MOLECULAR AND CELLULAR BIOLOGY, Feb. 2001, p. 814–826 Vol. 21, No. 3
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.3.814–826.2001

Genetic and Biochemical Analysis of the Yeast Plasma Membrane Ssy1p-Ptr3p-Ssy5p Sensor of Extracellular Amino Acids

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Received 13 September 2000/Returned for modification 17 October 2000/Accepted 13 November 2000

Ssy1p and Ptr3p are known components of a yeast plasma membrane system that functions to sense the presence of amino acids in the extracellular environment. In response to amino acids, this sensing system initiates metabolic signals that ultimately regulate the functional expression of several amino acid-metabolizing enzymes and transport proteins, including multiple, genetically distinct amino acid permeases. We have found that *SSY5* encodes a third component of this amino acid sensing system. Mutations in *SSY5* manifest phenotypes that are indistinguishable from those resulting from either single *ssy1* and *ptr3* mutations or *ssy5 ssy1* and *ssy5 ptr3* double mutations. Although Ssy5p is predicted to be a soluble protein, it exhibits properties indicating that it is a peripherally associated plasma membrane protein. Each of the three sensor components, Ssy1p, Ptr3p, and Ssy5p, adopts conformations and modifications that are dependent upon the availability of amino acids and on the presence of the other two components. These results suggest that these components function as part of a sensor complex localized to the plasma membrane. Consistent with a sensor complex, the overexpression of *SSY1* or the unique N-terminal extension of this amino acid permease homologue inactivates the amino acid sensor in a dominant-negative manner. Each of the components of the Ssy1p-Ptr3p-Ssy5p (SPS) signaling system undergoes rapid physical changes, reflected in altered electrophoretic mobility, when leucine is added to cells grown in media lacking amino acids. Furthermore, the levels of each SPS sensor component present in whole-cell extracts diminish upon leucine addition. The rapid physical alterations and reduced levels of sensor components are consistent with their being downregulated in response to amino acid availability. These results reveal the dynamic nature of the amino acid-initiated signals transduced by the SPS sensor.

The ability of *Saccharomyces cerevisiae* cells to rapidly respond and adapt to changing environmental conditions is essential for viability. A prerequisite for the generation of a proper physiological response is the ability to sense and subsequently transduce information regarding the extra- and intracellular environments. Sensor-initiated signals are used to make dynamic adjustments in patterns of gene expression and protein turnover, processes that enable cells to express the necessary components appropriate for prevailing conditions. For example, in response to nutrient availability, yeasts regulate the expression and activity of proteins involved in nutrient uptake and utilization. Recently, several plasma membrane-localized nutritional sensors that monitor nutrient availability in the extracellular environment have been identified in yeast. These include two glucose sensors, *SNF3* and *RGT2* (41, 49); a G-protein-coupled receptor that is activated by the presence of fermentable sugars, *GPR1* (35, 44, 67); a high-affinity ammonium transporter (*MEP2*) (45) that may function as an ammonium sensor (43); and an amino acid sensor, *SSY1* (19, 30, 33, 34). Little is known regarding whether these primary sensors function alone or in complexes together with other proteins, and the mechanisms by which these sensors transduce nutritionally derived signals remain to be elucidated.

SNF3 and *RGT2* encode unique members of the hexose transporter (HXT) family that possess unusually long C-terminal domains. These proteins are poorly expressed compared to the other known functional hexose transporters and pleiotropically affect the expression of multiple HXTs (41, 49). The expression of *SNF3* is repressed by the presence of glucose (10), whereas *RGT2* is constitutively expressed (49). Recent reports have shown that the cytoplasmically oriented C-terminal extensions of Snf3p and Rgt2p have important roles in signaling. The C terminus of Snf3p expressed as a soluble protein (62), or as a hybrid with the Hxt2p hexose transporter (48), can partly suppress phenotypes associated with an *snf3* deletion. The C-terminal extensions of Snf3p and Rgt2p physically interact with two homologous proteins, Std1p and Mth1p (37, 56). Std1p and Mth1p are suggested to act as repressors of Snf3p and Rgt2p target genes in the absence of signaling. Green fluorescent protein-Std1p fusion proteins are localized to the cell periphery and the cell nucleus; thus the possibility exists that Std1p mediates information directly from the cell surface to the nucleus (56).

SSY1 encodes a unique member of the amino acid permease protein family that functions as a sensor of extracellular amino acids (19, 30, 33, 34). Ssy1p has a unique 200-amino-acid N-terminal extension that is required for *SSY1* activity (34); however, its precise function remains obscure. Ssy1p is localized to the plasma membrane and is, as are the other members of the amino acid permease family, dependent upon the amino acid-specific packaging chaperone Shr3p (25, 42) to exit the endoplasmic reticulum (34). *SSY1* is required for the transcriptional

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TABLE 1. Characteristics of the *cerevisiae* strains used in this study

Strain	Genotype	Reference
cdc25H	*MAT***a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112 cdc25-2 GAL*$^+$	53
Isogenic derivatives of PLY1		
PLY1	*MAT***a** *ura3-52 his4Δ29*	42
PLY4	*MAT*α *ura3-52 his4Δ29 ade2Δ1::URA3*	42
PLAS10-8C	*MAT***a** *ura3-52 his4Δ29 shr4-10 (ssy5-410)*	This work
PLAS10-9A	*MAT*α *ura3-52 his4Δ29 shr4-10 (ssy5-410)*	This work
PLAS7-4C	*MAT***a** *ura3-52 his4Δ29 shr10-7 (ssy1-107)*	34
PLAS14-1A	*MAT***a** *ura3-52 his4Δ29 shr6-14 (ptr3-614)*	34
HKY37	*MAT***a** *ura3-52 his4Δ29 ssy1Δ13*	34
HKY71	*MAT***a** *ura3-52 his4Δ29 ssy5Δ1::hisG-URA3-kan*^{r}*-hisG*	This work
Isogenic derivatives of PLY126		
PLY126	*MAT***a** *ura3-52 lys2Δ201*	34
HKY20	*MAT***a** *ura3-52 lys2Δ201 ssy1Δ13*	34
HKY31	*MAT***a** *ura3-52 lys2Δ201 ptr3Δ15*	34
HKY33	*MAT***a** *ura3-52 lys2Δ201 ssy1Δ13 ptr3Δ15*	34
HKY75	*MAT***a** *ura3-52 lys2Δ201 ssy5Δ1::hisG-URA3-kan*^{r}*-hisG*	This work
HKY77	*MAT***a** *ura3-52 lys2Δ201 ssy5Δ2*	This work
HKY84	*MAT***a** *ura3-52 lys2Δ201 ssy1Δ13 ssy5Δ2*	This work
HKY85	*MAT***a** *ura3-52 lys2Δ201 ptr3Δ15 ssy5Δ2*	This work

induction of multiple genes encoding amino acid permeases (*AGP1, BAP2, BAP3, GNP1, VAP2,* and *TAT2*), the peptide transporter (*PTR2*), and arginase (*CAR1*) in response to extracellular amino acids (19, 30, 34). *SSY1*-mediated signals are also required for full transcriptional repression of the general amino acid permease (*GAP1*) on ammonium-based media in the presence of amino acids (34). Ssy1p is dependent upon *PTR3* to mediate amino acid-derived signals (34). Ptr3p is a peripherally associated plasma membrane protein and contains a domain that shares homology with amino acid permeases and Gcn4p (34).

Several factors required for transcription of *SSY1*-controlled genes have been identified, including *STP1, STP2*, and *ABF1* (15, 16) and *UGA35* (also known as *DAL81*) and *GRR1* (30). *STP1* and *STP2* were originally identified as genes required for pre-tRNA maturation (66). Abf1p is a general transcription factor involved in global gene activation and silencing (17, 54, 57). *UGA35*/*DAL81* encodes a nonspecific factor required for the full induction of several genes active in nitrogen utilization, including γ-aminobutyric acid and allophanate-inducible genes (8, 14, 64). Some of the *SSY1*-responsive genes, including *BAP2*, also carry promoter binding sites for Gcn4p and Leu3p (18). *GCN4* encodes a transcription factor that is translationally regulated by the general amino acid control pathway that monitors intracellular levels of free amino acids. The general control pathway coordinately upregulates biosynthetic genes and transporters in response to amino acid deprivation (28). Leu3p, a transcription factor that is capable of acting as a repressor or an inducer, participates in regulating the transcription of several genes within the branched-chain amino acid biosynthetic pathways (9, 65). *GRR1* was previously reported to be involved in glucose signaling and cell cycle control (5, 40). Grr1p is an F-box-containing component of discrete Skp1–Cullin–F-box (SCF) ubiquitin ligase complexes that mark proteins for degradation via the proteasome. Several F-box-containing proteins have been identified in yeast, and they are thought to provide specificity by recruiting distinct protein substrates to SCF complexes for ubiquitination (51). Presumably the inability of *grr1* mutants to properly respond to nutritional signals is a consequence of aberrant patterns of regulated protein degradation (32, 50). The large number of factors that affect the transcription of target genes indicates a complex network of regulatory processes that most likely integrate signals derived from different primary nutritional sensors.

