

The N-Terminal Regulatory Domain of Stp1p Is Modular and, Fused to an Artificial Transcription Factor, Confers Full Ssy1p-Ptr3p-Ssy5p Sensor Control

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Received 8 March 2004/Returned for modification 26 April 2004/Accepted 10 June 2004

Stp1p and Stp2p are homologous and redundant transcription factors that are synthesized as latent cytoplasmic proteins with N-terminal regulatory domains. In response to extracellular amino acids, the plasma membrane-localized Ssy1p-Ptr3p-Ssy5p (SPS) sensor induces an endoproteolytic processing event that cleaves away the N-terminal regulatory domains. The shorter forms of Stp1p and Stp2p are targeted to the nucleus, where they bind and activate the transcription of amino acid permease genes. A novel genetic screen, specifically designed to search for rare mutations that affect the SPS-sensing pathway, identified the F-box protein Grr1p as an obligatory factor required for Stp1p/Stp2p processing. Additionally, we have found that a null mutation in the *ASII* (amino acid sensor-independent) gene enables full-length unprocessed Stp1p/Stp2p to enter the nucleus and derepress SPS sensor-dependent genes. The N-terminal domains of Stp1p/Stp2p contain two conserved motifs that are required for proper nuclear exclusion and proteolytic processing. These motifs function in parallel; mutations that abolish processing inhibit signaling, whereas mutations that interfere with cytoplasmic retention result in constitutive derepression of SPS sensor-regulated genes independently of processing. The N-terminal domain of Stp1p is functionally autonomous and transferable to other transcription factors, where its presence confers *ASII*-dependent nuclear exclusion and SPS sensor-induced proteolytic processing.

All cells have the capacity to sense and respond to extracellular environmental cues by changing patterns of gene expression. Eukaryotic cells can regulate patterns of gene expression simply by controlling the movement of transcription factors across the nuclear envelope. For example, cells can synthesize transcription factors as latent forms that are excluded from entering the nucleus and, in response to discrete environmental cues, activate processes that induce their nuclear targeting. Several latent transcription factors that transfer regulatory information from the plasma membrane to the nucleus in metazoan cells have been described (9). Regulated latent transcription factors involved in signal transduction include the well-studied NF- κ B/Relish, Cubitus interruptus, and Notch proteins (4, 5, 20). In such instances, nuclear targeting not only is used to provide signals necessary for the correct timing of gene regulation but also provides the means to physically transfer signals from nonnuclear compartments to specific promoter sequences.

Fundamental to understanding regulated latent transcription factors is the elucidation of the mechanisms that direct these proteins to change compartments in a controlled fashion. This change may be achieved through a protein modification that regulates the activity of intrinsic nuclear localization determinants; nuclear localization sequences (NLS) are sequence motifs recognized by the nuclear import machinery and, in the opposing manner, nuclear export sequences (NES) are sequence motifs that mediate nuclear export. Although NLS and NES motifs are required for directed nuclear import and export, respectively, perhaps the simplest way to regulate nuclear

targeting is through physically tethering or anchoring latent precursor forms of transcription factors outside the nucleus. The first example of such a mechanism is the sterol regulatory element-binding protein (SREBP) (10). SREBP is an integral membrane protein that is anchored in the membranes of the early secretory pathway. The cytoplasmically oriented domain possessing transactivation activity is released from membranes in two successive rounds of proteolytic processing by site-specific membrane-bound proteases (38).

The unicellular budding yeast *Saccharomyces cerevisiae* was recently found to assess the availability of extracellular nutrients through sensors in the plasma membrane. Sensors, presumably functioning as receptors, that recognize small molecules, such as sugars or amino acids, have been identified (for a review, see reference 18). Ssy1p is a nutrient receptor that functions together with the two peripheral membrane-associated proteins, Ptr3p and Ssy5p, as a sensor of extracellular amino acids. This function was initially demonstrated by the observation that amino acid permease promoters are derepressed upon the addition of small amounts of amino acids (13). Amino acid induction requires all three sensor components, Ssy1p-Ptr3p-Ssy5p (SPS), and the F-box protein encoded by *GRR1* (7, 13, 17, 25, 26, 29). The participation of Grr1p in signal transduction is not well understood, but its role as a substrate specificity factor in the Skp1p-Cullin-F-box ubiquitin protein-ligase (SCF) complex suggests a role for ubiquitylation in SPS sensor signal transduction. Consistent with this suggestion, mutants of the SCF complex and ubiquitin have been shown to affect SPS sensor-dependent promoter derepression (7).

Based on several lines of evidence, the homologous zinc finger transcription factors Stp1p and Stp2p are redundant downstream effector components of the SPS sensor pathway.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
CAY29	<i>MATa ura3-52</i>	1
CAY91	<i>MATa ura3-52 ssy1Δ13::hisG</i>	1
CAY86	<i>MATa ura3-52 grr1Δ50::hphMX4</i>	1
CAY103	<i>MATa ura3-52 lys2Δ201 grr1Δ50::hphMX4</i>	This work
CAY107	<i>MATa ura3-52 lys2Δ201 asilΔ8::kanMX</i>	This work
CAY109	<i>MATa ura3-52 grr1Δ50::hphMX4 asi1Δ8::kanMX</i>	This work
CAY123	<i>MATa ura3-52 stp1Δ51::Agleu2 stp2Δ50::hphMX4</i>	1
CAY144	<i>MATa ura3-52 asilΔ8::kanMX</i>	This work
CAY152	<i>MATa ura3-52 stp1Δ51::Agleu2 stp2Δ50::hphMX4 asi1Δ8::kanMX</i>	This work
CAY161	<i>MATα ura3-52 ade2Δ51 stp2Δ50::hphMX4 gap1Δ::PAGP1-lacZ</i>	1
CAY206	<i>MATa ura3-52 ssy1Δ13::hisG asi1Δ8::kanMX</i>	This work
CAY224	<i>MATa ura3-52 gap1Δ::PAGP1-lacZ</i>	This work
CAY225	<i>MATα ura3-52 gap1Δ::PAGP1-lacZ</i>	This work
CAY228	<i>MATa ura3-52 gap1Δ::PAGP1-lacZ ade2Δ::PTR3-loxP-kanMX-loxP-SSY1</i>	This work
CAY229	<i>MATα ura3-52 gap1Δ::PAGP1-lacZ can1Δ::SSY5-natMX4-SHR3</i>	This work
CAY233	<i>MATα ura3-52 gap1Δ::PAGP1-lacZ ade2Δ::PTR3-loxP-kanMX-loxP-SSY1 can1Δ::SSY5-natMX4-SHR3</i>	This work
CAY241	<i>MATα ura3-52 his3Δ200 gap1Δ::PAGP1-lacZ ade2Δ::PTR3-loxP-kanMX-loxP-SSY1 can1Δ::SSY5-natMX4-SHR3</i>	This work
HKY20	<i>MATa ura3-52 lys2Δ201 ssy1Δ13::hisG</i>	29
HKY31	<i>MATa ura3-52 lys2Δ201 ptr3Δ15::hisG</i>	29
HKY77	<i>MATa ura3-52 lys2Δ201 ssy5Δ2::hisG</i>	17
PLY126	<i>MATa ura3-52 lys2Δ201</i>	29
PLY861	<i>MATa ura3-52 his3Δ200</i>	This work

Single deletions of either *STP1* or *STP2* partially impair SPS sensor signaling, whereas deletions of both fully abolish signaling (1, 12). Stp1p and Stp2p bind to specific upstream activating sequences (UAS) present within SPS sensor-regulated promoters (12, 33). Both Stp1p and Stp2p are synthesized as latent cytoplasmic factors that are activated by receptor-mediated processing (1). In response to the addition of amino acids and in a strictly SPS sensor-dependent manner, Stp1p and Stp2p are endoproteolytically cleaved. This event liberates the DNA-binding and transactivation domains from an approximately 10-kDa N-terminal fragment. The shorter forms of Stp1p and Stp2p accumulate in the nucleus, where they function to transactivate SPS sensor-regulated genes.

