Constitutive Signal Transduction by Mutant Ssy5p and Ptr3p Components of the SPS Amino Acid Sensor System in Saccharomyces cerevisiae

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Amino acids in the environment of Saccharomyces cerevisiae can transcriptionally activate a third of the amino acid permease genes through a signal that originates from the interaction between the extracellular amino acids and an integral plasma membrane protein, Ssy1p. Two plasma membrane-associated proteins, Ptr3p and Ssy5p, participate in the sensing, which results in cleavage of the transcription factors Stplp and Stp2p, removing 10 kDa of the N terminus of each of them. This confers the transcription factors with the ability to gain access to the nucleus and activate transcription of amino acid permease genes. To extend our understanding of the role of Ptr3p and Ssy5p in this amino acid sensing process, we have isolated constitutive gain-of-function mutants in these two components by using a genetic screening in which potassium uptake is made dependent on amino acid signaling. Mutants which exhibit inducer-independent processing of Stplp and activation of the amino acid permease gene AGP1 were obtained. For each component of the SPS complex, constitutive signaling by a mutant allele depended on the presence of wild-type alleles of the other two components. Despite the signaling in the absence of inducer, the processing of Stplp was more complete in the presence of inducer. Dose response assays showed that the median effective concentration for Stplp processing in the mutant cells was decreased; i.e., a lower inducer concentration is needed for signaling in the mutant cells. These results suggest that the three sensor components interact intimately in a complex rather than in separate reactions and support the notion that the three components function as a complex.

Differences in nutrient availability from one environment to another and changes in nutrient abundance that occur as a consequence of growth present fundamental challenges to free-living microbes. Prokaryotic and eukaryotic microorganisms have evolved nutrient-sensing systems that detect changes in extracellular nutrient concentrations and initiate signal transduction responses that alter the expression of relevant nutrient transporter genes (19). In the yeast Saccharomyces cerevisiae, for example, distinct sensing systems have been described for phosphate (15) and for glucose and amino acids (reviewed in reference 8).

In the sensing pathway described here, S. cerevisiae cells respond to extracellular amino acids by transcriptional induction of some of the amino acid transporters, such as Agp1p. Genetic studies have played a key role in identifying and dissecting key components of the pathway. Due to overlapping specificities of amino acid transporters, a mutant screening designed to identify mutations conferring resistance to inhibitors of branched-chain amino acids was successful in identifying the upstream factors that mediate amino acid signaling in this organism. Thus, SSY1, SSY5, and PTR3 were found to be required for the induction of downstream amino acid permease genes by amino acids (6, 10, 21, 23, 24) and encode the components of the so-called (12) SPS (Ssy1-Ptr3-Ssy5) sensor. Ssy1p seems to be the actual amino acid-sensing molecule.

Although it is a membrane protein resembling an amino acid permease, Ssy1p seems not to transport amino acids, yet it is required for amino acid-stimulated transcription of the inducible downstream permease genes. The discovery of mutations in SSY1 that confer constitutive signaling in the absence of extracellular amino acids (14) revealed that, even if Ssy1p might normally be able to transport small amounts of amino acids, transported amino acids seem not to constitute the intracellular signal that mediates downstream induction of the responsive permease genes.

SSY1, PTR3, and SSY5 are believed to be part of a membrane-associated signaling complex (12, 24). Consistent with this idea, both SSY5 and PTR3 remain essential for amino acid signaling even in cells expressing the SSY1 signaling constitutive alleles (14). A key function of the SPS sensor is the activation of the transcription factors Stplp (22) and Stp2p, which bind to the promoters (27) and induce increased transcription of the responsive amino acid permease genes. Andréasson and Ljungdahl (3, 4) found that activation of these transcription factors involves the proteolytic removal of an inhibitory amino terminal region. This processing normally requires all three components of SPS, but Abdel-Sater et al. (1) found that overexpression of an N-terminally tagged version of Ssy5p causes constitutive processing of Stplp and suggested that Ssy5p is a serine protease that carries out the cleavage. Full-length Stplp and Stp2p are retained in the cytoplasm in a manner dependent on the amino terminus of these transcription factors and on the function of the ASI1 through ASI3 genes (13). Removal of the amino terminus of Stplp or Stp2p
by SPS allows its transport to the nucleus and the consequent transcriptional response (3).

Although Ssy5p has been proposed to be the protease that cleaves Stp1p and Stp2p, other details of the molecular function of SPS remain largely unknown. For example, how does Ssy1p recognize amino acids? Is transport of amino acid by Ssy1p a part of the conformational change that activates the SPS? Do Ssy1p, Ssy5p, and Ptr3p actually form a complex? What are the functional dependency relationships between members of the SPS? What specific roles does each member play?