Although several of the downstream components required for the transmission of amino acid-induced signals have been identified, little is known regarding the primary component composition of the plasma membrane amino acid-sensing system. As previously indicated, this system not only depends upon Ssy1p, but also requires Ptr3p (34). Even less is known regarding the mechanisms associated with initial signaling events that occur within the sensing system in response to extracellular amino acids. We have focused our efforts on characterizing the proteins comprising the plasma membrane amino acid nutritional sensor. In this paper, we present results that identify Ssy5p as a third component of this signaling system. Mutations in *SSY5* have earlier been described to interfere with amino acid uptake (33). We show that *ssy5* mutants belong to the same phenotypic epistasis group as both *ssy1* and *ptr3* mutants. Ssy5p is a peripheral membrane protein that binds to the plasma membrane and is dependent upon *SSY1* and *PTR3* for wild-type expression. Additionally, Ssy1p and Ptr3p exhibit altered electrophoretic mobilities dependent on the presence of the other sensing components and on the availability of amino acids. Finally, we have documented the dynamic nature of amino acid-induced signaling and have found that each of the components of the Ssy1p1-Ptr3p-Ssy5p (SPS) signaling system is rapidly downregulated in response to amino acid availability.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study are listed in Table 1. Strains carrying a null mutation of *SSY5* were constructed as follows. PLY1 and

TABLE 2. Plasmids and oligonucleotides used in this study

Plasmid or oligonucleotide	Description or sequence	Reference
Plasmids		
pPL193	5-kb *Eco*RI fragment containing *PTR3* in pRS316	34
pPL341	*ptr3Δ14::hisG-URA3-kan*^{r}*-hisG* in pUC118 (ΔB-ΔH)	34
pPL356	3.9-kb *Spe*I-*Cla*I fragment containing *SSY1* in pRS316	34
pPL496	7.8-kb *Sau*3A fragment containing *SSY5* in pRS202	This work
pHK003	pPL356 with all *Xba*I sites removed	34
pHK010	*SSY1-HA1* in pRS316	34
pHK012	*SSY1* in pRS202	This work
pHK018	*PTR3-HA1* in pRS316	34
pHK026	*PTR3* in pRS202	This work
pHK031	*ssy1Δ12::hisG-URA3-kan*^{r}*-hisG* in pBluescript	This work
pHK035	4.9-kb *Bst*XI-*Bam*HI fragment containing *SSY5* in pRS316	This work
pHK036	Same as pHK035, except *SSY5* fragment inserted in opposite orientation	This work
pHK037	*SSY5* in pRS202	This work
pHK038	*SSY1-NT*(1–206) in pRS316	This work
pHK039	*SSY1-NT*(1–206) in pRS202	This work
pHK040	pPL193 with *Bam*HI site in MCS removed	This work
pHK041	pHK036 with *Bam*HI site in MCS removed	This work
pHK042	pHK040 with *Bam*HI site (b) introduced after ATG in *PTR3*	This work
pHK043	pHK041 with *Bam*HI site (b) introduced after ATG in *SSY5*	This work
pHK044	*PTR3 in pSOS*	This work
pHK045	*SSY5* in pSOS	This work
pHK047	pHK041 with *Bam*HI site (a) introduced after the ATG of *SSY5* ORF	This work
pHK048	*SSY5-c-myc* in pRS316	This work
pHK049	*ssy5Δ1::hisG-URA3-kan*^{r}*-hisG* in pRS316	This work
pHK050	*SSY5* in pAD40	This work
Oligonucleotides		
POL95-039	5′-GATTGAGTTGAATTCTTCTACCACTAC-3′	
POL96-021	5′-CCTGGCTGATTTCTAGATAGGGTTATATG-3′	
POL99-026	5′-GATATACCGATGGGATCCGTCAGATTTTTTGG-3′	
POL00-004	5′-GATATACCGATGGGGATCCTCAGATTTTTTGG-3′	
POL00-005	5′-TAGGCGTTCATGGGGATCCACTCTCATAG-3′	

PLY126 were transformed with a linear 9-kb *Xba*I fragment from pHK049 (see Table 2; plasmids are described in the following section) containing the *ssy5Δ1::hisG-URA3-hisG* deletion construct. Strain HKY75 was propagated on media containing 5-fluoroorotic acid (5-FOA) (26) to attain strain HKY77 carrying the unmarked *ssy5Δ2* deletion. Strain HKY82 was obtained by transforming HKY77 with a linear *Sal*I-*Spe*I fragment of pHK031 containing *ssy1Δ12::hisG-URA3-hisG*. Similarly, strain HKY83 was obtained by transforming HKY77 with a linear *Eco*RI fragment of pPL341 containing *ptr3Δ14::hisG-URA3-hisG* (34). Strains HKY82 and HKY83 were propagated on 5-FOA, resulting in strains HKY84 and HKY85 with unmarked *ssy1Δ13* and *ptr3Δ15* alleles, respectively. The correct integration of each gene replacement was confirmed by Southern analysis. The yeast strain cdc25H was obtained from Stratagene (La Jolla, Calif.).

Standard yeast media were prepared as described by Guthrie and Fink (26). Nonstandard synthetic medium with proline as the nitrogen source (SPD) was prepared as follows. Proline (4 g/liter) and Difco yeast nitrogen base (26.8 g/liter) were added together to make a 4× stock solution that was filter sterilized. Other components were autoclaved as separate stock solutions (40% glucose and 4% Difco Bacto agar). Stock solutions and sterile water were mixed to make a 2× solution containing 4% glucose, and an equal volume of molten 4% agar was added. Where required, SPD was supplemented as indicated (e.g., 30 mM L-histidine or L-methionine). The concentration of yeast nitrogen base in these synthetic media is fourfold higher than the amount used in other standard synthetic media. Yeast transformations were performed as described by Ito et al. (31) with 50 μg of heat-denatured calf thymus DNA. Transformants were selected on solid complete synthetic dextrose medium (SC) lacking either uracil, leucine, or lysine as required.

Plasmids. The plasmids and oligonucleotides used in this study are listed in Table 2. In separate reactions, a 4.9-kb *Bam*HI-*Bst*EII fragment from pPL496 containing *SSY5* was ligated to *Bam*HI-digested pRS316 (59) and pRS202 (13), resulting in pHK036 (CEN, ori1), pHK037 (CEN, ori2), and pHK037 (2μm, ori2). A blunt-ended 5-kb *Bam*HI-*Bgl*II fragment isolated from pSE1076 (1) containing a *hisG URA3 kan*^{r} *hisG* blaster cassette was inserted into *Bsr*GI-digested pHK036 made blunt by using T4 DNA polymerase. This *ssy5Δ* construct (pHK049) removes nucleotides (nt) −28 to +1108 (nucleotide designations are in relation to the first nucleotide of the initiator ATG codon). Plasmid pHK031 was constructed by inserting a *Sal*I-*Spe*I fragment from pHK030 carrying the *ssy1Δ12::hisG URA3 kan*^{r} *hisG* deletion allele (34) into *Sal*I-*Spe*I-digested pBluescript II KS(+) (Stratagene, La Jolla, Calif.). To remove the *Bam*HI sites within their multicloning sequences, plasmids pPL193 (34) and pHK036 were digested with *Bam*HI, made blunt with T4 DNA polymerase, and religated, creating pHK040 and pHK041, respectively. The *SSY5*–c-*myc* epitope-tagged allele in pHK048 was constructed in two steps. First, a *Bam*HI site (reading frame a) was introduced immediately after the ATG initiation codon of *SSY5* by using site-directed mutagenesis (36, 63) with single-stranded pHK041 as a template and oligonucleotide POL99-026 as mutagenic primer, resulting in pHK047. In step 2, a *Bam*HI-flanked cloning cassette, encoding the c-myc epitope reiterated three times (c-myc^3) was inserted into the unique *Bam*HI site of pHK047, creating pHK048. The construction of plasmids encoding SOS hybrid proteins required multiple steps. Site-directed mutagenesis with single-stranded pHK040 and pHK041 as a template and mutagenic primers POL00-005 and POL00-004 was used to create unique *Bam*HI sites (reading frame b) immediately following the ATG of *PTR3* and the ATG of *SSY5*, resulting in plasmids pHK042 and pHK043, respectively. The vectors contained in the CytoTrap kit were obtained from Stratagene. The *Bam*HI-*Sal*I fragments from plasmids pHK042 and pHK043 were inserted into *Bam*HI-*Sal*I-digested pSOS (Stratagene), creating pHK044 and pHK045. Plasmid pHK050 was constructed by moving the *Sac*I-*Sal*I fragment from pHK035 containing *SSY5* into *Sac*I-*Sal*I-digested pAD40 (obtained from M. Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). In a single reaction, two *Xba*I sites were introduced into the *SSY1* coding sequence with single-stranded pHK003 as a template (34) and mutagenic primers POL95-039 and POL96-021. The resulting plasmid was digested with *Xba*I to release the internal fragment of *SSY1* and religated, resulting in pHK038. pHK038 encodes only the first 206 N-terminal amino acids of Ssy1p. The 2μm plasmids pHK039

TABLE 3. Concentrations of basic amino acids in whole cells and vacuoles of the wild-type and *ssy5*, *ssy1*, and *ptr3* mutant strains[a]

Amino acid	Concn of wild type (PLY1)		Concn of *ssy5-410* (PLAS10-8C)		Fold	Concn of *ssy1-107* (PLAS7-4C)		Fold	Concn of *ptr3-614* (PLAS14-1A)		Fold
	WC	Vac	WC	Vac		WC	Vac		WC	Vac	
Histidine	27	24	58	52	2.2	72	62	2.6	102	78	3.5
Arginine	87	81	268	256	3.2	294	266	3.3	429	371	4.6
Lysine	82	80	89	87	1.1	89	85	1.1	100	95	1.2

[a] Concentrations are nanomoles per 10^8 cells. Fold, fold wild-type vacuolar amino acid concentration; WC, whole cells; Vac, vacuoles.

and pHK012 were constructed by inserting the *Sac*II-*Kpn*I fragments from pPL356 (34) or pHK038 into *Sac*II-*Kpn*I-digested pRS202, respectively. Plasmid pHK026 (2μm *PTR3*) was created by ligating a *Sal*I-*Sac*II fragment containing *PTR3* obtained from pPL193 into *Sal*I-*Sac*II-digested pRS202.