In this study, we directly tested the requirement of the proteolytic processing of Stp1p/Stp2p. We found that the N-terminal domains of Stp1p/Stp2p contain two conserved motifs that are required for SPS sensor-dependent regulation. These motifs are functionally distinct and function in parallel to ensure proper nuclear exclusion and proteolytic processing. We also showed that Grr1p is required for the proteolytic processing of Stp1p/Stp2p and that Asi1p, an integral membrane protein, functions to restrict the entry of full-length unprocessed forms of Stp1p and Stp2p into the nucleus. Finally, the N-terminal regulatory domain of Stp1p is transferable and confers full SPS sensor pathway and Asi1p control when fused to an unrelated synthetic transcription factor. Our data clearly indicate that Stp1p and Stp2p are maintained as latent cytoplasmic transcription factors merely because of motifs within their N-terminal regulatory regions.

MATERIALS AND METHODS

Media. Standard media, including yeast extract-peptone-dextrose (YPD) medium and ammonia-based synthetic minimal dextrose (SD) medium, supplemented as required to enable the growth of auxotrophic strains, were prepared as described previously (11). Ammonia-based synthetic complex dextrose (SC)

medium was prepared as described previously (1). When needed, L-leucine was added at a concentration of 1.3 mM to induce the SPS sensor, and L-glutamate was added at a final concentration of 1 mM to SD medium. When needed, 5-fluoroortoc acid (5-FOA) was added to SC medium (1 g/liter). Media were made solid with 2% (wt/vol) Bacto Agar (Difco). Antibiotic selections were made on solid YPD medium supplemented with 200 mg of G418 (Invitrogen, Carlsbad, Calif.)/liter, 100 mg of clonNAT (Werner Bioagents, Jena, Germany)/liter, or 300 mg of hygromycin B (Duchefa, Haarlem, The Netherlands)/liter. Sensitivity to azetidine-2-carboxylic acid (AzC) was tested at a final concentration of AzC of 1 mM on solid leucine-supplemented SD medium.

Yeast strains. All yeast strains used in this work (Table 1) are isogenic descendants of the S288c-derived strain AA255/PLY115 (3). Generation of the deletion alleles *asi1Δ8::kanMX*, *grr1Δ50::hphMX4*, *stp1Δ51::Agleu2*, *stp2Δ50::hphMX4*, and *gap1Δ::PAGP-lacZ* has been described elsewhere (1, 16). CAY224 and CAY225 are meiotic segregants obtained from a cross between CAY161 and PLY126. CAY228 was constructed by transforming CAY224 with two PCR products, one containing a 3' portion of *kanMX* (23) fused to *SSY1* flanked by 3' *ADE2* genomic sequences and one containing 5' *ADE2* genomic sequences fused to *PTR3* flanked by a 5' portion of *kanMX*. Homologous sequences within the region of overlap between the two amplified portions of *kanMX* facilitated the simultaneous targeting of both fragments to the *ADE2* locus (see Fig. 3A). Transformants were selected on G418-supplemented medium, and *ade2Δ* (red) colonies carrying *PTR3* and *SSY1* sequences were identified. Similarly, CAY229 was obtained by transforming CAY225 with two PCR products, one encompassing *SSY5* fused to a 5' portion of *natMX4* (22) and one containing a 3' portion of *natMX4* fused to *SHR3*. Overhangs in the appropriate primers facilitated targeting to the *CAN1* locus (see Fig. 3A). Transformants were selected on clonNAT-supplemented medium and screened for canavanine resistance.

The function of each of the targeted integration alleles was tested by complementation analysis. CAY228 and CAY229 were individually crossed to strains carrying *URA3*-marked deletions of *SSY1*, *PTR3*, *SSY5*, and *SHR3*. The resulting diploids were sporulated, and multiple meiotic segregants containing the integration alleles (*Ade*⁻/G418^r or Can^r/Nat^r) and the respective endogenous deletion alleles (*Ura*⁺) were analyzed. In all four cases, each integrated allele complemented the AzC resistance (AzC) exhibited by the individual *ssy1Δ*, *ptr3Δ*, *ssy5Δ*, and *shr3Δ* alleles. CAY233 is a meiotic segregant, containing both pairs of integration alleles, and was obtained by crossing CAY228 and CAY229. CAY241 is a *his3* derivative of CAY233 and was obtained as a meiotic segregant from a cross between PLY861 and CAY233.

Plasmids. The plasmids used in this study are listed in Table 2. The mutagenic oligonucleotides and PCR primers used are available on request. Plasmid

TABLE 2. Plasmids used in this study

Plasmid	Description	Reference or source
pCA027	pRS316 (<i>URA3</i>) containing <i>STP1Δ131</i>	1
pCA030	pRS317 (<i>LYS2</i>) containing <i>PAGP1-lacZ</i>	This work
pCA047	pRS316 (<i>URA3</i>) containing <i>STP1-HA</i>	1
pCA120	pRS316 (<i>URA3</i>) containing <i>STP1-133</i>	This work
pCA122	pRS317 (<i>LYS2</i>) containing <i>STP1-HA</i>	This work
pCA127	pRS316 (<i>URA3</i>) containing <i>STP1-101-HA</i>	This work
pCA128	pRS316 (<i>URA3</i>) containing <i>STP1-103-HA</i>	This work
pCA129	pRS316 (<i>URA3</i>) containing <i>stp1-102-HA</i>	This work
pCA135	pRS316 (<i>URA3</i>) containing <i>STP1-102,133</i>	This work
pCA160	2 μ m <i>LYS2 PADH1 ΔSphI</i>	This work
pCA161	2 μ m <i>LYS2 PADH1</i> expressing <i>Stp1₍₁₋₁₂₅₎-LexA-AD</i>	This work
pCA171	pRS313 (<i>HIS3</i>) containing <i>STP1-HA</i>	This work
pCA212	pRS316 (<i>URA3</i>) containing <i>GRR1</i>	This work
pHK010	pRS316 (<i>URA3</i>) containing <i>SSY1</i>	29
pSH18-34	2 μ m <i>URA3</i> containing <i>Op_{lexA}-lacZ</i>	24
pSSY1-102	pRS316 (<i>URA3</i>) containing <i>SSY1-102</i>	19

pCA030 was created by ligating a 7.2-kb HindIII/SalI fragment obtained from YCpAGP1-LacZ (25) with similarly restricted pRS317. The insert of pCA047 was subcloned as an EcoRI/SmaI fragment into pRS317, creating pCA122. Plasmid pCA171 carrying *HIS3* was isolated from yeast cells cotransformed with EagI/XhoI-restricted pRS313 and PvuI-restricted pCA047. Plasmid pCA120 was created by single-stranded mutagenesis of pCA047 with oligonucleotide 1 (30). Plasmids pCA127, pCA128, and pCA129 were created by single-stranded mutagenesis of pCA047 with oligonucleotides 2 to 4, respectively. Plasmid pCA135 was created by single-stranded mutagenesis of pCA047 with oligonucleotides 5 and 6. Plasmid pCA161 is a derivative of the commonly used two-hybrid bait plasmid pEG202 (24) with the following two modifications. First, the *HIS3* marker was replaced by *LYS2* amplified from pRS317 with primers 7 and 8. Second, the first 125 codons of *STP1* were introduced in frame (with primers 9 and 10 and pCA029 as a template) (1) with the *lexA* gene fused to the activation domain (AD) of VP16 (with primers 11 and 12 and pCMXVP16 as a template) (a kind gift from T. Perlmann) (8). Plasmid pCA160 is a derivative of pCA161 created by deleting part of the *ADH1* promoter and the entire *lexA* gene fusion by restriction with SphI and religation. Plasmid pCA212 was created by homologous recombination in yeast cells; a *GRR1*-containing PCR product (primers 13 and 14) amplified with S288c genomic DNA as a template was cotransformed with KpnI/SacI-restricted pRS316.