We previously devised a genetic screening that allowed the identification of mutations in SPSY that result in constitutive signaling (14). The observation that Ssy5p and Ptr3p remain essential for signaling in these cells is consistent with a model in which all three proteins function interdependently or with one in which Ssy5p and Ptr3p function downstream of Ssy1p through separate interactions. The isolation of mutants containing constitutively signaling forms of Ssy5p and/or Ptr3p could help to distinguish between these alternatives by testing whether they are functionally dependent on Ssy1p. Establishing these relationships would also allow us to place additional constraints on a functional model of the SPS sensor and perhaps provide clues to its structural constraints. In this report, we describe the results of a genetic screening designed to identify constitutively signaling alleles of SSY5 and PTR3. The isolated alleles result in amino acid-independent processing of Stp1p and thus account for the constitutive expression of SPS-responsive permease genes. We have tested the dependency of these gain-of-function mutations on each of the components of the SPS sensor, and we discuss the significance of the results with regard to the mechanism of SPS signaling.

**MATERIALS AND METHODS**

**Media.** Standard glucose-based media synthetic dextrose (SD; synthetic minimal), synthetic complete (SC), and yeast-peptone-dextrose complex media were prepared as described previously (31). However, amino acid concentrations in SC medium were as specified previously elsewhere (17). Selections for kanR-marked strains were made on solid yeast-peptone-dextrose complex medium supplemented with 300 μg/liter G418 (Sigma).

**Yeast strains.** Strains M5077 (syr5Δ) and M5078 (ptr3Δ) were created in the genetic background of strain M4955, which was designed (14) to grow on SD medium in an amino acid signaling-dependent way (Table 1). They were constructed using the loxP-kanMX-loxP gene disruption technology with plasmid pUG6 (18) as follows. For deletion of PTR3, the primer pair PTR3pUG6.frm (5′-AACT GAT TAG TAC CTT ATC AGC CCT GAA AAC AGG CAT GGT CTA AAA CCG CTA ATG GAT TGC GGG CGG GTG C-3′) and PTR3pUG6.rev (5′-GAT GAT TGC TTC TCT TAA TAC TCT TAT AAC AGG CAT GCC TAC TCT GCA GGT GGA GAT TGG TTT TAG CAG ACG CGA TAC-3′) was used for PCR amplification of the deletion cassette from plasmid pUG6. For deletion of SSSY, the primer pair Ssy5pUG6.frm (5′-GTA CAG AAA ACG TAA ATA TAC AAT GGG TTC GTA TCA TGA CTG TTT GGG-3′) and Ssy5pUG6.rev (5′-5′-GAT GAT TGG TTA AAT AAC TTC AAA GAA GCT GAT CCA TCA TCT AGT TGT GGA TCG GAT AGG CCA CTA GAG GTG-3′) were used for PCR. Amplified-deletion cassettes, and selection on G418 yielded strains M5078 (syr5Δ) and M5078 (ptr3Δ).

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

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| M4955  | MATα trk1::HIS3 trk2::HIS3 ura3-tp1 pAGP1-KAT1 | 14 |
| M5077  | M4958 syr5::kanMX | This work |
| M5078  | M4959 ptr3::kanMX | This work |
| M4054  | MATα SUC2 mal gal2 CUP1 ura3 gap1Δ | 17 |
| M4723  | MATα SUC2 mal gal2 CUP1 ura3 gap1Δ ptr3Δ | P. S. Nielsen |
| M4724  | MATα SUC2 mal gal2 CUP1 ura3 gap1Δ ssy5Δ | P. S. Nielsen |
| M4871  | MATα SUC2 mal gal2 CUP1 ura3 gap1Δ ssy5ΔAAG-3′ | This work |
| M5359  | M4054 AGP1::PAGP1- lacZ | This work |
| M5360  | M4723 AGP1::PAGP1- lacZ | This work |
| M5361  | M4729 AGP1::PAGP1- lacZ | This work |
| M5380  | M4871 AGP1::PAGP1- lacZ | This work |
| M5443  | M4723 STP1::ZZ-kanMX | This work |
| M5444  | M4724 STP1::ZZ-kanMX | This work |
| M5445  | M4871 STP1::ZZ-kanMX | This work |
| M5447  | M4054 STP1::ZZ-kanMX | This work |