Genetic analysis. Strain PLY1 was used to isolate spontaneous mutants resistant to 30 mM histidine (42). The super-high-histidine-resistant (*shr*) mutants were backcrossed to PLY4 (*MAT*α *his4Δ29 ura3-52 ade2Δ1::URA3*), an isogenic derivative of PLY1. Tetrad analysis indicated that the mutant phenotypes segregated 2:2. Strain PLAS10-8C (*shr4-10*) was obtained as a meiotic segregant from one of these crosses. *SHR4* was cloned by complementation of the 30 mM histidine-resistant phenotype; strain PLAS10-8C (*shr4-10*) was transformed with a plasmid library (pRS202 library, obtained from Philip Hieter [13]), and Ura^+ transformants unable to grow on selective medium containing 30 mM histidine were identified. Complementing plasmids were isolated and further analyzed. Plasmid pHK035, which contains a 4.9-kb *Bst*EII-*Bam*HI insert with a single open reading frame (ORF) (YJL156c), fully complemented *shr4* mutant alleles.

Northern analysis. Steady-state levels of *AGP1* and *PTR2* mRNA (see Fig. 2) were determined in strains PLY126 and HKY77 transformed with YCp405 and pRS316. Cells from overnight cultures grown in SD were harvested, washed once, and resuspended in 10× volume of fresh SD at an optical density at 600 nm (OD_{600}) of 0.2. Cultures were grown to an OD_{600} of 0.8, one-half of the culture was induced by addition of leucine to a concentration of 0.15 mM, and an identical aliquot of water was added to the remaining half of the culture (uninduced control). After 45 min, 30 ml of cells was harvested, RNA was prepared, and Northern analysis was performed with aliquots (10 μg) of RNA as described previously (34). *SSY5* mRNA levels were analyzed in strains HKY77, HKY84, and HKY85 transformed with pHK048 and YCp405 (Fig. 4B). Cells were grown overnight in SD or SD supplemented with 1.3 mM leucine, washed once, and resuspended in a 10× volume of fresh medium at an OD_{600} of 0.2. RNA was isolated when cultures reached a cell density of an OD_{600} of 0.8. Radioactive probes were prepared with the following template DNA fragments: a 1.2-kb *Eco*RV internal fragment of *SSY5* and a 339-bp PCR fragment from *AGP1* amplified with oligonucleotide primer pairs POL00-006 and POL00-007. Additionally, the previously described 569-bp PCR fragment of *CAR1*, 530-bp PCR-fragment from *PTR2*, and a 1.65-kb *Bam*HI-*Hin*dIII fragment containing *ACT1* were used (34). The DNA fragments were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham, United Kingdom) by using a random-primed DNA labeling kit (MBI Fermentas Molecular Biology) and purified by using Bio-Rad Bio Spin columns. After hybridization, blots were rinsed once with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS), washed two times with 5× SSC–0.1% SDS, one time with 1× SSC–0.1% SDS, and one time with 0.5× SSC–0.1% SDS if required. Washings were performed at 55°C for 20 min. After washings, blots were visualized and quantified with a Fujix Bio-Image Analyzer BAS1500 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Protein manipulations. Protein was determined by the method of Markwell et al. (46). Whole-cell protein was examined with lysates prepared from 100 ml of cells by the glass-bead method described by Chang and Slayman (11). The membrane association of Ssy5p in strain HKY77 transformed with plasmids pHK048 and YCp405 was examined as follows. An aliquot of a lysate (100 μg of protein) in low-salt BB buffer (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM Tris [pH 7.5]) was diluted 1:1 with either H_2O, 1.6 M urea, or 2 mM EDTA; mixed; and incubated on ice for 30 min. Samples were centrifuged at 100,000 × *g* for 45 min at 4°C, and protein pellets were resuspended in 2× sample buffer containing low-salt BB buffer. After sonication and denaturation and then incubation for 10 min at 37°C, aliquots (10 μg of protein) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by immunoblotting.

The levels of expression and electrophoretic properties of Ssy5p, Ssy1p, and Ptr3p were examined in whole-cell lysates prepared from strains HKY77 (pHK048, YCp405), HKY84(pHK048, YCp405), HKY85(pHK048, YCp405), HKY20(pHK010, YCp405), HKY33(pHK010, YCp405), HKY84(pHK010, YCp405), HKY31(pHK018, YCp405), HKY33(pHK018, YCp405), and HKY85 (pHK018, YCp405). In each case, lysates were prepared from 100 ml of cells grown to an OD_{600} of 0.8 in SD or SC lacking uracil and lysine, as indicated. After denaturation of protein preparations (10 min at 37°C for Ssy5p and Ssy1p, 3 min at 95°C for Ptr3p) in sample buffer, aliquots (10 to 20 μg of protein) were resolved by SDS-PAGE and analyzed by immunoblotting.

Immunoblots were probed with 1:1,000 dilutions of monoclonal antibodies recognizing the hemagglutinin (HA) (12CA5) or c-myc (9E10) epitopes; or rabbit anti-Pma1p diluted 1:3,000 as described by Klasson et al. (34). Chemiluminescent signals were visualized by enhanced chemiluminescence (ECL-PLUS system; Amersham) and quantitated by using the LAS1000 system (Fuji Photo Film Co., Ltd.).

Amino acid pool size determination. Whole-cell and vacuolar amino acid pool concentrations were determined with cells grown in YPD to an OD_{600} of ≈1 essentially as described by Ohsumi et al. (47). Appropriate quantities of cultures (3×10^8 cells) were harvested by centrifugation, and cell pellets were washed twice with 1.5 ml of water and resuspended in 1.5 ml of AA buffer (0.6 M sorbitol, 2.5 mM potassium phosphate buffer [pH 6]) containing 10 mM glucose. For the determination of vacuolar amino acid pools, the cells were resuspended in the same buffer containing 0.8 mM $CuCl_2$ and incubated for 10 min at 30°C. One-milliliter aliquots of cell suspensions were filtered (Whatman GF/F filters), and filters were washed four times with AA buffer. The washed filters were boiled in 3 ml of water for 15 min, and 1-ml aliquots were centrifuged to remove particles of filter. The concentrations of amino acids in 30-μl aliquots were determined.

Time course experiments. Cells from overnight cultures of strains HKY31 (pHK018, YCp405), HKY77(pHK048, YCp405), and HKY20(pHK010, YCp405) grown in SD were washed once and resuspended in a 10× volume of fresh SD to an OD_{600} of 0.1 to 0.2. After cultures reached a cell density of OD_{600} of 0.5, the cultures were split into two equal volumes. One-half of the cultures received an addition of L-leucine to a concentration of 1.3 mM (induced); the other half received an aliquot of water (uninduced control). Subsamples (130 ml) were withdrawn immediately prior to the addition of L-leucine ($t = 0$) and at 10, 30, 60, 120, and 180 min after L-leucine addition. Subsamples were rapidly chilled on ice, total cell protein was prepared from 100 ml of culture, and RNA was isolated from 30 ml of culture.

RESULTS

***ssy5* mutations result in histidine resistance and increased vacuolar pools of arginine and histidine.** Yeast strains carrying mutations in *SHR4* were isolated in a genetic selection for *shr* mutants resistant to 30 mM histidine (42). *SHR4* was cloned by complementation of the recessive 30 mM histidine-resistant phenotype of strain PLAS10-8C (*shr4-10*) (Materials and Methods). Subsequent sequence analysis indicated that *SHR4* is identical to ORF YJL156c, previously identified as *SSY5* (33). In addition to exhibiting resistance to 30 mM histidine, *ssy5* mutant strains have increased vacuolar pools of arginine and histidine (Table 3). Compared to the wild-type strain PLY1, the vacuolar levels of histidine and arginine in mutant strain PLAS10-8C (*ssy5-410*) were increased by two- and three-fold, respectively. In contrast, the levels of lysine remained unchanged. The observed increases in vacuolar amino acid pools resulting from the *ssy5* mutation are similar to those

observed in strains carrying mutations in *SSY1* and *PTR3* (Table 3) (34).

A deletion allele of *SSY5* was created by replacing the major portion of the coding region with the selectable *URA3* marker. This construct, *ssy5Δ1::hisG-URA3 kan*^{r} *hisG*, was introduced into haploid strains PLY1 (*ura3-52 his4Δ29*) and PLY126 (*ura3-52 lys2Δ201*) by transformation. Viable transformants were readily obtained, indicating that *SSY5* is not an essential gene. The resulting *ssy5Δ1* null mutant strains, HKY71 and HKY75, were resistant to 30 mM histidine. A diploid resulting from crossing HKY71 and the wild-type strain PLY4 did not grow in the presence of toxic levels of histidine, indicating that the *ssy5* null mutation is recessive. In contrast, the diploid strain obtained by crossing strains HKY71 (*MAT*a *ura3-52 his4Δ29 ssy5Δ1*) and PLAS10-9A [*MAT*α *ura3-52 his4Δ29 shr4-10* (*ssy5-410*)] grew well on medium containing 30 mM histidine. This diploid was sporulated, and meiotic segregants were analyzed by tetrad analysis. In all cases, the mutant phenotype segregated 4:0; all spore-derived colonies from 15 tetrads exhibited resistance to toxic levels of histidine. These results indicate that *SSY5* and *SHR4* are identical and that the originally isolated *shr4* alleles are likely to be loss-of-function mutations.

***ssy5* null mutant strains exhibit levels of resistance to toxic amino acids and azetidine carboxylate similar to those of *ssy1* and *ptr3* mutants.** The growth characteristics of isogenic wild-type, *ssy1Δ13, ptr3Δ15, ssy5Δ2, ssy1Δ13 ptr3Δ15, ssy5Δ2 ssy1Δ13*, and *ssy5Δ2 ptr3Δ15* strains were examined. All strains grew well on SPD and SD (Fig. 1A and D, respectively). Only the growth of the wild-type strain was inhibited on medium containing toxic levels of histidine (Fig. 1B), methionine (Fig. 1C), or L-azetidine-2-carboxylate (Fig. 1E). L-Azetidine-2-carboxylate is a proline analogue (38) that pleiotropically inhibits multiple amino acid permeases, including the general amino acid permease (*GAP1*) (29). In contrast to the wild-type strain, the *ssy5Δ2, ssy1Δ13*, and *ptr3Δ15* mutant strains grew equally well on each of the selective media and formed colonies of similar size. Thus, the *ssy5* null mutation manifests identical levels of resistance to either *ssy1* or *ptr3* null mutations. Additionally, strains carrying the possible double mutant combinations *ssy1Δ13 ptr3Δ15, ssy5Δ2 ssy1Δ13*, and *ssy5Δ2 ptr3Δ15* did not exhibit any additive affects.