Isolation of *grr1* mutants. Twenty independent colonies of CAY241 were inoculated into 5 ml of SD medium supplemented with adenine, glutamate, histidine, and uracil. The cultures were continuously shaken and grown to saturation for 36 h at 30°C. A 100- μ l aliquot of each culture was plated on solid SD medium supplemented with adenine, glutamate, histidine, leucine, uracil, and AzC and incubated for 7 days at 30°C; between 50 and 200 AzC-resistant colonies formed on each of the 20 plates. The colonies were screened for β -galactosidase activity by using a 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) overlay assay. Six AzC-resistant colonies with no detectable X-Gal staining were isolated.

β -Galactosidase activity assays. β -Galactosidase activity was determined with *N*-lauroyl-sarcosine-permeabilized cells (28). After preincubation of cells in 800 μ l of 0.2% (wt/vol) sodium *N*-lauroyl-sarcosine Z buffer at 30°C for 15 min, a 160- μ l aliquot of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) solution (4 mg/ml) was added and the tubes were mixed by inversion. The reaction was stopped by the addition of 400 μ l of 1 M Na₂CO₃, and the tubes were centrifuged at 12,000 \times g for 5 min. The absorbance of the supernatant was measured at 420 nm. Semiquantitative measurements of β -galactosidase activity were routinely made by using equally turbid cell suspensions (optical density at 600 nm, 1) diluted 1:1 in 0.4 M potassium phosphate buffer (pH 7) containing 0.2% (wt/vol) sodium *N*-lauroyl-sarcosine and 0.2 mg of X-Gal/ml. Cell suspensions were incubated at 30°C until blue color was detectable. A similar buffer containing 0.5% (wt/vol) low-melting-point agarose was used as an overlay to assess the β -galactosidase activity of whole colonies.

Immunoblot analysis. Whole-cell extracts were prepared under denaturing conditions with NaOH and trichloroacetic acid treatment as described previously (35). Extracted proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and analyzed by immunoblotting. Immunoblots were incubated with primary antibody diluted in blocking buffer as follows: 12CA5 ascitic fluid (antihemagglutinin [HA] monoclonal antibody),

1:1,000; and anti-LexA polyclonal antibody (Invitrogen), 1:5,000. Immunoreactive bands were visualized by chemiluminescence detection (SuperSignal West Dura extended-duration substrate; Pierce) of horseradish peroxidase conjugated to secondary antibodies (anti-mouse immunoglobulin from sheep and anti-rabbit immunoglobulin from donkey; Amersham) and quantified by using an LAS1000 system (Fuji Photo Film Co. Ltd).

RESULTS

The N-terminal domains of Stp1p and Stp2p contain conserved sequence elements. Based on current understanding of the SPS sensor signal transduction pathway (Fig. 1A), the N-terminal regulatory domains of Stp1p and Stp2p possess sequences that prevent their movement into the nucleus and protease recognition and cleavage sites (1). We sought to identify such elements by comparing the sequences of the first 125 N-terminal amino acids from the closely related orthologues of Stp1p and Stp2p from *S. cerevisiae*, *S. bayanus*, *S. mikatae*, and *S. paradoxus* (27). These domains are highly conserved between orthologues of Stp1p (60% identity) and Stp2p (55% identity). However, the degree of conservation is markedly reduced when Stp1p and Stp2p sequences are compared. Sequence alignments of all Stp1p and Stp2p homologues enabled two regions containing stretches of conserved amino acid residues to be identified (Fig. 1B). Region I is rich in branched-chain amino acids and is situated in the middle of the regulatory domain defined by the large in-frame deletion giving rise to the constitutive *STP1Δ131* allele (Fig. 1C, REG) (1). Region II corresponds to the predicted site of proteolytic processing (Fig. 1C, scissor). The level of conservation within these regions and their locations suggest that they have important roles in the regulation of Stp1p and Stp2p nuclear exclusion (region I) and endoproteolytic processing (region II).

A mutation that blocks proteolytic processing inhibits SPS signal transduction. The first 3 amino acids of region II are conserved in all Stp1p and Stp2p homologues (Fig. 1B). To test the importance of these amino acids, residues L65, F66, and P67 were individually replaced with alanine in an initially functional protein carrying an HA tag at the extreme C terminus. An *stp1Δ stp2Δ* mutant strain was transformed with plasmids expressing wild-type Stp1p or alanine substitution L65A, F66A, and P67A mutant proteins. Cells were grown in amino-acid-free SD medium, and Stp1p cleavage in whole-cell extracts prepared before and 30 min after induction by leucine was monitored. Leucine induced the proteolytic processing of wild-type Stp1p (Fig. 2A, left panel, compare lanes 1 and 5). Alanine substitution mutations L65A and P67A had negligible effects on processing; the mutant proteins were proteolytically processed as effectively as wild-type Stp1p (Fig. 2A, left panel, compare lanes 2 and 6 and lanes 4 and 8). In contrast, leucine did not induce the proteolytic processing of the F66A mutant protein (Fig. 2A, left panel, compare lanes 3 and 7). The steady-state level of the F66A mutant protein was similar to that of wild-type Stp1p, suggesting that the alanine substitution does not grossly affect protein stability.

The availability of a noncleavable mutant form of Stp1p enabled us to test whether proteolytic processing is obligatorily coupled to the transduction of SPS sensor-initiated signals. A growth assay based on the amino acid-induced uptake of the toxic proline analogue AzC was used to monitor Stp1p activity. AzC is transported into cells by Agp1p and Gnp1p (Fig. 1A);