**Plasmids.** pPEP10 was constructed as follows. For deletion of PTR3, the primer pair PTR3pUG6.frm (5′-AGA CAC AAC AAT ATT ATT CGG CTA GAG ACC TCT CTA CTA CGT GAT TGG TTT TAG CAG ACG CGA TAC-3′) and PTR3pUG6.rev (5′-5′-GAT GAT TGG TTA AAT AAC TTC AAA GAA GCT GAT CCA TCA TCT AGT TGT GGA TCG GAT AGG CCA CTA GAG GTG-3′) was used for PCR to generate deletion cassettes. Strains with deletion cassettes were transformed into the resulting PCR product, selecting on G418, and yielding strains M5443, M5444, M5445, and M5447, respectively. Correct integration was verified by PCR using primers Ssy5pUG6.frm and Ssy5pUG6.rev, which yield a 387-bp deletion product. Strains M5359, M5360, M5361, and M5380 were constructed by transformation of M4054, M4723, M4724, and M4871, respectively, with plasmid pPEP10 linearized with BmgBI, which cuts the AGP1 promoter 387 bp upstream from the start codon. Integrants were selected on G418.

Stp1p was tagged (29) at the C terminus in the genome with an immunoglobulin G-binding domain (Z) of protein A (36). A PCR fragment encoding a doublet of domain Z, a kanMX cassette, and 50-bp overlaps to STP1, was amplified from plasmid pFZ (36) as follows. Strains M4054, M4723, M4724, and M4871, respectively, with plasmid pPEP10 linearized with BmgBI, which cuts the promoter 387 bp upstream from the start codon. Integrants were selected on G418, and yielding strains M5443, M5444, M5445, and M5447, respectively. Correct integration was verified by PCR using primers Ssy5pUG6.frm and Ssy5pUG6.rev, which yield a 387-bp deletion product. Strains M5359, M5360, M5361, and M5380 were constructed by transformation of M4054, M4723, M4724, and M4871, respectively, with plasmid pPEP10 linearized with BmgBI, which cuts the AGP1 promoter 387 bp upstream from the start codon. Integrants were selected on G418.

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region, whereas the PTR3 terminator PCR fragment contains 114 bp of the coding region and 287 bp of the terminator region. The fragments were digested with EcoRI-BamHI and XbaI-BamHI, respectively, and inserted into pRS316 digested with EcoRI-XbaI. Plasmid pPEP11 can be linearized with BamHI, which cuts between the promoter and terminator, allowing recombination with a PCR-mutagenized fragment of the entire PTR3 ORF.

The AGP1-lacZ reporting plasmid pEP15 was created by inserting a 995-bp AGP1 promoter fragment, made by PCR using the primers AGP1-3′ (5′-GTC GAC GAG CCC TCT ACC ACA CCA TGG 3′) and AGP1-2′ (5′-TGG GAC AAG CTA CTT TGT TCT ATA TTA TTT GAC CAG 3′), into the SalI-EcoRI sites of plasmid pYC-Z130 (28). The 518-bp FseI CEN-ARS fragment was removed from pEP15 to form the integrating AGP1-lacZ-reporting plasmid pPEP17.

Plasmids pSSY5 and pPTR3 (23; also referred to as pMB5 and pMB2, respectively [5]) contain a 3-kb HindIII-SacI fragment with SSY5 and a 5-kb XbaI and PstI fragment with PTR3, respectively, inserted into pRS316 (CEN/URA3) (32).

Mutagenesis of the SSY5 and PTR3 coding regions. Libraries of PCR fragments of SSY5 and PTR3 with randomly introduced mutations were constructed with Mutazyme polymerase (Stratagene) and primers SSY5-1′ (5′-CTA CGT TGG TAA ACT CGA TAT ACC G 3′) and SSY5-2′ (5′-CCA TCT AGT TGT GGA TCA ATG TCC 3′) and primers PTR3-1′ (5′-GGT AGC AAA TAC ACA ACT GAC 3′) and PTR3-3′ (5′-TGT ATA CTT GTA CTA CCA TAC 3′), using plasmids pssy5 and pPTR3, respectively, as templates and the conditions recommended by the manufacturer. Custom DNA sequencing of the isolated mutant plasmids was carried out by MWG Biotech (Germany). We observed a difference between the sequence of the SSY5 gene cloned (23) from the YCP50 library (30) and the sequence in the Saccharomyces Genome Database (SGD: http://www.yeastgenome.org/). Thus, the A nucleotide in codon 685 was deleted (5, 10). Deletion of this A base (SGD; http://www.yeastgenome.org/) resulted in a Ssy5p that is 12 amino acids longer than the version in the SGD. The extended Ssy5p has a C terminus that is identical to those of other trk1 and trk2 species (SGD).