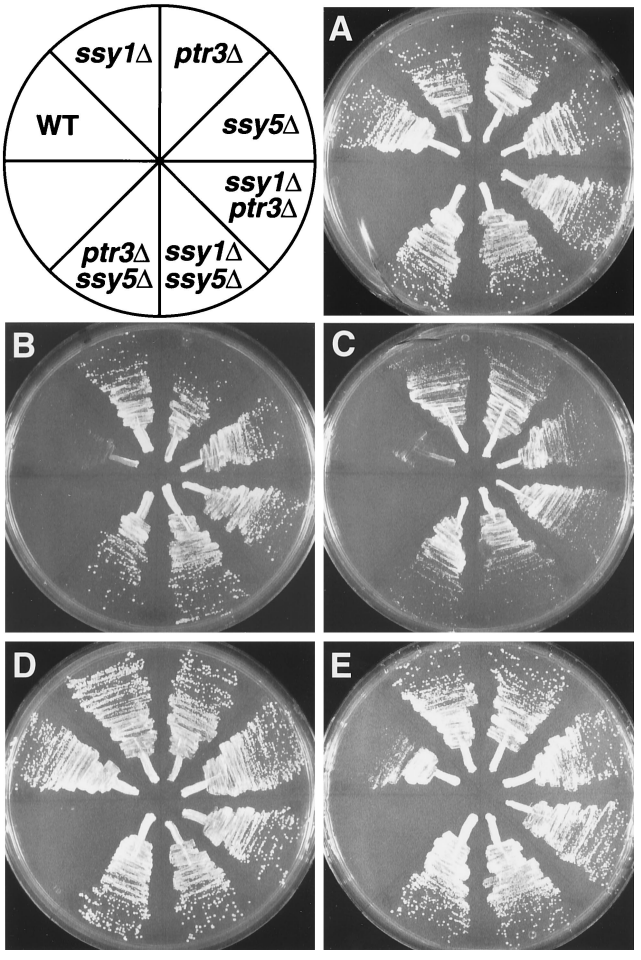

FIG. 1. Growth characteristics of strains carrying single and double mutant combinations of *ssy5*, *ssy1*, and *ptr3* null alleles. Strains PLY126 (wild type [WT]), HKY20 (*ssy1Δ13*), HKY31 (*ptr3Δ15*), HKY77 (*ssy5Δ2*), HKY33 (*ssy1Δ13 ptr3Δ15*), HKY84 (*ssy5Δ2 ssy1Δ13*), and HKY85 (*ssy5Δ2 ptr3Δ15*) were streaked onto the following media: SPD (plus uracil and lysine) (A), SPD (plus uracil and lysine) containing 30 mM L-histidine (B), SPD (plus uracil and lysine) containing 30 mM L-methionine (C), SD (plus uracil and lysine) (D), and SD (plus uracil and lysine) containing 100 μg of L-azetidine-2-carboxylate ml^{-1} (E). Plates were incubated for 3 days at room temperature and photographed.

***SSY5* is required for amino acid-induced expression of permeases.** Wild-type (PLY126) and *ssy5Δ2* (HKY77) strains carrying plasmids pRS316 and YCp405, which complement the *ura3-52* and *lys2Δ201* auxotrophies, respectively, were grown on SD medium without nutritional supplements to an OD_{600} of 0.8. Total RNA was isolated 45 min after the addition of 0.15 mM leucine or an equal volume of water, and the transcript levels of the broad-range amino acid permease (*AGP1*) (30) and the peptide transporter (*PTR2*) (52) were determined by Northern analysis (Fig. 2). The levels of expression were quantitated by phosphorimaging, and *ACT1* transcript levels were used to standardize quantitations. *AGP1* transcripts were detected in wild-type cells (Fig. 2A, lanes 1 and 2), but not in *ssy5* null mutant cells (Fig. 2A, lanes 3 and 4). The lack of detectable *AGP1* transcripts in *ssy5* mutant cells indicates that *SSY5* is required to maintain the basal *AGP1* expression observed in wild-type cells grown in the absence of exogenously added amino acids (Fig. 2, compare lanes 1 and 3). When leucine was added to wild-type cells, *AGP1* mRNA levels increased by fivefold (Fig. 2A, compare lanes 1 and 2). Similarly, leucine-induced wild-type cells have approximately 20-fold more *PTR2* transcripts than uninduced cells (Fig. 2B, compare lanes 1 and 2). In contrast, when leucine was added to *ssy5Δ2* cells, the levels of *AGP1* transcripts did not increase (Fig. 2A, lane 4), and *PTR2* (Fig. 2B, lane 4) did not accumulate to wild-type levels. These results indicate that *ssy5* mutations mimic the observed transcriptional defects exhibited by *ssy1* and *ptr3* mutations (4, 19, 30, 34).

Ssy5p is a peripheral membrane protein that associates with the plasma membrane. *SSY5* encodes a 76-kDa protein comprised of 687 amino acids that does not share significant sequence homology with other known proteins. A functional epitope-tagged version of Ssy5p was created by introducing the

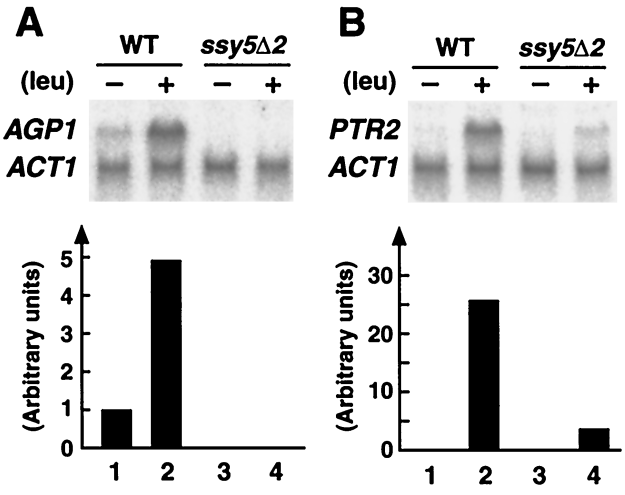

FIG. 2. Expression of *AGP1* and *PTR2* in wild-type (WT) and *ssy5* null mutant strains. Strains PLY126 (wild type) and HKY77 (*ssy5Δ2*) were grown in SD to an OD_{600} of 0.8, and total RNA was isolated 45 min after the addition of water (lanes 1 and 3) or 0.15 mM leucine (lanes 2 and 4). Expression levels of *AGP1* (A) and *PTR2* (B) were determined by Northern analysis and quantitated by phosphorimaging. The levels of actin (*ACT1*) transcript were used to standardize quantitations (lower panels). The relative expression levels were normalized to the expression levels in the uninduced wild-type strain.

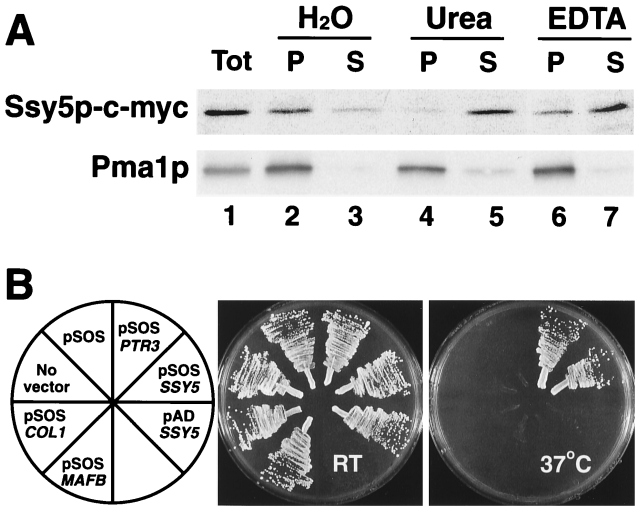

FIG. 3. *SSY5* encodes a peripherally associated PM protein. (A) The membrane association of Ssy5p was examined by using whole-cell lysates prepared from strain HKY77 expressing *SSY5-c-myc*. Aliquots of total protein lysate (Tot) were diluted 1:1 with H_2O, 1.6 M urea, or 2 mM EDTA; mixed; and incubated on ice for 30 min. Membrane pellet (P) and soluble (S) fractions, obtained after centrifugation at 100,000 × *g* for 45 min at 4°C, were resolved by SDS-PAGE and analyzed by immunoblotting. As a control, the membrane association of the PM ATPase (Pma1p) was monitored. (B) The ability of Ssy5p to associate with the PM was assessed by using the SOS membrane recruitment system. Strains cdc25H (No vector) and cdc25H transformed with plasmids pSOS, pSOS-*PTR3* (pHK044), pSOS-*SSY5* (pHK045), pAD-*SSY5* (pHK050), pSOS-*Col1*, and pSOS-*MAFB* were grown on YPD. Culture plates were incubated at room temperature (RT [permissive]) and 37°C (nonpermissive) as indicated, and after 4 days, the plates were photographed.

c-myc epitope at the extreme N terminus (see Materials and Methods). The *SSY5-c-myc* allele was judged to be functional based on its ability to complement the 30 mM histidine-resistant phenotype of *ssy5* mutants. The level of Ssy5p-c-myc present in whole-cell extracts was examined by SDS-PAGE and immunoblotting. Ssy5p-c-myc migrated as a 67-kDa protein, significantly faster than its predicted molecular weight, and the signal strength of the immunoreactive Ssy5p-c-myc band was rather weak. The low levels of Ssy5p within extracts are consistent with the low codon index bias (0.017) of the *SSY5* ORF.