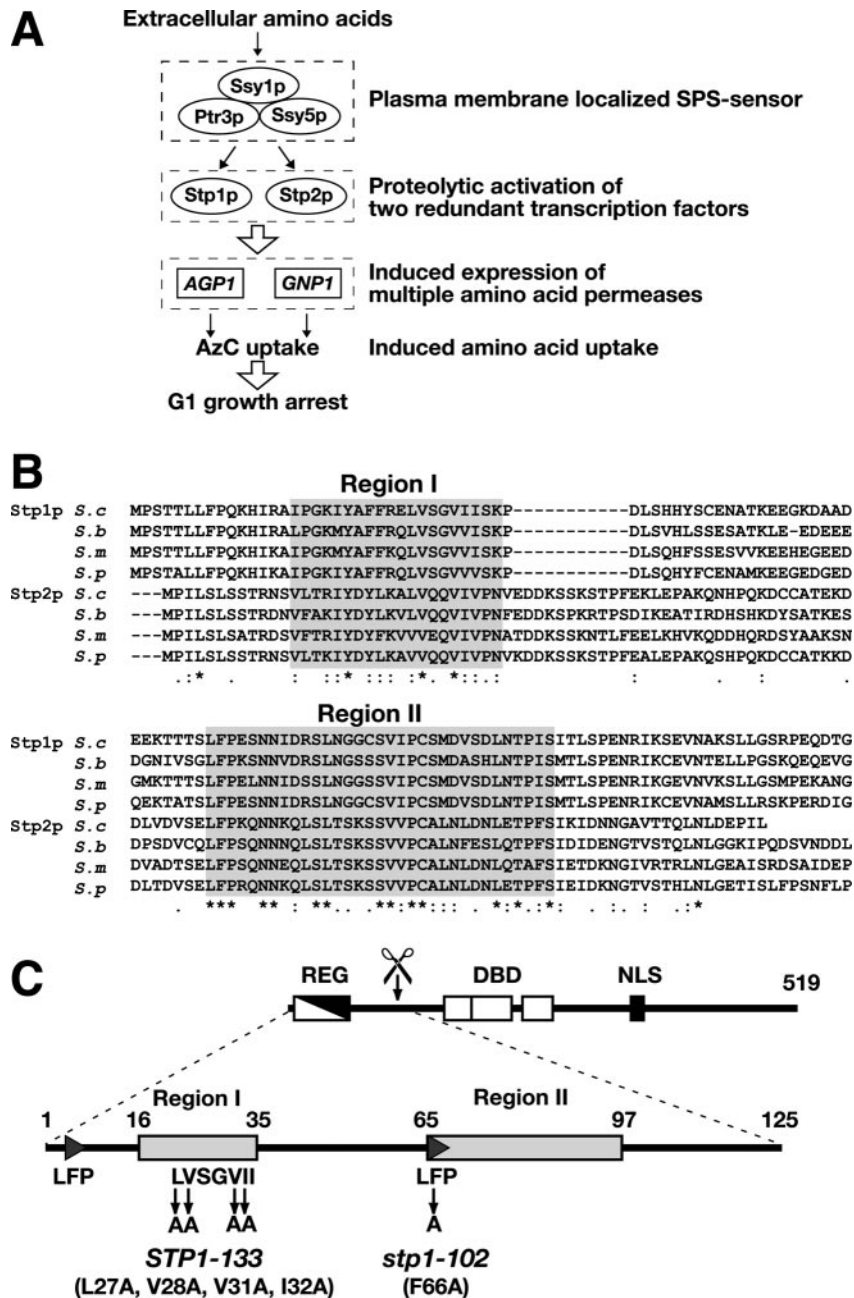


FIG. 1. The N-terminal regulatory domains of the Stp1p and Stp2p orthologues contain two distinct regions of sequence conservation. (A) Schematic diagram of the SPS sensor pathway. Extracellular amino acids activate the SPS sensor, induce the endoproteolytic processing of transcription factors Stp1p and Stp2p, and thereby induce the expression of multiple amino acid permease genes. Consequently, cells grown in the presence of extracellular amino acids exhibit robust amino acid uptake and are sensitive to toxic amino acid analogues, e.g., AzC. (B) The first 125 amino acid residues from Stp1p/Stp2p (*S.c.*) and their orthologues in *S. bayanus* (*S.b.*), *S. mikatae* (*S.m.*), and *S. paradoxus* (*S.p.*) were aligned by using the CLUSTAL W algorithm (37). Conserved (asterisks) and similar (colons and periods) residues are marked. Regions I and II (gray boxes) are defined by stretches of enhanced sequence conservation. (C) Schematic representation of Stp1p. The locations of the inhibitory domain (REG), defined by the breakpoint of the *STP1Δ133* allele (1), the DNA-binding domains (DBD), and the putative NLS are depicted in the full-length Stp1p (519 amino acids). Region I (amino acids 16 to 35), region II (amino acids 65 to 97), and the alanine substitution mutations of the *STP1-133* and *stp1-102* alleles are indicated in the enlargement of the N-terminal domain (amino acids 1 to 125).

the genes encoding these amino acid permeases are transcribed in an Stp1p- or Stp2p-dependent manner (2). We examined the growth on SD medium and SD medium supplemented with AzC of an *stp1Δ stp2Δ* mutant strain transformed with a control vector or plasmids expressing wild-type Stp1p or

L65A, F66A, and P67A mutant proteins. All strains exhibited robust growth on control SD medium (Fig. 2A, upper right panel). However, on SD medium supplemented with AzC, only cells carrying the control vector or the plasmid expressing the F66A mutant protein were able to grow (Fig. 2A, lower right

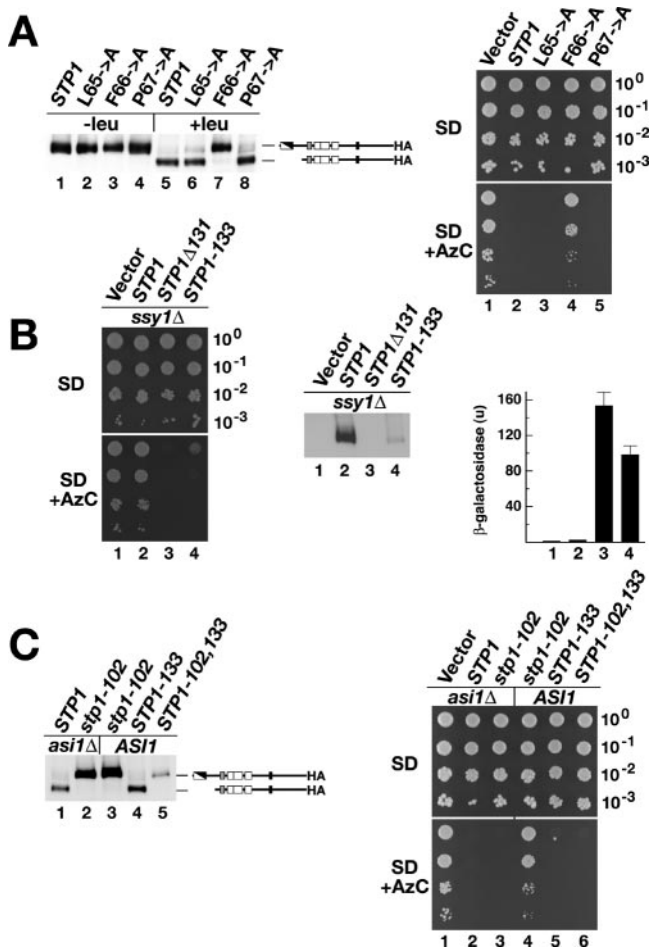


FIG. 2. Mutations in regions I and II of Stp1p demonstrate the modular nature of the N-terminal inhibitory domain. (A) The *stp1-102* allele encodes a mutant protein that is not endoproteolytically processed. (Left panel) Immunoblot analysis of protein extracts from strain CAY123 (*stp1* Δ *stp2* Δ) transformed with plasmids carrying wild-type *STP1* (pCA047) or mutant allele L65→A (pCA127), F66→A (pCA129), or P67→A (pCA128). Cells were grown in SD medium (-leu) and, where indicated, leucine was added 30 min prior to harvest (+leu). Extracts were resolved by SDS-PAGE and immunoblotted with anti-HA. The immunoreactive forms of Stp1p present in the cell extracts are schematically represented at their corresponding positions of migration. (Right panel) Tenfold dilution series of cell suspensions of strain CAY123 (*stp1* Δ *stp2* Δ) transformed with pRS316 (Vector), pCA047 (*STP1*), pCA127 (L65→A), pCA129 (F66→A), or pCA128 (P67→A) were spotted on plates containing SD medium (top) and SD medium supplemented with leucine and AzC (bottom). (B) The *STP1-133* allele encodes a constitutively active protein. (Left panel) Tenfold dilution series of cell suspensions of an *ssy1* Δ strain (HKY20) cotransformed with pCA030 (*PAG1-lacZ*) and either pRS316 (Vector), pCA047 (*STP1*), pCA027 (*STP1* Δ 131), or pCA120 (*STP1-133*) were spotted on plates containing SD medium (top) and SD medium supplemented with leucine and AzC (bottom). (Middle panel) Immunoblot analysis of protein extracts from HKY20 transformants grown in SD medium. Extracts were resolved by SDS-PAGE and immunoblotted with anti-HA. (Right panel) β -Galactosidase activity (Miller units, with standard deviations [$n = 4$]) present in each strain was quantified to monitor the expression of the *PAG1-lacZ* reporter construct. (C) The *STP1-133* mutation and the *asi1* Δ allele can suppress noncleavable *stp1-102*. (Left panel) Immunoblot analysis of protein extracts from strains CAY152 (*asi1* Δ) and CAY123 (*ASI1*) transformed with a plasmid carrying wild-type *STP1* (pCA047) or mutant allele *stp1-102* (pCA129), *STP1-133* (pCA120), or *STP1-102,133* (pCA135). Cells were grown in SD medium, and leucine was added 30 min prior