Isolation of constitutively signaling SSY5 and PTR3 mutants. Strain M5077 (ptr1Δ ptr2Δ ssy5Δ) harboring plasmid pAGP1-KAT1 (14) was transformed with 0.5 μg pPEP10 linearized with BamHI and 0.5 μg Mutazyme-amplified SSY5 ORF by using a Gietz lab yeast transformation kit and procedure II (Genomics Office, Buffalo, NY). The transformation reaction was plated on SD medium without uracil and tryptophan but supplemented with 100 mM KCl to allow expression of the inserted SSY5 alleles. After 20 h at 30°C, transformants were replica plated to SD medium. After 2 to 4 days at 30°C, fast-growing, potentially constitutive SSY5 mutants appeared on the SD plates. Mutants were streaked to single colonies on SD plates, and plasmid DNA was rescued using a yeast DNA extraction reagent kit (Ferrieeck, Rockford, Illinois). Escherichia coli strain DH5α was transformed with the yeast DNA preparations, and SSY5-containing plasmids were identified by colony PCR screening using primers SSY5-3′ (5′-CCA AGG ACC TTA CTT GGT GCT GAT ACC C-3′) and SSY5-4′ (5′-GGG TTC GTA TCA TGA CTT GGG-3′). The plasmids were reintroduced into yeast strain M5077 and analyzed for their growth phenotype on SD plates and on SD plates supplemented with 0.2 mM leucine or 100 mM KCl.

PTR3 mutants were isolated in the same manner, using strain M5078 (ptr1Δ ptr2Δ ptr3Δ) as the screening strain and pPEP11 linearized with BamHI as the receptor plasmid. The PTR3 ORF mutant library was generated with Mutazyme as described above, using primers PTR3-3′ (5′-GGG ATT CCT TAA ACC AAC TGG GCT ACC G-3′) and PTR3-4′ (5′-CGG GAT CCG TAT TGC ACA TGT GAT TTCG-3′). β-Galactosidase activity was determined by using exponential-phase yeast cells harvested from cultures inoculated at an optical density at 600 nm (OD600) of 0.2 from overnight SD cultures and grown to an OD600 of 0.8 in SD medium. Soluble protein extracts were prepared from cell pellets using the Y-PER reagent procedure from Ferrieeck. The β-galactosidase activity of the lysates was then determined (5, 10).

Qualitative β-galactosidase assays were carried out by resuspending cells grown on SD plates supplemented with leucine (2 mM) in buffer Z (5, 10) containing 0.2% (wt/vol) Na-N-lauryl-sarcosine and 0.2 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Cell suspensions were incubated at 30°C overnight or until a blue color was detectable.

Dose-response analysis. Yeast cultures were grown exponentially overnight at 30°C in SD medium. When an OD600 of 0.3 to 0.5 was reached, 5-ml aliquots were exposed to leucine at a concentration ranging from 5 nM to 1 mM. Samples of 1.5 ml were withdrawn after 10 min, and proteins were extracted as described below.

Western analysis. Proteins were extracted under denaturing conditions with NaOH and β-mercaptoethanol from exponentially growing yeast cells and precipitated with trichloroacetic acid (9). Proteins were separated on NuPage 4 to 12% Bis-Tris gradient gels (Invitrogen) and blotted onto Immobilon polyvinylidene difluoride membranes (Invitrogen). Protein blots were incubated with a complex of horseradish peroxidase antibody and horseradish peroxidase (Rabbit PAP Z0113; DakoCytomation) in a 1:10,000 dilution in primary antibody diluent (Invitrogen), and chemiluminescence was detected using ECL Plus (Amersham Biosciences) and a Storm 860 apparatus. Quantification of chemiluminescence was carried out with ImageQuant software, version 5.0.

RESULTS

Genetic screening for constitutively signaling SSY5 and PTR3 mutants. Constitutively signaling SSY5 and PTR3 mutants were isolated using the KAT1 potassium channel reporter system for amino acid sensing developed by Gaber et al. (14). This reporter system makes use of a yeast host strain that is impaired in potassium import by deletion of the TRK1 and TRK2 genes and a reporter construct in which transcription of the gene for the Arabidopsis thaliana KAT1 potassium channel is controlled by a target promoter of SPS signaling. The selected promoter normally controls the expression of the AGP1 amino acid permease gene. trk1Δ trk2Δ cells grow poorly on standard minimal medium (SD medium), in which the potassium concentration is 7 mM. However, when these cells contain the reporter plasmid (pAGP1-KAT1), normal growth is restored by addition of leucine, since this amino acid induces SPS sensor-mediated activation of the AGP1 promoter, which leads to production of the KAT1 potassium channel and, consequently, increased potassium import.