Ssy5p is predicted to be a hydrophilic protein that lacks identifiable transmembrane domains and N-terminal ER targeting signal sequences. However, Ssy5p-c-myc was found to be enriched in the membrane fraction of whole-cell lysates together with the integral plasma membrane ATPase (Pma1p) (Fig. 3A, lanes 2 and 3), Ssy5p-c-myc could be displaced from membranes by treatment with urea (Fig. 3A, lanes 4 and 5) and the chelating agent EDTA (Fig. 3A, lanes 6 and 7). Pma1p was not extracted from the membrane under these conditions. These results suggest that Ssy5p is a peripherally associated membrane protein.

We examined whether Ssy5p was able to associate with the plasma membrane by using the Sos recruitment system (3). The Sos recruitment system exploits the ability of the human Cdc25p homologue, h-SOSp, to suppress the temperature-sensitive *cdc25-2* mutation in strain cdc25H (53). Fusion proteins that direct h-SOSp to the cytosolic face of the plasma membrane enable cdc25H cells (*cdc25-2*) to grow at 37°C (2). Strain cdc25H was transformed with pSOS, pSOS-*PTR3* (pHK044), pSOS-*SSY5* (pHK045), and pAD40-*SSY5* (pHK050) and negative control plasmids pSOS-*Coll* (murine type IV collagenase, amino acids 148 to 357) (12) and pSOS-*MAFB* (full-length MafB) (3). Transformants were selected at room temperature on SC (no Leu). Leu^+ transformants were streaked on to two YPD plates. One plate was incubated at room temperature, and the other was incubated at 37°C. After 4 days, the plates were photographed (Fig. 3B). All transformants grew at similar rates on the YPD plate incubated at room temperature. Only transformants carrying plasmids expressing h-SOSp as a fusion protein with either Ssy5p (pSOS-*SSY5*) or Ptr3p (pSOS-*PTR3*) grew at 37°C. Transformants carrying the other plasmids were unable to form colonies at the nonpermissive temperature.

These results indicate that h-SOSp expressed alone (pSOS), or fused to control proteins that do not interact with the plasma membrane (PM), such as transcription factor MafB (pSOS-*MAFB*) and collagenase 1 (pSOS-*Coll*), is unable to suppress the *cdc25-2* mutation. Additionally, transformants carrying pAD-*SSY5* did not grow at 37°C, indicating that by itself, Ssy5p is not able to activate the essential Ras signaling pathway. These findings demonstrate that h-SOSp fusion proteins containing either Ssy5p or Ptr3p associate with the PM, thereby enabling h-SOSp to carry out its function. The ability of Ssy5p and Ptr3p to recruit h-SOSp to the PM is consistent with the finding that Ssy5p fractionates as a peripheral membrane protein (Fig. 3A) and previous localization studies regarding Ptr3p (34).

***SSY1* and *PTR3* are required for the proper expression of Ssy5p.** We compared the levels and electrophoretic properties of Ssy5p-c-myc in whole-cell extracts isolated from wild-type

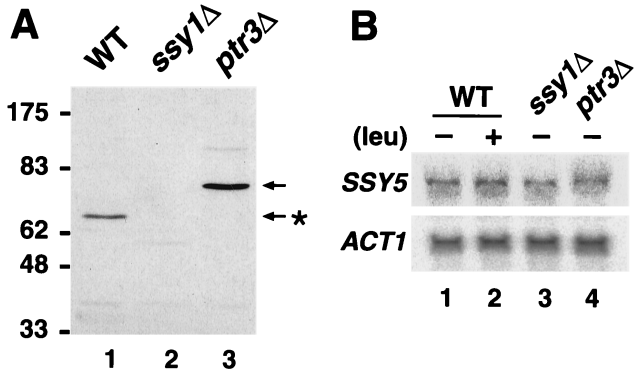

FIG. 4. Functional expression of Ssy5p requires *SSY1* and *PTR3*. (A) Strains HKY77 (wild type [WT]; lane 1), HKY84 (*ssy1Δ13*; lane 2), and HKY85 (*ptr3Δ15*; lane3) expressing *SSY5-c-myc* were grown in SD to an OD_{600} of 0.8, and the levels of Ssy5p-c-myc were analyzed in whole-cell lysates by SDS-PAGE and immunoblotting. Prior to electrophoresis, samples were denatured for 10 min at 37°C. (B) Total RNA was prepared from strains HKY77 (wild-type; lanes 1 and 2), HKY84 (*ssy1Δ13*; lane 3), and HKY85 (*ptr3Δ15*; lane 4) expressing *SSY5-c-myc* grown to an OD_{600} of 0.8 in SD (lanes 1, 3, and 4) or SD supplemented with 1.3 mM leucine (lane 2). The levels of *SSY5* mRNA were analyzed by Northern blotting, and the levels of actin (*ACT1*) transcripts were used to control loading.

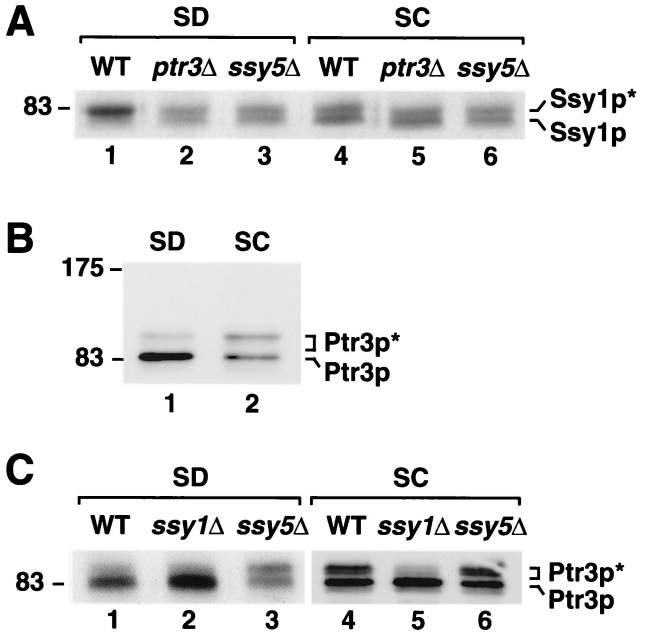

FIG. 5. SPS sensor component interactions. (A) The pattern of Ssy1p migration during SDS-PAGE is dependent upon Ptr3p and Ssy5p and is altered in response to amino acids. Whole-cell lysates were prepared from strains HKY20 (wild-type [WT]; lanes 1 and 4), HKY33 (*ptr3Δ15*; lanes 2 and 5), and HKY84 (*ssy5Δ2*; lanes 3 and 6) transformed with pHK010 (*SSY1-HA1*) grown in SD (lanes 1 to 3) and SC (lanes 4 to 6) to an OD_{600} of 0.8. The levels of Ssy1p-HA1 in extracts were analyzed by SDS-PAGE and immunoblotting. (B) The electrophoretic mobility of Ptr3p is altered in response to amino acids. Whole-cell lysates were prepared from wild-type strain HKY31 transformed with pHK018 (*PTR3-HA1*) grown in SD (lane 1) and SC (lane 2) to an OD_{600} of 0.8. The levels of Ptr3p-HA1 in extracts were analyzed by SDS-PAGE and immunoblotting. (C) The pattern of Ptr3p migration during SDS-PAGE is dependent upon Ssy1p and Ssy5p. Whole-cell lysates were prepared from strains HKY31 (wild type; lanes 1 and 4), HKY33 (*ssy1Δ*; lanes 2 and 5), and HKY85 (*ssy5Δ*; lanes 3 and 6) transformed with pHK018 (*PTR3-HA1*) grown in SD (lanes 1 to 3) and SC (lanes 4 to 6) to an OD_{600} of 0.8. The levels of Ptr3p-HA1 in extracts were analyzed by SDS-PAGE and immunoblotting. Note that lane 2 in panel B and lane 4 in panel C are derived from the same sample. A longer exposure time was used for a more detailed analysis of the protein bands in lanes 4 to 6.

(HKY77), *ssy1Δ13* (HKY84), and *ptr3Δ15* (HKY85) null mutant strains (Fig. 4A). As previously indicated, Ssy5p-c-myc migrates as a 67-kDa protein (Ssy5p*) in extracts isolated from wild-type cells (Fig. 4A, lane 1). We were unable to detect Ssy5p-c-myc in extracts prepared from *ssy1* null mutant cells (Fig. 4A, lane 2). In extracts derived from *ptr3* null mutants, Ssy5p-c-myc migrated as a 76-kDa protein, a mobility that corresponds to the predicted molecular mass of Ssy5p (Fig. 4A, lane 3). Although the data presented in Fig. 4 were obtained by using extracts isolated from strains grown in SD medium without amino acids, similar observations regarding the behavior of Ssy5p-c-myc in *ssy1* and *ptr3* null mutants were made when strains were grown in SC medium. Faint immunoreactive protein bands, corresponding to twice the molecular weight of the expressed Ssy5p, were observed in extracts prepared from wild-type and *ptr3Δ* cells (most easily seen in Fig. 4A, lane 3). Consistent with Ssy5p being a membrane protein, the intensity of the slower-migrating bands varied, dependent upon the denaturing conditions used, and increased when samples were subjected to higher denaturing temperatures.

We examined the possibility that the inability to detect Ssy5p-c-myc in extracts of *ssy1Δ* null mutants was due to the lack of transcription of *SSY5* in these mutants. Northern blot analysis showed that the amounts of *SSY5-c-myc* mRNA were similar in both wild-type and *ssy1Δ* cells (Fig. 4B, compare lanes 1 and 3). This finding indicates that *SSY5* transcription is independent of sensor function, a conclusion that is supported by the fact that *SSY5* is transcribed normally in the absence of *PTR3* (Fig. 4B, lane 4). Furthermore, wild-type cells grown in the presence of leucine, at concentrations known to affect the transcription of amino acid permease genes (e.g., *AGP1* [see Fig. 2]), did not exhibit altered levels of *SSY5* expression (Fig. 4B, lanes 1 and 2). Together the data presented in both panels of Fig. 4 indicate that Ssy5p is unstable in the absence of Ssy1p and that Ssy5p is likely to be posttranscriptionally modified in a Ptr3p-dependent manner.