to harvest. Extracts were resolved by SDS-PAGE and immunoblotted with anti-HA. (Right panel) Tenfold dilution series of cell suspensions of strains CAY152 (*stp1* Δ *stp2* Δ *asi1* Δ) and CAY123 (*stp1* Δ *stp2* Δ *ASI1*) transformed with pRS316 (Vector), pCA047 (*STP1*), pCA129 (*stp1-102*), pCA120 (*STP1-133*), or pCA135 (*STP1-102,133*) were spotted on plates containing SD medium (top) and SD medium supplemented with leucine and AzC (bottom).

panel, lanes 1 and 4). The growth of cells expressing the F66A mutant protein demonstrated that this protein is unable to transduce SPS sensor signals. We named the noncleavable and noninducible F66A mutant allele *stp1-102* (Fig. 1C). The lack of growth on SD medium supplemented with AzC of cells carrying plasmids expressing wild-type Stp1p or L65A and P67A mutant proteins (Fig. 2A, right panel, lanes 2, 3, and 5) indicates that these proteins are functional, a finding consistent with their amino acid-induced processing.

Alanine mutations in conserved region I constitutively activate Stp1p. We replaced multiple residues in the branched-chain amino acid cluster with alanine (Fig. 1B, region I). One allele with four alanine substitutions, *STP1-133* (Fig. 1C), resulted in phenotypes consistent with constitutive signaling. It suppressed AzC resistance exhibited by an *ssy1* Δ strain (Fig. 2B, left panels, compare lanes 2 and 4) and activated the expression of β -galactosidase from a *PAG1-lacZ* reporter plasmid almost to the extent of the constitutive *STP1* Δ 131 allele (Fig. 2B, right panel, compare bar 2 with bars 3 and 4). The steady-state levels of full-length Stp1-133p present in SD medium-grown *ssy1* Δ cells were substantially lower than those in the same cells expressing wild-type Stp1p (Fig. 2B, middle panel, lanes 2 and 4), suggesting that the *STP1-133* allele may result in misfolding of the N-terminal domain and destabilization of the protein. The fact that the *STP1-133* allele phenocopies the characterized *STP1* Δ 131 allele (1) indicates that residues affected by the mutations are important in the retention of unprocessed Stp1p outside the nucleus.

Proteolytic processing is not a prerequisite for transactivation, and full-length Stp1p activates transcription when inappropriately induced to enter the nucleus. Loss-of-function mutations in the *AS11* gene enable the constitutive expression of Stp1p/Stp2p-dependent genes even in the absence of a functional SPS sensor (16). We previously showed that Stp1p/Stp2p processing does not occur in cells lacking a functional SPS sensor (1); thus, the constitutive expression of Stp1p/Stp2p-dependent genes in *asi1* Δ mutants without SPS sensor activity predicts that full-length Stp1p/Stp2p can activate transcription. To examine this possibility, we analyzed the status of Stp1p processing and AzC-resistant growth of *asi1* Δ mutant and *AS11* wild-type strains. An *stp1* Δ *stp2* Δ *asi1* Δ strain was transformed with a control plasmid (vector) or plasmids expressing either *STP1* or the noncleavable *stp1-102* allele. Immunoblot analysis revealed that Stp1p processing was not affected by the loss of *AS11*; upon leucine induction, the shorter cleaved form was readily detected (Fig. 2C, left panel, lane 1). In contrast, all of the noncleavable Stp1-102p mutant protein migrated at the position of the full-length unprocessed form (Fig. 2C, left panel, lane 2). The activities of the expressed proteins were examined as AzC resistance. All transformed strains formed colonies of equal sizes on SD medium (Fig. 2C, upper right

to harvest. Extracts were resolved by SDS-PAGE and immunoblotted with anti-HA. (Right panel) Tenfold dilution series of cell suspensions of strains CAY152 (*stp1* Δ *stp2* Δ *asi1* Δ) and CAY123 (*stp1* Δ *stp2* Δ *ASI1*) transformed with pRS316 (Vector), pCA047 (*STP1*), pCA129 (*stp1-102*), pCA120 (*STP1-133*), or pCA135 (*STP1-102,133*) were spotted on plates containing SD medium (top) and SD medium supplemented with leucine and AzC (bottom).

panel). Cells transformed with the empty plasmid (vector) readily formed colonies on AzC-containing medium (Fig. 2C, lower right panel, lane 1), whereas cells carrying a plasmid expressing wild-type *STP1* (Fig. 2C, lower right panel, lane 2) or the *stp1-102* allele did not form colonies (Fig. 2C, lower right panel, lane 3). The lack of growth of the *STP1*-expressing strain is consistent with the observed processing of Stp1p. The finding that *stp1Δ stp2Δ asi1Δ* cells expressing noncleavable Stp1-102p are unable to grow in the presence of AzC clearly demonstrates that unprocessed Stp1p is able to function as a transactivator if it is able to enter the nucleus.

The observation that the *stp1-102* allele encodes a nonprocessed protein that is functional when expressed in an *asi1Δ stp1Δ stp2Δ* strain but not in an *stp1Δ stp2Δ* strain (Fig. 2C, right panels, lanes 4 and 3, respectively) enabled us to examine the epistasis of mutations in conserved regions I and II (Fig. 1B). We combined the recessive *stp1-102* and the dominant *STP1-133* mutations into a single gene, creating the double-mutant *STP1-102,133* allele. An *stp1Δ stp2Δ* strain was transformed with plasmids expressing noncleavable Stp1-102p, constitutively active Stp1-133p, or double-mutant Stp1-102,133p. Immunoblot analysis showed that Stp1-133p was processed normally but that Stp1-102,133p was not (Fig. 2C, left panel, compare lanes 4 and 5). Cells transformed with the constitutively active *STP1-133* allele exhibited AzC sensitivity and failed to form colonies on SD medium supplemented with AzC (Fig. 2C, right panels, lane 5). Also, cells carrying the double-mutant *STP1-102,133* allele were unable to grow in the presence of AzC (Fig. 2C, right panels, lane 6). These results demonstrate that in the absence of a functional inhibitory domain (region I), cleavage is not required for unprocessed Stp1p to transactivate gene expression. Thus, it can be concluded that amino acid-induced cleavage is required merely to sever the inhibitory domain from the transactivating portion of Stp1p and that cytoplasmic retention and endoproteolytic activation are functionally separable and distinct processes.