Similar to the previous isolation of a mutation ssy1 allele that constitutively activates the AGP1 promoter (14), here, we present the isolation of constitutive SSY5 and PTR3 mutants using the KAT1 reporter system. The SSY5 ORF was subjected to mutagenesis by error-prone PCR amplification as described in Materials and Methods. Yeast strain M5077 (ptr1Δ ptr2Δ ssy5Δ/pAGP1-KAT1) was transformed with a mixture of the mutagenized DNA and linearized pPEP10, a centromere-based, URA3-based plasmid that contains sequence overlaps with the mutagenized DNA. Subsequent homologous recombination in vivo ensured insertion of the mutagenized copies of the SSY5 ORF into pPEP10. Among the approximately 9,000 uracil-independent transformants screened, 24 potentially constitutive SSY5 mutants were identified by their early appearance on SD plates. Plasmid DNA was isolated from seven of these mutants and reintroduced into strain M5077 (ptr1Δ ptr2Δ ssy5Δ/pAGP1-KAT1). The transformants grew rapidly on SD medium, indicating that this trait was associated with the plasmid-borne mutant SSY5 allele. DNA sequencing revealed that the mutant plasmid pSSY5-6 harbored a single base pair substitution in the SSY5 ORF, whereas each of the other mutants contained alterations at several positions. The mutation in plasmid pSSY5-6 was a transition from G to A in codon 512 (GAA to AAA), resulting in a Glu-to-Lys substitution (E512K) in Ssy5p.

The M5077/pSSY5-6 transformant was analyzed for its growth phenotype by using SD medium and SD medium supplemented with 0.2 mM leucine or 100 mM KCl. M5077/pSSY5-6 cells formed colonies within 3 days of incubation at 30°C on SD medium, whereas M5077 transformed with plasmid pSSY5-6...
of the mutants were affected at multiple sites. The PTR3

to identify constitutive alleles of PTR3 potentially constitutive

mutant single base pair exchange in the

activity in cell extracts of strains M5360 (ptr3

KCl (SD

vector) were spotted onto minimal medium (SD); minimal medium

SSY5 gene) or the vector without insert

SSY5-6, pPTR3-5, and pPTR3-17 were introduced into M5361 (ssy5Δ AGP1::
lacZ) and M5360 (ptr3Δ AGP1::lacZ) cells, and β-galactosidase levels in these cells grown in SD were measured and compared to those expressing wild-type SSY5 and PTR3 alleles (Fig. 1B). The levels of β-galactosidase in the mutant cells were significantly higher than those in the wild-type cells, showing that the mutant SSY5 and PTR3 alleles constitutively activate the AGP1 promoter. When the cells were grown in SD supplemented with leucine, the wild-type and the mutant SSY5 and PTR3 alleles activated the AGP1 promoter to the same extent as that observed with cells expressing mutant alleles in the absence of leucine (data not shown).

Constitutive SSY5 and PTR3 alleles confer increased Stp1p processing in the absence of amino acids. Proteolytic removal of ca. 10 kDa of the N terminus of the Stp1p (or Stp2p) transcription factor is a necessary step for the induction of amino acid-responsive permease genes (3). To analyze the SSY5 and PTR3 mutants in a more direct way than using the AGP1::lacZ reporter, we examined the effect on Stp1p processing by Western blot analysis of cells in which Stp1p is produced as a fusion protein with a C-terminal addition of a 15.5-kDa tandem doublet of an immunoglobulin G-binding domain (Z) of Staphylococcus aureus protein A (see Materials and Methods). Stp1p processing in ssy5Δ and ptr3Δ cells transformed with plasmids that express either wild-type or constitutive mutant alleles of SSY5 or PTR3, respectively, was measured (Fig. 2). In M5444 (ssy5Δ, STP1::ZZ) cells expressing a plasmid-borne wild-type SSY5 gene, the steady-state level of processed Stp1p-XX is ~12% of total Stp1p-XX in the absence of amino acids. Similarly, in wild-type cells (strain M5447; STP1::ZZ), Stp1p processing is 14% (data not shown). However, expression of SSY5-6 in M5444 (ssy5Δ) resulted in a 2.5-fold increase in Stp1p processing. In M5443 (ptr3Δ) cells harboring the plasmid-borne wild-type PTR3 gene, the steady-state level of processed Stp1p was 8% of total Stp1p-XX. However, cells expressing PTR3-5 or PTR3-17 exhibited processed Stp1p-XX levels of 33% and 27%, respectively. Stp1p-XX processing in M5445 (ssy1Δ) cells expressing the constitutive SSY1-102 allele (14) was found to be 48%, whereas cells harboring the wild-type SSY1 allele have a processing level of only 2% (data not shown). Collectively, our results show that the
constitutive SSY5, PTR3, and SSY1 mutants have increased steady-state levels of processed Stp1p in the absence of extracellular amino acids.