Ssy1p and Ptr3p exhibit altered electrophoretic properties dependent upon amino acid availability and sensor component interactions. Our finding that the functional expression of Ssy5p requires both *SSY1* and *PTR3* (Fig. 4) prompted us to examine the levels and electrophoretic properties of Ssy1p and Ptr3p. Whole-cell extracts containing functional epitope-tagged Ssy1p (Ssy1p-HA1) (34) were isolated from wild-type strains grown in media without (SD) and with (SC) added amino acids. In addition, extracts were prepared from various mutant strains lacking sensor function. In wild-type (HKY20) cells grown in SD (Fig. 5A, lane 1), Ssy1p migrates as a single major band (Ssy1p*). In cells grown in SC, a second lower band (Ssy1p) becomes evident, and the intensity of the upper Ssy1p* band is clearly diminished (Fig. 5A, lane 4). The pattern of Ssy1p staining observed in *ptr3* and *ssy5* null mutant strains is

similar to that observed in wild-type strains grown in SC (Fig. 5A, compare lanes 5 and 6 with lane 4). This pattern is also seen in mutant strains grown in the absence of amino acids (Fig. 5A, lanes 2 and 3).

The expression of functional epitope-tagged Ptr3p (Ptr3p-HA1) (34) was similarly examined. Multiple forms of Ptr3p were observed in extracts obtained from all strains, regardless of the amino acid content of the growth medium. In SD-grown wild-type cells (HKY31), the majority of Ptr3p is present in a faster-migrating band (Ptr3p); however, a faint and slower-migrating band (Ptr3p*) is also evident (Fig. 5B, lane 1). The difference in apparent molecular mass between the slower- and faster-migrating forms of Ptr3p is approximately 15 to 20 kDa. In cells grown on SC, the relative intensity of the upper Ptr3p* band increased, and that of the lower band diminished (Fig. 5B, lane 2). When immunoblots were exposed for longer times (Fig. 5C, lane 4), the upper band was shown to be comprised of at least two bands. In SC-grown *ssy1* null mutant cells, Ptr3p exhibited the same pattern of expression observed in both SD-grown wild-type cells and *ssy1* null mutant cells (Fig. 5C, compare lane 5 with lanes 1 and 2). The slower-migrating bands (Ptr3p*) were barely detectable in *SSY1*-deleted cells (Fig. 5C, lanes 2 and 5). In contrast, the electrophoretic properties of Ptr3p in SD-grown *ssy5* null mutant cells appear similar to those observed in SC-grown wild-type cells (Fig. 5C, compare lane 3 with lane 4).

The data presented in Fig. 5 indicate that Ssy1p and Ptr3p are differentially modified and that multiple forms of Ssy1p and Ptr3p exist in cells. The electrophoretic properties of the slower-migrating forms of Ssy1p and Ptr3p, Ssy1p* and Ptr3p*, respectively, are likely to result from posttranslational modifications. We examined the possibility that Ssy1p* and Ptr3p* were phosphorylated by incubating whole-cell extracts in the presence of various amounts of alkaline phosphatase (25 to 250 U per mg of protein). Under the conditions used, phosphatase treatment did not diminish the intensity of the Ssy1p* or Ptr3p* bands. We also examined the possibility that the large difference in the apparent molecular weight of Ptr3p and Ptr3p* was due to ubiquitination by overexpressing c-myc-tagged ubiquitin (22). We did not observe an upward shift of either Ptr3p or Ptr3p* in strains overexpressing the tagged ubiquitin construct. Although these results appear to rule out the possibility of phosphate and ubiquitin modification, they are negative in nature and thus need to be confirmed by further experimentation. Finally, we have observed that the total levels of Ssy1p and Ptr3p are consistently reduced in extracts from cells grown in the presence of amino acids. The quantitative analysis of the immunoreactive bands in Fig. 5 indicates that the levels of Ssy1p and Ptr3p are reduced by 15 and 50%, respectively, in SC-grown cells (Fig. 5A, lanes 1 and 4; B, lanes 1 and 2).

Overexpression of Ssy1p or the N terminus of Ssy1p disrupts amino acid sensor function. We examined the effects of individually overproducing *SSY1, PTR3*, and *SSY5*. The wild-type strain PLY1 was separately transformed with 2μm-based plasmids containing these genes as inserts. Ura^+ transformants were selected on SC − Ura, cells derived from single colonies were resuspended in water, and dilution series were analyzed on minimal SPD medium supplemented with 0.16 mM histidine (SPD) or toxic levels of histidine (SPD plus 5 mM His).

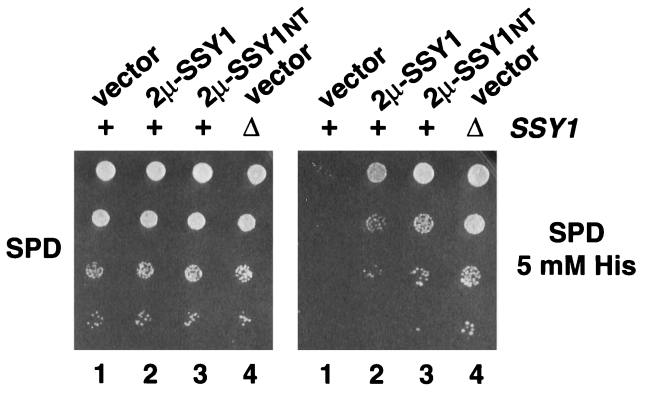

FIG. 6. Overexpression of Ssy1p or the N terminus of Ssy1p exerts a dominant-negative effect on amino acid sensor function. Dilution series of strain PLY1 transformed with pRS202 (vector; lane 1), pHK012 (2μm-*SSY1*; lane 2), pHK039 (2μm-$SSY1_{NT}$; lane 3), and strain HKY37 (*ssy1*Δ*13*) transformed with pRS202 (vector; lane 4) were spotted onto SPD and SPD supplemented with 5 mM histidine as indicated. Culture plates were incubated for 4 days at room temperature and photographed.

Transformants carrying plasmids *PTR3* (pHK026) or *SSY5* (pHK037) grew well on SPD containing 0.16 mM histidine, indicating that the overexpression of Ptr3p and Ssy5p did not have any deleterious effects on growth; however, these strains were unable to grow on SPD supplemented with toxic levels of histidine.

In contrast, strains carrying *SSY1* (pHK012) grew well on SPD and on SPD containing 5 mM histidine (Fig. 6, dilution series 2). Similarly, transformants overexpressing only the first 206 N-terminal amino acids of Ssy1p (pHK039, 2μm-$SSY1_{NT}$) grew well on both SPD and SPD (plus 5 mM His) (Fig. 6, dilution series 3). Transformants overexpressing the N-terminal domain grew nearly as well as the *ssy1* null mutant strain (Fig. 6, dilution series 4). The ability of these transformants to grow in the presence of toxic levels of histidine indicates that the overexpression of Ssy1p or the extended hydrophilic N-terminal portion of Ssy1p disrupts sensor function. The fact that plasmids pHK012 and pHK039 enabled the growth of wild-type cells, which are otherwise unable to grow on SPD containing 5 mM histidine (Fig. 6, dilution series 1), demonstrates that overexpression of Ssy1p or the N-terminal region of Ssy1p exerts dominant-negative effects. The expression of only the N terminus of Ssy1p was not able to suppress *ssy1* mutant alleles. These results are consistent with out previous findings that the N terminus of Ssy1p, the portion of Ssy1p absent from the other members of the amino acid permease gene family, has an important role in sensor function (34).

Time course of amino acid sensor-dependent transcriptional induction. Previous work has demonstrated that leucine is a potent inducer of *PTR2* (4) and *CAR1* (21) transcription. We have shown that the leucine-induced transcription of these genes is dependent on all three sensor components, *SSY1, PTR3*, and *SSY5* (Fig. 2) (34). The time course of leucine-induced transcription of *PTR2* and *CAR1* was examined. The level of *PTR2* transcripts increased almost 40-fold within 30 min after cells received an aliquot of 1.3 mM leucine (Fig. 7A, lanes 2 to 6). After 30 min, the level of *PTR2* transcripts gradually decreased, and after 180 min, the level of *PTR2*