A novel genetic screen identifies recessive mutations in *GRR1* that inactivate the SPS sensor pathway. Mutational analysis of the N-terminal region of Stp1p identified the endoproteolytic processing of Stp1p/Stp2p as the key regulatory step in the SPS sensor pathway and prompted us to search for additional factors that may be involved in the processing step. To enable the isolation of rare mutations affecting the SPS sensor pathway, we created a haploid strain harboring functional duplicated alleles of *SSY1*, *PTR3*, and *SSY5* integrated into the genome (see Materials and Methods) (Fig. 3A). We also chose to duplicate *SHR3*, a gene encoding a membrane-localized chaperone that is specifically required for the exit of Ssy1p from the endoplasmic reticulum (21, 29). By making our starting strain merodiploid for these genes, our aim was to eliminate the re-isolation of recessive loss-of-function mutations in previously identified SPS sensor components. The starting strain also carried an *AGPI*-promoted *lacZ* reporter gene integrated into the genome, leading to the expression of β-galactosidase when the SPS sensor pathway is intact. In contrast, cells with a mutant SPS sensor pathway will lack the expression of detectable β-galactosidase activity.

Signaling through the SPS sensor pathway leads to the transcription of *AGPI* and *GNPI*, resulting in AzC import and cell cycle arrest (Fig. 1A). Consequently, mutations abolishing SPS

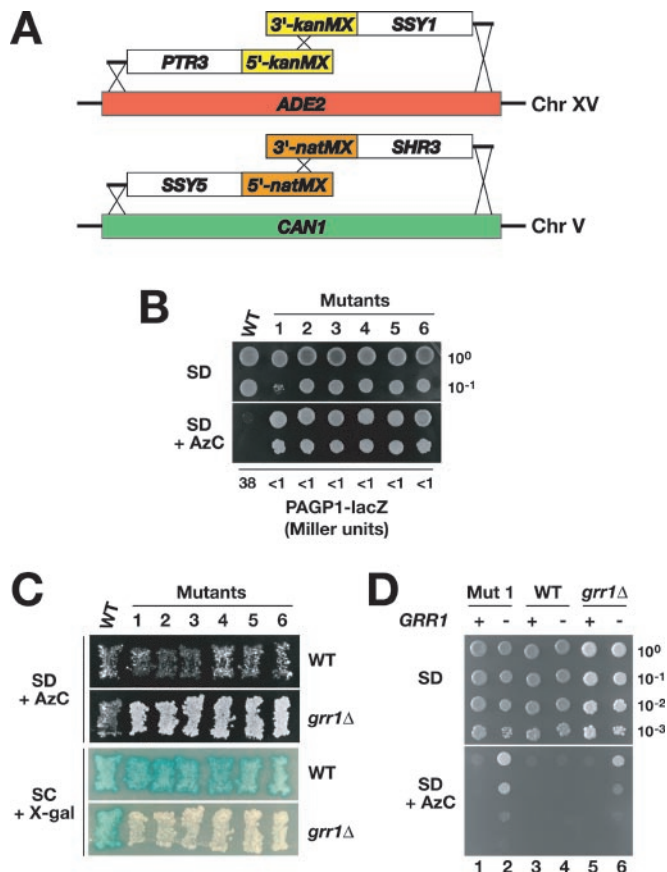


FIG. 3. A genetic screen identifies *GRR1* as an obligate component of the amino acid-induced SPS sensor pathway. (A) Merodiploid strains carrying duplicated genes encoding the known SPS sensor components and *SHR3* were constructed by integrating *PTR3* and *SSY1* into the *ADE2* locus on chromosome (Chr) XV and *SSY5* and *SHR3* into the *CAN1* locus on chromosome V, creating the *ade2Δ::PTR3-loxP-kanMX-loxP-SSY1* and *can1Δ::SSY5-natMX-SHR3* alleles, respectively. (B) Tenfold dilution series of strain CAY241 (wild type [WT]) and six spontaneous AzC-resistant mutants lacking detectable β-galactosidase activity were applied as drops to solid SD medium (top) and SD medium containing leucine and AzC (bottom). The plates were incubated at 30°C for 7 days and photographed. β-Galactosidase activity (Miller units) present in each strain was quantified to monitor the expression of the integrated *PAGP1-lacZ* reporter construct. (C) Complementation analysis of diploid strains generated by mating CAY241 (WT) and AzC-resistant LacZ-negative mutants (mutants 1 to 6) with PLY126 (WT) and CAY103 (*grr1Δ*). The diploid strains were patched on SD medium supplemented with leucine, uracil, and AzC (top) and on SC medium (bottom). The plates were incubated at 30°C for 2 days, and β-galactosidase activity was visualized on SC medium by using an X-Gal overlay. (D) Tenfold dilution series of cell suspensions of mutant 1 (Mut 1), a WT strain (CAY241), or a *grr1Δ* strain (CAY86) transformed with pRS316 (-) or pCA212 (+) were spotted on plates containing SD medium (top) and SD medium supplemented with leucine and AzC (bottom). The plates were incubated at 30°C for 4 days and photographed.

sensor signaling make cells AzC resistant. Spontaneously arising AzC-resistant colonies were obtained by plating saturated cultures of the merodiploid starting strain on solid SD medium supplemented with AzC. Six slowly growing AzC-resistant colonies that were small and flat and that remained white after incubation with an X-Gal overlay were identified (Fig. 3B).

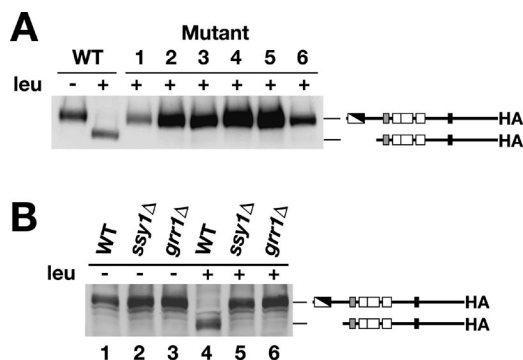


FIG. 4. Loss-of-function mutations in *GRR1* inhibit the endoproteolytic processing of Stp1p. (A) Immunoblot analysis of whole-cell extracts from a wild-type (WT) strain (CAY241) and AzC-resistant LacZ-negative mutant strains (Fig. 3) transformed with pCA171 (*STP1-HA*). Cells were grown in SD medium supplemented with adenine and uracil. Leucine (leu) was added 30 min prior to harvest. (B) Immunoblot analysis of whole-cell extracts from a WT strain (CAY29), an *ssy1Δ* strain (CAY91), and a *grr1Δ* strain (CAY86) transformed with pCA047 (*STP1-HA*). Cells were grown in SD medium. Leucine (leu) was added 30 min prior to harvest.

Under all growth conditions, these six mutants consistently grew at greatly reduced rates compared to the wild-type starting strain. Microscopic analysis revealed that in all instances, the mutant cells were elongate and remained attached to one another, forming branched chains of cells. Consistent with these latter observations, the mutants exhibited a flocculent growth phenotype in liquid cultures.

The growth phenotypes of our newly identified mutant strains closely resemble those exhibited by *grr1* mutant strains (14). Complementation analysis was used to address whether the mutations in our strains were indeed allelic with *GRR1*. All six strains were found to carry recessive mutations; diploids resulting from crosses with the wild-type strain were AzC sensitive and expressed robust β -galactosidase activity (Fig. 3C). In contrast, diploids from crosses with the *grr1Δ* tester strain were AzC resistant and lacked β -galactosidase activity (Fig. 3C). The lack of complementation of both phenotypes is consistent with the mutations being allelic with *GRR1*. Genetic linkage was established by sporulating the two diploids obtained from crosses with mutant 1; strict 2:2 segregation was observed in tetrads derived from the cross with the wild-type strain, and no AzC-sensitive spores were recovered from the cross with the *grr1Δ* strain. Additionally, plasmid-borne *GRR1* complemented the AzC resistance exhibited by mutant 1 (Fig. 3D, compare lanes 1 and 2 with lanes 5 and 6). We conclude that the mutants isolated carry recessive mutations in *GRR1*.