Constitutive SSY5 and PTR3 mutants confer a gain of function. To test for dominance, the constitutive SSY5 and PTR3 alleles were introduced on centromere-based plasmids into strain M5359, which is wild type with regard to the SPS sensor components but has an AGP1 promoter-β-galactosidase construct integrated at the AGP1 locus. The levels of β-galactosidase activity in these transformants grown in the absence of leucine are presented in Fig. 3A. The β-galactosidase activity in M5359 cells expressing the SSY5 and PTR3 mutant alleles was significantly increased compared to control cells with wild-type SSY5 and PTR3 alleles on the plasmids. Thus, the SSY5 and PTR3 mutants confer gain of function in SPS-mediated signaling.

We also supplemented these data with the more direct analysis of Stp1p processing in wild-type (M5447, STP1::ZZ) cells expressing the various constitutive alleles (Fig. 3B). The level of Stp1p processing in the mutants was significantly increased compared to that in wild-type cells. Moreover, it can be seen that similar levels of Stp1p processing are obtained when the mutant alleles are expressed either in the M5447 wild-type strain or in the ssy5Δ and ptr3Δ deletion strains (compare Fig. 3B with Fig. 2).

Constitutive signaling by SSY5 or PTR3 mutants is dependent on all components of the SPS sensor. Amino acid signaling normally requires all three SPS sensor components, Ssy1p, Ptr3p, and Ssy5p (6, 10, 21, 23, 24). To test whether the constitutive SSY5 mutant is able to circumvent SSY1 and PTR3 function, SSY5-6 was introduced into strains M5380 (ssy1Δ) and M5360 (ptr3Δ) harboring an integrated AGP1::lacZ reporter. Amino acid signaling in these cells was revealed by the blue coloration that resulted from β-galactosidase activity in X-Gal-containing buffer (Fig. 4). The absence of detectable β-galactosidase activity in SSY1- or PTR3-deleted cells expressing SSY5-6 shows that the mutant Ssy5 protein is dependent on Ssy1p and Ptr3p.

We similarly failed to detect β-galactosidase activity in M5380 (ssy1Δ) and M5361 (ssy5Δ) expressing the PTR3-5 and PTR3-17 mutations (Fig. 4). These results show that constitutive signaling by the isolated PTR3 mutants is unable to bypass the need for functional Ssy1p and Ssy5p; in other words, the constitutive signaling in these mutants is dependent on SSY1 and SSY5.

Constitutive SSY5, PTR3, and SSY1 mutants require less inducer to give strong signaling. To further characterize the constitutive SSY5 and PTR3 mutants presented in this paper and the SSY1-102 mutant previously described (14), we analyzed the pattern of Stp1p processing in response to different concentrations of leucine. The data collected from these experiments were used to estimate a median effective concentration (EC50), which is the concentration of leucine in the medium that results in 50% processed Stp1 protein: the EC50 value is also a reflection of the apparent Ke for leucine binding to Ssy1p. The resulting dose-response curves for a wild-type strain and strains with the Ssy5-E512K, Ptr3-Q439R, Ptr3-T435K, and Ssy1-T382K mutant sensor components are shown in Fig. 5.

To obtain a control EC50 value for leucine in a SPS sensor wild-type background, we used strain M5444 (ssy5Δ) complemented by the wild-type SSY5 allele on a pRS316 centromere-based plasmid (pSSY5). As shown in Fig. 5, the EC50 value was...
determined to be 11.9 μM; the same EC₅₀ value was also found using wild-type strain M5447 in which the three SPS-encoding genes were present in one copy at their normal chromosomal location (for three experiments, one of which is shown in Fig. 5 [upper panel], the EC₅₀ was 12.25 and the standard error of the mean was 0.92). It may be noted that the Stp1p processing reaches a level of only about 60 to 70%. Plating for single colonies on uracil-containing medium and replica plating of these onto uracil-free medium showed that this is due to the loss of pSSY5 in about one-third of the cells in the culture, a value typical for the pRS316 vector (32). In contrast, Stp1p processing typically reaches 100% in the EC₅₀ determinations carried out with wild-type strain M5447 (29). Since we obtain the same EC₅₀ value for the M5444/pSSY5 and M5447 cells, we consider the system in which the gene for a SPS sensor component is deleted on the chromosome and complemented by the corresponding wild-type gene or constitutive mutant

![FIG. 4. Constitutive signaling by the SSY5-6 and PTR3-5 mutants is dependent on the two other components of the SPS sensor. Strains carrying the indicated deletions and transformed with the centromere-based plasmid pRS316 carrying the indicated mutant genes were grown in SD medium and permeabilized. Exposure to the substrate X-Gal yielded a blue color where activation of the AGP1 promoter had resulted in the presence of β-galactosidase. (A) Strains M5380 (ssy1Δ agr1::lacZ) and M5361 (ssy5Δ agr1::lacZ). (B) Strains M5360 (ptr3Δ agr1::lacZ) and M5361. (C) Strains M5380 and 5360. (D) Strains 5361 and 5360.](image)