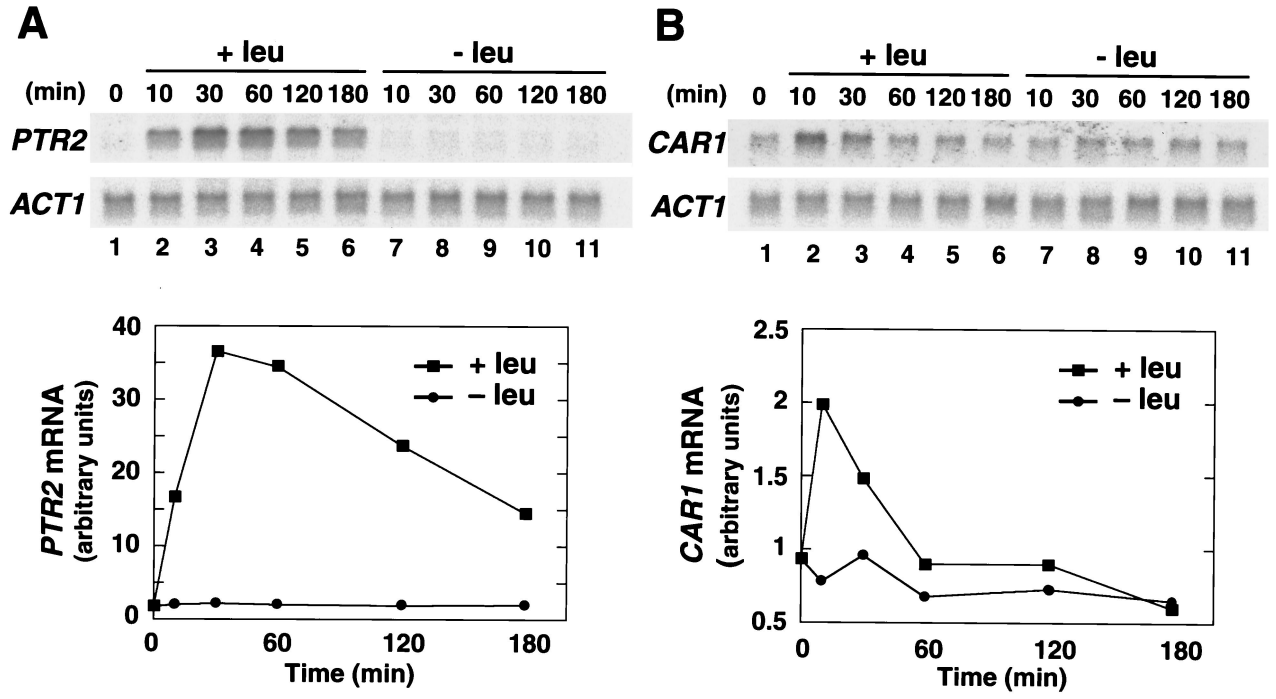

FIG. 7. Time course of L-leucine-induced transcription of *PTR2* and *CAR1*. A liquid culture of strain HKY77 carrying plasmid pHK048 (*SSY5-c-myc*) was grown in SD to an OD_{600} of 0.5, and the culture was split into two parts ($t = 0$). One-half of the cell culture received an aliquot of L-leucine (+leu; lanes 2 to 6) to a final concentration of 1.3 mM, and the other received an equal volume of water (−leu; lanes 7 to 11). Both cultures were incubated at 30°C for an additional 180 min, and at the times indicated, subsamples were withdrawn and total RNA was isolated. The levels of *PTR2* (A) and *CAR1* (B) expression were analyzed by Northern blotting (upper panels), and after background correction, signal strengths (arbitrary units) relative to the levels of actin mRNA (*ACT1*) were quantitated (lower panels).

transcripts was down to half of the maximum level. The time course of leucine-induced *CAR1* transcription (Fig. 7B, lanes 2 to 6) exhibited a faster response; however, in general, the pattern of induction was similar. Ten minutes after the addition of leucine, the level of *CAR1* transcripts increased twofold, and after 60 min, *CAR1* transcripts were restored to basal levels. No increase in *PTR2* or *CAR1* transcription was observed in parallel uninduced cultures (Fig. 7, lanes 7 to 11). Thus, in the presence of inducing amino acids, cells transiently upregulate the expression of *PTR2* and *CAR1*. Similar results regarding the transcription of the branched-chained amino acid transporter genes *BAP2* and *BAP3* have been reported (15).

Time course of amino acid-induced sensor component modifications and sensor downregulation. We examined whether the observed amino acid-induced changes in the electrophoretic mobility of Ssy1p and Ptr3p (Fig. 5) and Ssy5p occur in a similar time frame as sensor-dependent transcriptional induction (Fig. 7). Strains expressing *SSY1-HA1, PTR3-HA1*, and *SSY5-c-myc* were grown in liquid cultures of SD to an OD_{600} of 0.5. Each culture was divided into two culture flasks: one received an aliquot of leucine to a final concentration of 1.3 mM, and the other received an equal volume of water. At various times, subsamples were removed, whole-cell lysates were prepared, and the levels and electrophoretic characteristics of Ssy1p-HA1, Ptr3p-HA1, and Ssy5p-c-myc were analyzed by immunoblotting (Fig. 8).

At time zero (Fig. 8, lane 1) and in a control culture that did not receive an aliquot of leucine (Fig. 8, lane 7), Ssy1p is predominantly expressed in its slower-migrating form (Ssy1p*), Ptr3p is principally present in its faster-migrating from, and Ssy5p is readily detected in its Ptr3p-dependent processed form (Ssy5p*). After the addition of leucine, the levels of immunologically detectable Ssy5p rapidly decreased; after 5 min, 25% of Ssy5p remained; and after 60 min, Ssy5p levels were 10-fold lower than in the starting culture. The levels of Ssy1p and Ptr3p also decreased—however, at a markedly slower rate and to a lesser extent than Ssy5p. After 120 min, the total levels of Ssy1p and Ptr3p were twofold lower than in the starting culture. It should be noted that the quantitations graphically presented in Fig. 8B were calculated based on the combined signal strength of both Ssy1p and Ssyp1* and Ptr3p and Ptr3p*, respectively. Two hours after leucine was added, the sensor components exhibited similar characteristics to those isolated from SC-grown cells (Fig. 5). The level of each component was reduced, Ssy1p* was not the predominant species of Ssy1p, and the levels of Ptr3p* were similar to those of Ptr3p. Thus the addition of leucine induced rapid and long-term changes that apparently result in the down regulation of all three sensor components.

DISCUSSION

We have found that *SSY5* encodes a third component of the yeast plasma membrane sensor of extracellular amino acids. Ssy5p functions together with the two previously characterized sensor components, Ssy1p1 and Ptr3p, to regulate diverse met-

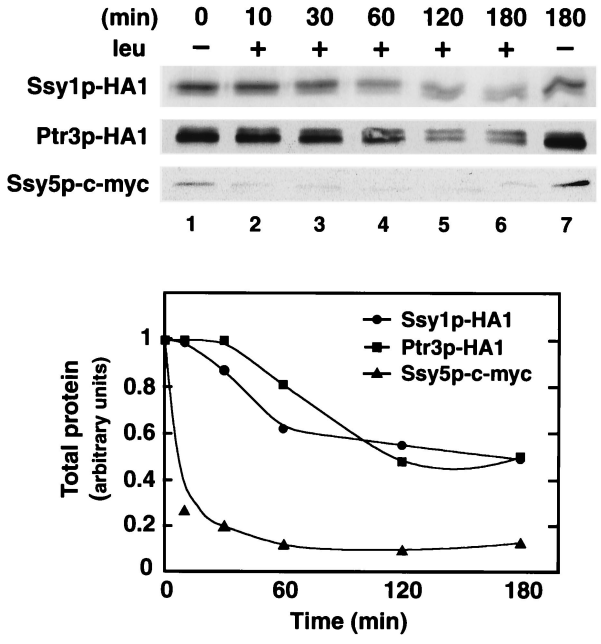

FIG. 8. Time course analysis of the physical alterations to Ssy1p, Ptr3p, and Ssy5p after induction of sensor function by L-leucine. Strains HKY20, HKY33, and HKY77 expressing *SSY1-HA1*, *PTR3-HA1*, and *SSY5-c-myc*, respectively, were grown in liquid cultures of SD to an OD_{600} of 0.5 ($t = 0$; lane 1). At $t = 0$, each culture received an aliquot of L-leucine to a final concentration of 1.3 mM (lanes 2 to 6). At the times indicated, subsamples were removed, whole-cell lysates were prepared, and proteins were analyzed by immunoblotting (upper panel). Lane 7 shows protein levels present in an uninduced control culture similarly incubated for 180 min. The corresponding chemiluminescent signals were quantitated (lower panel).

abolic processes important for proper amino acid uptake and compartmentalization. The conclusion that Ssy5p is a component of the plasma membrane amino acid sensor is based on several observations. First, mutations in *SSY5* belong to the same epistasis group as *ssy1* and *ptr3* mutations. Ssy5 mutants exhibit similar increases in vacuolar pools of histidine and arginine (Table 3). *ssy5* null mutants display identical levels of resistance to toxic amino acids and azetidine carboxylate, and the *ssy5*Δ *ssy1*Δ, *ssy5*Δ *ptr3*Δ, and *ssy1*Δ *ptr3*Δ double mutant strains exhibit levels of resistance identical to those of each of the single mutant strains. Additionally, *SSY5* is required for amino acid-induced transcription of two genes, *AGP1* and *PTR2*, known to be controlled by *SSY1* and *PTR3* (Fig. 2). The resistance to toxic amino acids and amino acid analogues is likely to be a consequence of the altered uptake and increased capacity to compartmentalize amino acids (Table 3) (34). Second, functional epitope-tagged Ssy5p-c-myc fractionates as a peripherally bound membrane protein, and h-SOS-Ssy5p fusion proteins are recruited to the cytosolic face of the plasma membrane in an Ssy5p-dependent manner (Fig. 3). The observation that Ssy5p, which is predicted to be a soluble protein, is able to associate with the plasma membrane, directly implicates this protein as a constituent of this sensing system. Third, the proper expression of Ssy5p, but not the transcription of *SSY5*, requires both *SSY1* and *PTR3* (Fig. 4). Finally, the electrophoretic properties of both Ssy1p and Ptr3p are altered in *ssy5* null mutant strains (Fig. 5A and C).

The demonstrated ability of Ssy1p, Ptr3p, and Ssy5p to localize to the plasma membrane is consistent with the possibility that these components associate within a sensor complex, although there is as yet no direct evidence for a physical association between them. Of the three identified sensor components, Ssy1p is the only integral membrane-spanning component; thus, Ssy1p is likely to be the component that transmits signals across the PM. The in vivo membrane topology of the general amino acid permease (Gap1p) has recently been determined; both the N- and C-terminal domains are oriented towards the cytoplasm (24). Based upon the sequence and structural homology that exists between Ssy1p and the other members of the amino acid permease gene family members, it is likely that the N terminus of Ssy1p is also cytoplasmically oriented. The large N-terminal extension of Ssy1p may serve to organize the assembly of the other peripheral membrane components. Ssy5p is a strong candidate for directly interacting with Ssy1p. In the absence of Ssy1p, Ssy5p is unstable, as evidenced by our inability to detect Ssy5p in protein lysates derived from *ssy1* null mutants (Fig. 4A). The fact that Ssy5p is degraded in the absence of Ssy1p prevented us from directly assessing whether its localization to the PM is dependent upon Ssy1p. We have previously found that the Ptr3p localizes to the plasma membrane independently of Ssy1p (34).