***GRR1* is required for the amino acid-induced endoproteolytic processing of Stp1p.** We previously reported that *grr1Δ* mutations do not impair the proteolytic processing of Stp1p, suggesting that Grr1p functions downstream of this regulatory event (1). We investigated whether our newly isolated *grr1* mutant alleles affected the proteolytic processing of Stp1p. For wild-type extracts, the full-length form of Stp1p-HA was present in the noninduced control, and the cleaved 10-kDa lower-molecular-mass form was observed after leucine induction (Fig. 4A). In contrast, only the nonprocessed form of

Stp1p-HA was found in leucine-induced extracts from the newly isolated *grr1* mutants (Fig. 4A).

These unexpected results prompted us to repeat the analysis with a *grr1Δ* mutant strain. The cleavage of Stp1p-HA in a wild-type strain with an intact SPS sensor and that in an *ssy1Δ* mutant strain lacking the receptor component of the SPS sensor were monitored and served as positive and negative controls, respectively. Leucine-induced processing of Stp1p-HA was apparent in the wild-type strain (Fig. 4B, compares lane 1 and 4), whereas no processing was detected in the *ssy1Δ* strain (Fig. 4B, compare lanes 2 and 5). Consistent with our analysis of newly isolated *grr1* mutant strains, no processing was observed in the *grr1Δ* mutant strain (Fig. 4B, compare lanes 3 and 6). Together, these results indicate, contrary to what we previously reported, that *GRR1* is required for the proteolytic processing of Stp1p/Stp2p.

Constitutive activation of the Ssy1p receptor does not bypass the *GRR1* requirement. *SSY1-102* encodes a dominant mutant form of Ssy1p that constitutively activates the expression of SPS sensor-regulated genes even in the absence of inducing amino acids (19). We tested whether *SSY1-102* could suppress the Stp1p-processing defect in *grr1Δ* cells. Control wild-type and *grr1Δ* strains carrying a plasmid expressing Stp1p-HA were transformed with *SSY1*- or *SSY1-102*-containing plasmids and analyzed by immunoblotting. As previously shown, leucine induced Stp1p processing in wild-type cells (Fig. 5A, compare lanes 1 and 5) but not in *grr1Δ* cells (Fig. 5A, compare lanes 3 and 7). In uninduced wild-type cells expressing *SSY1-102*, a portion of immunodetectable Stp1p was found in its processed form (Fig. 5A, lane 2). Interestingly, leucine induction increased the amount of processed Stp1p to a level similar to that observed in *SSY1*-expressing cells (Fig. 5A, compare lanes 5 and 6). These results account for the constitutive amino acid-independent activation of SPS sensor-regulated genes in *SSY1-102*-expressing cells. Furthermore, these findings confirm that the proteolytic processing of Stp1p is a direct consequence of the amino acid-induced activation of Ssy1p and is not due to an indirect effect related to amino acid uptake (19). Thus, the constitutive nature of Ssy1-102p signaling appears to be mechanistically similar to amino acid-induced wild-type Ssy1p signaling. The Stp1p-processing defect in the *grr1Δ* strain could not be suppressed by the introduction of the *SSY1-102* allele even in the presence of leucine (Fig. 5A, compare lanes 4 and 8).

The dominant activated allele of *STP1* (*STP1Δ133*) and the recessive *asi1Δ* mutation bypass the requirement for *GRR1*. If the lack of Stp1p/Stp2p processing is the underlying reason for the signaling defect in *grr1* mutant strains, mutations activating the pathway downstream of the processing step should exert a suppressing effect. To test this possibility, we examined whether the recessive *asi1Δ* and dominant activated *STP1Δ133* alleles could bypass the need for *GRR1*. Wild-type, *ssy1Δ*, and *grr1Δ* cells, with and without the *asi1Δ* and *STP1Δ133* alleles, were spotted on solid SD medium and SD medium supplemented with AzC. All strains grew on the control plate containing SD medium (Fig. 5B, upper panel). In the presence of AzC, the wild-type strain exhibited no growth, whereas the *ssy1Δ* and *grr1Δ* strains formed colonies, indicating that these strains are defective in SPS sensor signaling (Fig. 5B, lower panel, lanes 2 and 3). The introduction of either *asi1Δ* or

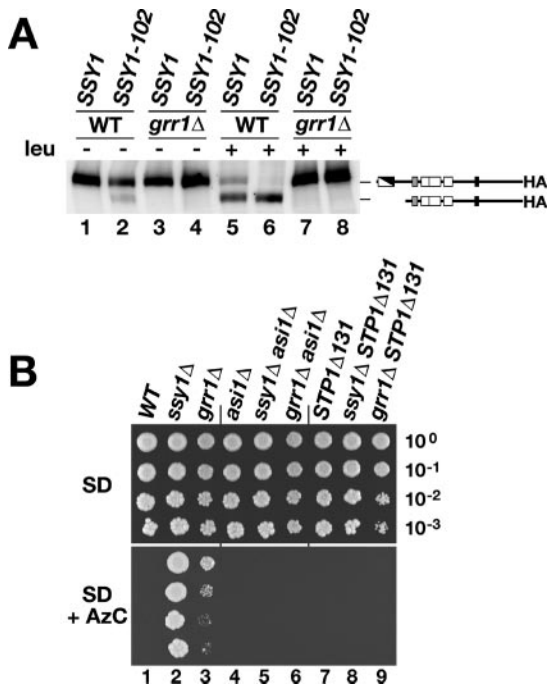


FIG. 5. Epistasis analysis of *grr1Δ* and SPS-sensing pathway mutations. (A) Immunoblot analysis of whole-cell extracts from a wild-type (WT) strain (PLY126) and a *grr1Δ* strain (CAY103) cotransformed with pCA122 (*STP1-HA*) and either pHK010 (*SSY1*) or pSSY1-102 (*SSY1-102*). Cells were grown in SD medium, and leucine (leu) was added 30 min prior to harvest. (B) Tenfold dilution series of cell suspensions of WT (CAY29), *ssy1Δ* (CAY91), *grr1Δ* (CAY86), *asi1Δ* (CAY144), *ssy1Δ asi1Δ* (CAY206), and *grr1Δ asi1Δ* (CAY109) strains carrying plasmid pRS316 and *STP1Δ131* (CAY29), *ssy1Δ STP1Δ131* (CAY91), and *grr1Δ STP1Δ131* (CAY86) strains carrying plasmid pCA027 were spotted on plates containing SD medium (top) and SD medium supplemented with AzC (bottom). The plates were incubated at 30°C.

STP1Δ133 mutations suppressed the loss of SPS sensor activity, as evidenced by the lack of growth of *ssy1Δ asi1Δ* and *ssy1Δ STP1Δ133* strains (Fig. 5B, lower panel, compare lane 2 with lanes 5 and 8). The *asi1Δ* and *STP1Δ133* mutations also suppressed the *grr1Δ* allele (Fig. 5B, lower panel, compare lane 3 with lanes 6 and 9). The epistasis analysis indicates that the defect in SPS sensor signaling exhibited by *grr1Δ* cells is identical to that observed in cells carrying loss-of-function mutations in any of SPS sensor component genes (*SSY1*, *PTR3*, or *SSY5*).