![FIG. 5. The constitutive SPS sensor mutants require less inducer to give strong signaling. Cells growing exponentially in SD medium were exposed to leucine at various concentrations, and Stp1p-ZZ processing was monitored. EC₅₀ (apparent Kₐ) was calculated using SigmaPlot 2000 by fitting the data to y = y₀ + a(x/b + x), where b is EC₅₀. In each panel for the mutants, the indicated values for the mean and the standard error of the mean for EC₅₀ are derived from three experiments carried out in identical ways on different days; data points and the fitted curve represent one of the three experiments. The indicated mutations were present as inserts in the centromere-based and URA3-based vector pRS316 in ura3 strains deleted for the corresponding wild-type (wt) SSY5, PTR3, or SSY1 gene, respectively. In the upper panel, the chromosomal SSY5 deletion was complemented by a wild-type SSY5 gene harbored in pRS316 (pSSY5).](image)
gene on a plasmid to be a reliable experimental system for determining EC\textsubscript{50} values.

Compared to the wild type, a 3.7-, 1.6-, 2.0-, and 9.0-fold reduction in EC\textsubscript{50} is observed for Ssy5-E512K, Ptr3-Q439R, Ptr3-T435K and Ssy1-T382K, respectively. The most dramatic reduction in EC\textsubscript{50} is found for Ssy1-T382K, which is believed to interact directly with the extracellular inducer. A more moderate reduction in EC\textsubscript{50} is found for the SSY5 and PTR3 mutant proteins. Nevertheless, we conclude that the leucine concentration needed to induce a strong signaling response is reduced in each of the mutants.

Thus, the mutational changes of Ptr3p and Ssy5p make these proteins more prone to trigger the onset of Stp1p processing, and they also increase the apparent affinity between Ssy1p and extracellular ligand, presumably by affecting an equilibrium between a nonsignaling and a signaling conformation. This effect supports the notion that the Ssy1, Ssy5, and Ptr3 proteins interact intimately in a complex.

**DISCUSSION**

In *S. cerevisiae*, the ability to sense the presence of extracellular amino acids and to initiate signal transduction, leading to increased transcription of amino acid permease genes, depends on the function of several genes, including SSY1, PTR3, and SSY5 (6, 10, 12, 21, 23, 24). While Ssy1p resembles members of the amino acid permease family, it is apparently unable to transport significant quantities of amino acids. Thus, Ssy1p is believed to act as a receptor, initiating signal transduction in a manner dependent on Ptr3p and Ssy5p. That these three proteins are not only functionally but also physically associated is supported by various experimental data. Firstly, several tagged versions of Ptr3p and Ssy5p were localized to the plasma membrane despite their lack of typical membrane-spanning domains (12, 20, 24). Secondly, overexpression of the cytoplasmic, N-terminal domain of Ssy1p inhibits sensor function, a finding consistent with formation of defective, nonsignaling complexes consisting of the Ssy1p N-terminal domain and protein(s) normally binding to Ssy1p (7, 12). Thirdly, two-hybrid experiments are consistent with the existence of a complex between the three proteins. Thus, Ptr3p was found to interact with itself and with Ssy5p (7), and Ssy1p was found to interact with Ptr3p (35). It is important to note, however, that all of these data address primarily the interactions necessary for signaling but are not critically concerned with whether Ssy1p, Ptr3p, and Ssy5p actually function in a complex and what the functional properties of such a complex could be. The present work sheds some light on this issue.

The putative complex comprising Ssy1p, Ptr3p, and Ssy5p has been termed the SPS sensor (12). The three proteins mediate amino acid-dependent activation of the transcription factors Stp1p and Stp2p (3, 4), and their individual roles are beginning to emerge through analysis of genetically altered members of the putative complex. The notion that extracellular amino acid directly interacts with Ssy1p gained support from the isolation of constitutively signaling and hyperresponsive SSY1 mutants (14). Thus, whether or not Ssy1p can normally transport small amounts of amino acid, imported amino acids per se are not required for signaling. These mutations also significantly increased the apparent affinity for amino acids, providing further support for the hypothesis that Ssy1p functions by direct interaction with extracellular amino acids.