We further investigated the functional relationships between the SPS sensor components and made several observations that indicate that these three components do indeed intimately interact with one another. We have found that each of the components displays physical properties that are dependent upon the availability of amino acids and on the presence of the other two components. In wild-type cells grown in SD, Ssy1p* is the predominating form of Ssy1p, whereas in *ptr3*Δ or *ssy5*Δ null mutants it is not (Fig. 5A). Thus, in mutant cells lacking either *PTR3* and *SSY5*, Ssy1p exhibits characteristics that mimic those of Ssy1p isolated from wild-type cells grown in the presence of amino acids. Additionally, we have consistently observed that cells lacking either Ptr3p and Ssy5p express less Ssy1p (Fig. 5A). Ptr3p migrates as several bands that exhibit significant differences in mobility on SDS gels (Fig. 5B). The presence of the slower-migrating Ptr3p* species in amino acid-containing medium is dependent on *SSY1*, but not *SSY5* (Fig. 5C); however, in SD-grown cells lacking *SSY5*, Ptr3p exhibits characteristics identical to those of SC-grown wild-type cells (Fig. 5C). In wild-type cells, Ssy5p is apparently proteolytically modified in a *PTR3*-dependent manner (Fig. 4A). Because the c-myc epitope used to visualize Ssy5p is located within the N terminus, the Ptr3p-dependent processing event is likely to occur within the C-terminal portion of Ssy5p. The inability to detect Ssy5p in whole-cell extracts prepared from strains carrying *ssy1* mutations (Fig. 4A) is presumably the consequence of a proteolytic cleavage event that minimally removes its N terminus.

The observation that overexpression of the first 206 amino acids comprising the N-terminal extension of Ssy1p disrupts sensor function in a dominant-negative manner (Fig. 6) is also consistent with the existence of a multicomponent sensor complex. This finding clearly shows that the N-terminal domain of Ssy1p has an important role in sensor function, a conclusion supported by our previous observation that small in-frame mutations within the N-terminal domain abolish signaling (34).

These results suggest that functional SPS sensor complexes assemble with a precise stoichiometry. Consequently, the overproduction of the N-terminus Ssy1p would interfere with SPS sensor function by forming nonproductive complexes with proteins normally interacting with Ssy1p. These nonproductive interactions would effectively decrease the availability of the limiting components to form functional sensor complexes. Similarly, dominant-negative phenotypes associated with the mutations in the cytoplasmically oriented C terminus of the G-protein-coupled alpha-factor receptor (*STE2*) have been observed to exert their effects by sequestering G-proteins (20, 39). The overexpression of full-length Ssy1p also exhibited dominant-negative effects; this unexpected observation may be the result of a fraction of Ssy1p being mislocalized. Accordingly, limiting sensor components would be sequestered at inappropriate intracellular membranes.

When leucine is added to wild-type cells grown in medium without supplementary amino acids, the transcription of *PTR2* and *CAR1* is transiently induced (Fig. 7). The response is quite rapid; within 10 min, there is a 15-fold induction of *PTR2* and a 2-fold induction of *CAR1*. After reaching maximum levels (*PTR2*, 30 min; *CAR1*, 10 min), their transcript levels slowly adjust back to basal levels. Similar patterns of induction have been reported for the branched-chain amino acid permeases (*BAP2* and *BAP3*) (15). Concurrent with its effect on transcription, leucine stimulates the components of the SPS sensor to become physically modified and causes the levels of each of the SPS sensor components to diminish (Fig. 8). The rapid physical alterations and reduced levels of sensor components are consistent with their being downregulated in response to amino acid availability. It is important to note that in cells grown in medium supplemented with amino acids, the downregulated sensor components are necessary to maintain the steady-state transcript levels of *AGP1* and *PTR2* (Fig. 2) and the glutamine permease (*GNP1*) (34). Additionally, the downregulated SPS sensor is required to fully repress the functional expression of Gap1p (34).

Regarding the leucine-induced mobility changes and downregulation of SPS sensor components (Fig. 8), we have defined three sensor states, each associated with defined patterns of component expression (see Fig. 9 for a schematic presentation). In the absence of extracellular amino acids, the state I, or preactivation, conformation, Ssy1p migrates predominantly in its slower-migrating form (Ssy1p*), Ptr3p is primarily present in its faster-migrating form, and Ssy5p is readily detected in its Ptr3p-dependent low-molecular-mass processed form (Ssy5p*). Within minutes after the addition of leucine, rapid changes are observed, resulting in the transient state IIa sensor complex (Fig. 9). The characteristics of each component within the state IIa sensor correspond to those observed after 10 min of leucine addition (Fig. 8). The state IIa sensor is defined by the low levels of Ssy5p; the electrophoretic characteristics of the other two components appear unchanged. With increasing time (30- and 60-min time points) (Fig. 8), shifts in the migration of Ssy1p and Ptr3p become increasingly obvious (Fig. 9, state IIb conformation). Within the transitional state IIb complex, the levels of Ssy1p* decrease and the relative proportion of the slower-migrating form of Ptr3p (Ptr3p*) increases. The state III configuration, evident 120 min after leucine addition, is indistinguishable from the downregulated sensor conformation

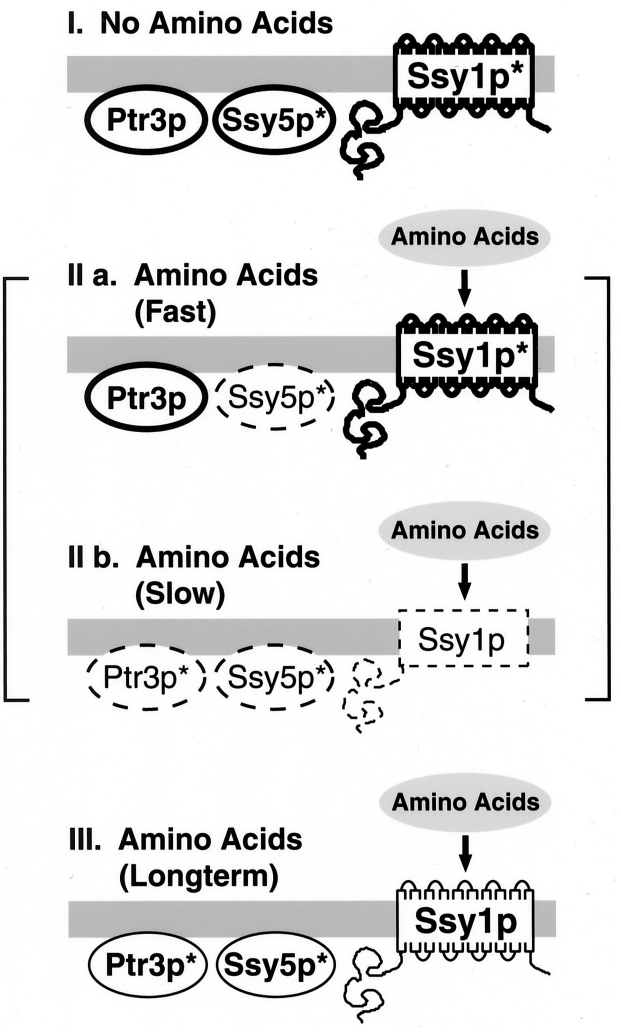

FIG. 9. Schematic diagram summarizing the dynamic characteristics of the SPS sensor component interactions. The state I sensor is the complex present in cells grown in the absence of amino acids. The SPS components are present in high levels, represented by the heavy outlines. In analogy to the G-protein-coupled α-factor receptor complex in *MAT***a** cells (39), the state I conformation may represent a preactivation complex. States IIa and IIb are transient complexes that rapidly form when cells grown in the absence of amino acids are induced by amino acids. The components in the transient state IIa and IIb sensor undergoing dynamic changes in expression levels are represented by the dashed outlines. The state III conformation is the downregulated complex, and the diminished levels of the components are represented by light outlines. For additional details, see text.

that exists in cells grown in SC media. It is possible that the downregulated sensor conformation is actually comprised of a mixed sensor population; the patterns of bands associated with the state I conformation are still evident in the state III sensor.

In our analysis to date, we have defined three components of a plasma membrane sensor of extracellular amino acids. Further biochemical and genetic analysis is necessary to ascertain if these components represent the entire primary sensing complex or if other components exist. The isolation of dominant constitutively activated alleles in any of the genes encoding SPS sensor components would enable epistasis relationships to be better defined. Because the SPS sensor components are

localized to the plasma membrane, it is possible that the SPS sensor components, particularly Ssy1p, are subject to regulation by posttranslational mechanisms known to regulate amino acid permeases and other metabolite transporters. In response to ammonium, Gap1p is dephosphorylated and polyubiquitinated (27, 60). Similar mechanisms, as well as substrate-induced feedback inhibition, operate to regulate the uracil permease (23, 58). Finally, other proteins associated with the plasma membrane regulate amino acid uptake. For example, the target of rapamycin pathway, in a manner resembling the SPS sensor, inversely regulates the activity of general and specific amino acid permeases (6, 7, 55). ScRheb (YCR027c) is a member of a new class of farnesylated small G-proteins of the Ras superfamily, that negatively regulates the uptake of arginine by Can1p (61). This regulation is thought to occur at the plasma membrane directly with or through other proteins interacting with the Can1p permease. The fact that yeast plasma membrane nutrient sensors have only recently been discovered reveals how little is understood regarding the molecular signals that enable yeast to adapt to constantly changing environments. Many more novel discoveries can be expected.

ACKNOWLEDGMENTS

We thank Annalena Moliner for technical assistance during the cloning of *SHR4* and Carolyn Slayman for the generous gift of Pma1p antibodies. We thank members of the Ljungdahl laboratory and members of Morten Kielland-Brandt's group at the Carlsberg Laboratory (Copenhagen) for fruitful discussions.

This work was supported by the Ludwig Institute for Cancer Research. The cooperative research agreement between LICR-Stockholm Branch and Fuji Photo Film (Europe) is gratefully acknowledged.

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