrulline. Thus, the inducer concentration needed to induce a strong signaling response is reduced 10-fold by the T382H mutation. Because Ssy1$^{382H}$ exhibited a general increase in sensitivity to different amino acids, we favor a model in which the T382H mutation results in a shift in equilibrium between the nonsignaling and signaling-competent conformations of Ssy1. Taken together with our results showing that mutations at T382 confer constitutive signaling, the data strongly support the notion that Ssy1 functions as a receptor rather than a transporter and that Ssy1 interacts directly with the extracellular signaling molecules to initiate signaling.

DISCUSSION

The goal of this investigation was to elucidate the role of Ssy1 in amino acid signaling by selecting for mutations in SSY1
that confer inducer-independent signaling. We circumvented the potential difficulties of a genetic screen that might be sensitive to, or dependent on, transport of the signaling molecules themselves by use of the AGP1::KAT1 reporter. Potassium uptake, and thus growth, was made dependent on amino acid signaling but independent of the uptake of amino acids by the various permeases. By knocking out SSY1 in this background and then introducing PCR-mutagenized SSY1, mutations were focused on the amino acid sensor itself. Because both loss-of-function and gain-of-function mutations in SSY1 were easily distinguished, the AGP1::KAT1 reporter system should prove useful for obtaining similar mutations in other components of the SPS system and perhaps in other signaling systems as well.

SSY1 has been hypothesized to function at the head of the amino acid signaling pathway largely because of the fact that it is an integral plasma membrane protein that resembles amino acid permeases and is required for signaling, yet it is apparently unable to transport amino acids. A similar situation has been described for the glucose sensors Snf3 and Rgt2. These are proteins that resemble glucose transporters and are required for glucose sensing yet apparently do not transport glucose (20, 24). Indeed, constitutive inducer-independent signaling is an integral plasma membrane protein that resembles amino acid permeases and is required for signaling, yet it is apparently unable to transport amino acids. A similar situation has been described for the glucose sensors Snf3 and Rgt2 (23). In addition, a mutation in SSY1 has been reported (4) that abolishes the response to a high concentration of phenylalanine while the response to a high concentration of leucine is maintained; this mutation could be affected in the relative potency of amino acids, as suggested by the authors, or it could be generally hyporesponsive. Amino acid and glucose sensors may detect the extracellular presence of their respective nutrients directly and initiate signal transduction through interactions with other components of the signaling complex. However, the inability to detect amino acid or glucose uptake does not rule out the possibility that Ssy1 or Snf3/Rgt2 can indeed transport small quantities of the nutrients. If this is so, signaling might actually rely on the intracellular detection of these signaling molecules by other proteins. In the case of amino acid signaling, Ptr3 and Ssy5 would be excellent candidates as they have been shown to be intimately associated with Ssy1 and are responsible for mediating downstream effects (2, 8). Thus, a key aspect that we wished to resolve was whether it is the extracellular or intracellular presence of amino acids that is required for signaling by Ssy1.

The discovery that mutation of SSY1 codon 382 from threonine to lysine (or arginine) results in constitutive signaling in the absence of extracellular amino acids reveals that Ssy1 can initiate signaling in the absence of an intracellular, i.e., transported, inducer. These results effectively preclude a model in which any other downstream factor is required to recognize an internalized inducer and are consistent with the notion that Ssy1 functions as the protein that directly senses an inducer. Although the argument could still be made that some as yet unidentified factor is the actual amino acid sensor and that constitutive forms of Ssy1 simply bypass the need for this putative factor, SSY1 mutations such as T382H, which confer a 10-fold increase in the apparent affinity for an inducer, argue strongly against this. The simplest interpretation of the occurrence of hyperresponsive mutations such as T382H and T382L is that Ssy1 interacts directly with extracellular amino acids as part of the signaling mechanism. Thus, some alleles of SSY1 can confer constitutive signaling while others increase its ligand sensitivity. Taken together, our data support a model in which Ssy1 functions as a receptor, initiating signaling through direct interaction with extracellular amino acids. Constitutive signaling requires Ptr3 and Ssy5, indicating that Ssy1T382K is unable to induce AGP1 in the absence of the full SPS complex. Thus, as far as we can tell, Ssy1T382K confers normal, albeit constitutive activation of the SPS complex and the appropriate downstream transcription factors.

The sequence conservation between Ssy1 and other members of the large family of amino acid permeases in S. cerevisiae indicates that Ssy1 may have evolved from an ancestral transporter. If this is so, signal transduction by this sensor may rely on structural and functional aspects intrinsic to bona fide transporters. This is likely to include the abilities to bind amino acids and undergo subsequent conformational changes that are then translated into productive interactions with downstream factors such as Ptr3 and Ssy5. As mentioned, this might also be accompanied by undetected amino acid transport. The evolutionary recruitment of sensors from transporters appears to have occurred at least twice in fungi, but such sensors have not been described in other eukaryotes. Nutrient availability for free-living fungi can change drastically, over many orders of magnitude, in extracellular concentration. Thus, microorganisms such as S. cerevisiae appear to have adapted to such wide disparity in nutrient availability through the evolutionary accruement of at least 15 different amino acid permease genes and at least 17 sugar transporters. Within each family, individual transporters exhibit substrate specificities and affinities that overlap those of other members, suggesting that different transporters are best able to cope with specific conditions of nutrient availability. In the case of the HXT family, this has been largely borne out with regard to the differential expression of high-, moderate-, and low-affinity transporters under suitable conditions of glucose availability (23). The fact that constitutive mutations have now been identified in both glucose and amino acid sensors has helped to place constraints on both the nature of the intracellular signals and models of how these sensors might function. Gain-of-function mutations in downstream factors can also be very informative, as illustrated by a constitutively active truncated version of Stp1 (2).

ACKNOWLEDGMENTS

We thank Lisbeth F. Petersen for skillful technical assistance and Eckhard Boles and Peter S. Nielsen for generously supplying plasmids and strains.

The experiments described in this report were initiated in the laboratory of M.C.K.-B. by R.F.G. during a sabbatical leave granted by Northwestern University. This work was supported by a grant from the National Science Foundation (R.F.G.).

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