Concerning Ssy5p, recent evidence suggests that this protein is a trypsin-like serine protease whose activity is required for the cleavage and consequent activation of Stp1p and Stp2p (1, 2). So far, the function of Ptr3p is not known; however, a scan of the Ptr3 protein sequence against the protein signatures in the InterPro databases using the InterProScan tool (26), suggests that Ptr3p may contain WD40 repeats (23, 25) in the C-terminal region. A comparison of the Ptr3p sequence to WD40 repeat models in the Superfamily server (16) aligned the Ptr3p sequences from residues K364 to C605 with the models, suggesting five WD40 repeats in Ptr3p. Since repeated WD40 motifs are known to act as a site for protein-protein interactions (16, 25), the proposed WD40 motifs may be involved in interactions with Ssy1p and Ssy5p that regulate the activation of the Ssy5p proteolytic properties upon binding of amino acids to Ssy1p. Interestingly, the constitutive PTR3 mutants result in the amino substitutions T435K and Q439R, which map within the proposed WD40 repeat region. Whether Ptr3p indeed belongs to the WD40 repeat superfamily must await structural data for the protein.

In this report, using essentially the same genetic screening that yielded SSY1 constitutive alleles, we show that gain-of-function mutations in PTR3 and SSY5 that confer robust activation of a downstream amino acid permease gene, even in the absence of extracellular amino acids, can be isolated.

We obtained several different constitutive alleles of both PTR3 and SSY5 (described above and data not shown) and, in each case, activation of the downstream reporter genes depended on the presence of the complete SPS sensor. Thus, PTR3-5 and PTR3-17 exhibited no activation in ssy1Δ or ssy5Δ cells, and SSY5-6 exhibited no activation in ssy1Δ or ptr3Δ cells. These results support the hypothesis that the three proteins actually function in a complex, since they are not easily reconciled with a model in which sequential signaling steps between the three proteins are separable.

The dose-response relationships for signaling in the mutants are highly relevant for the understanding of the interactions in the complex. Notably, we have shown that in the PTR3 and SSY5 constitutive mutants, the apparent affinity for leucine, as measured by the EC\textsubscript{50} for induction of Stp1p processing, was altered. The EC\textsubscript{50} for PTR3-5 and PTR3-17 cells (7.5 and 6.0 μM, respectively) and for SSY5-6 cells (3.2 μM) were reduced compared to that for wild-type cells (11.9 μM), although not to the extent observed with cells expressing the constitutive SSY1/102 (1.3 μM). If Ptr3p and Ssy5p functioned individually downstream of Ssy1p, one would not expect constitutive mutations to affect the EC\textsubscript{50} in this manner. Rather, these results are easily explained by assuming that Ssy1p, Ptr3p, and Ssy5p function in a highly interdependent, conformationally coordinated manner.

The evidence suggesting that Ssy5p acts as the protease that activates Stp1p and Stp2p (1, 2) is consistent with Ssy5p functioning as the most downstream component of the SPS sensor. If so, one might expect that some constitutive alleles of SSY5 could confer activity independent of Ssy1p and Ptr3p. Fortunately, Per Ljungdahl et al. generated a constitutively active allele of SSY5 that functions even in the absence of the other members of the SPS sensor, not through point mutation but by
the addition of six tandem copies of the hemagglutinin epitope tag (HA<sub>6</sub>) to the amino terminus of Ssy5p (2).

The different behavior of HA<sub>6</sub>-Ssy5p and Ssy5-E512K (encoded by SSY5-6) is thought provoking and may be accounted for as follows. The proteolytic activity of Ssy5p appears to be internally inhibited. The inhibition can be counteracted by interaction with Ssy1p and Ptr3p in a way dependent on extracellular amino acid binding to Ssy1p. In this picture, HA<sub>6</sub>-Ssy5p is not properly inhibited and can act without Ssy1p and Ptr3p. Ssy5-E512K, on the other hand, is affected in the interaction with the partners in the complex. Interestingly, the Ssy5-E512K substitution is positioned in the protease region of Ssy5p close to amino acid D545, which has been proposed to be part of the trypsin-like serine protease catalytic triad (H465, D545, and S640) in Ssy5p (1, 2). An understanding of how this mutation confers constitutivity and maintains Ssy1p and Ptr3p dependence must await interaction studies of the SPS sensor components.

The recent finding that casein kinase I is necessary for the endoproteolytic processing of Stp1p (1, 34) adds to the complexity of the signal transduction mechanism of the SPS sensor. In addition, the involvement of Grr1p, a component of the SCFGrr1 ubiquitin ligase complex, remains a puzzling issue in the signal transduction system of the SPS sensor. We wish to emphasize that our results, by their very nature, cannot be regarded as proof of the existence of the complex of Ssy1p, Ptr3p, and Ssy5p. However, the previous two-hybrid and other data suggesting the existence of such a complex have, by the present and very different approach, been supported and complemented with data that say more about dynamics and functionality than can two-hybrid interactions.

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