The N terminus of Stp1p is modular and can be transferred to regulate the activity of an artificial transcription factor. We examined the possibility that the first 125 residues of Stp1p [Stp1₍₁₋₁₂₅₎], including the inhibitory motif (region I) and the cleavage sites (region II), could be transferred to confer amino acid-induced regulation of an artificial transcription factor. The N terminus of Stp1p was fused to a well-characterized synthetic transcription factor comprised of the bacterial LexA DNA-binding protein fused to the strong viral VP16 transcriptional AD. The LexA DNA-binding domain possesses an intrinsic NLS and is constitutively nuclear when expressed in yeast cells (34). A wild-type strain and strains carrying deletions of the SPS sensor components *GRR1* and *AS11* were

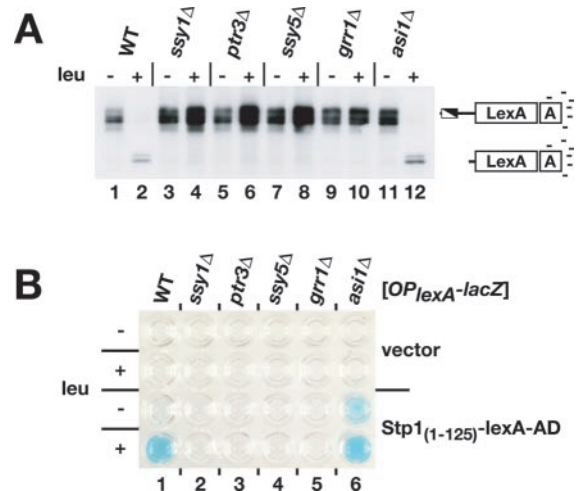


FIG. 6. The N-terminal regulatory domain of Stp1p is transferable and confers full SPS sensor control of an artificial transcription factor. (A) Immunoblot analysis of protein extracts from wild-type (WT) (PLY126), *ssy1Δ* (HKY20), *ptr3Δ* (HKY31), *ssy5Δ* (HKY77), *grr1Δ* (CAY103), and *asi1Δ* (CAY107) strains transformed with pCA161 [Stp1₍₁₋₁₂₅₎-LexA-AD] and grown in SD medium with or without leucine (leu). Extracts were resolved by SDS-PAGE and immunoblotted with anti-LexA antibodies. (B) Strains from panel A carrying pSH18-34 (*Op_{lexA}-lacZ*) and either pCA160 (vector) or pCA161 [Stp1₍₁₋₁₂₅₎-LexA-AD] were grown in SD medium with or without leucine (leu). The levels of X-Gal staining resulting from the expression of *lexA* operator-promoted β -galactosidase in permeabilized cells were assessed.

transformed with a plasmid expressing the Stp1₍₁₋₁₂₅₎-LexA-AD fusion protein to assess the full spectrum of SPS sensor pathway regulation.

First, we examined whether the Stp1₍₁₋₁₂₅₎-LexA-AD fusion protein was subject to amino acid-induced SPS sensor-dependent processing. Extracts were prepared from transformed strains and analyzed with anti-LexA antibodies by immunoblotting. In cells grown in SD medium, in the absence of an inducing amino acid, bands corresponding to the full-length Stp1₍₁₋₁₂₅₎-LexA-AD fusion protein were readily detected (Fig. 6A, lane 1). When cells were grown in the presence of leucine, processing was observed in the wild-type and *asi1Δ* mutant cells (Fig. 6A, lanes 2 and 12). In these cells, the full-length Stp1₍₁₋₁₂₅₎-LexA-AD fusion protein was detected only weakly, and more intense, faster-migrating bands were observed. No processing was observed in leucine-induced *ssy1Δ*, *ptr3Δ*, *ssy5Δ*, or *grr1Δ* mutant cells (Fig. 6A, lanes 3 to 10).

Next, the ability of the Stp1₍₁₋₁₂₅₎-LexA-AD fusion protein to activate a *lexA* operator-controlled *lacZ* reporter gene (*Op_{lexA}-lacZ*) was assessed. A plasmid-borne *Op_{lexA}-lacZ* reporter gene was introduced into strains carrying a vector without an insert or a plasmid expressing the Stp1₍₁₋₁₂₅₎-LexA-AD fusion protein. Leucine-induced promoter activation could be detected readily in wild-type cells transformed with Stp1₍₁₋₁₂₅₎-LexA-AD; a strong blue X-Gal precipitate formed when cells were grown in leucine-containing SD medium (Fig. 6B, lane 1). Derepression of the promoter was dependent on SPS sensor signaling; no blue precipitate was detected when Stp1₍₁₋₁₂₅₎-LexA-AD was expressed in *ssy1Δ*, *ptr3Δ*, *ssy5Δ*, or *grr1Δ* mutant

cells (Fig. 6B, lanes 2 to 5). Importantly, the regulation of Stp1₍₁₋₁₂₅₎-LexA-AD derepressed the reporter even in the absence of amino acids in an *asi1Δ* strain (Fig. 6B, lane 6). These results indicate that Stp1₍₁₋₁₂₅₎ functions as a modular domain that can be transferred to an artificial transcription factor to faithfully confer full amino acid-induced SPS sensor-dependent regulation. Thus, the N-terminal domain is sufficient for both nuclear exclusion and protease recognition.

DISCUSSION

Prior to this report, the only known mechanism directly regulating the activity of Stp1p/Stp2p was the amino acid-induced SPS sensor-dependent endoproteolytic removal of their N-terminal domains (1). By comparing the N-terminal sequences of Stp1p/Stp2p from *senso stricto* strains of *Saccharomyces*, we identified two conserved sequence motifs, designated region I and region II (Fig. 1B). The functions of these motifs were assessed by using targeted alanine substitutions in Stp1p. A mutant protein (Stp1-102p) with a single alanine substitution at the start of homology region II (F66A) was not processed in response to amino acid induction (Fig. 2A). *stp1-102* did not complement an *stp1Δ stp2Δ* double mutant, demonstrating that proteolytic removal of the inhibitory domain is strictly required for Stp1p/Stp2p activity. We could exclude the possibility that the lack of transcriptional induction by Stp1-102p was due to a folding defect or a general loss of function, since second site-suppressing mutations placed in *cis* (*STP1-133*) or in *trans* (*asi1Δ*) enabled the unprocessed mutant form of Stp1p to enter the nucleus and transactivate gene expression (Fig. 2C). The dominant nature and constitutive activity of the *STP1-133* allele, which carries mutations within region I, suggest that this motif normally facilitates the cytoplasmic retention of unprocessed Stp1p. The observation that Stp1-133p is proteolytically processed (Fig. 2C), despite the presence of a nonfunctional inhibitory domain, indicates that the N-terminal regulatory domain of Stp1p is modular.

These results confirm that the processing of Stp1p/Stp2p is indeed the major regulatory event that transduces SPS sensor-initiated signals, generated at the plasma membrane, to the nucleus and to UAS_{aa}-containing promoters. Our analysis revealed that specific and functionally separable motifs within the N-terminal domain provide the molecular basis for the latent nuclear exclusion and amino acid-induced processing of Stp1p/Stp2p. Our current model for Stp1p/Stp2p regulation is presented in Fig. 7. Newly translated Stp1p/Stp2p is excluded from the nucleus by two parallel activities. The primary mechanism is linked to the inhibitory motif designated region I, which appears to anchor the unprocessed full-length forms to an as-yet-undefined cytoplasmic determinant. The secondary mechanism is dependent on the action of Asi1p.

Forsberg et al. previously showed that recessive mutations in *ASII* constitutively derepress the transcription of SPS sensor-regulated genes in the absence of a functional SPS sensor (16). Remarkably, the lack of Asi1p function enables full-length Stp1-102p to enter the nucleus, indicating that the N-terminal regulatory domains in the native forms of Stp1p/Stp2p do not impair promoter binding or transactivation potential. These findings indicate that Asi1p normally functions to negatively regulate the transactivation potential of unprocessed Stp1p/

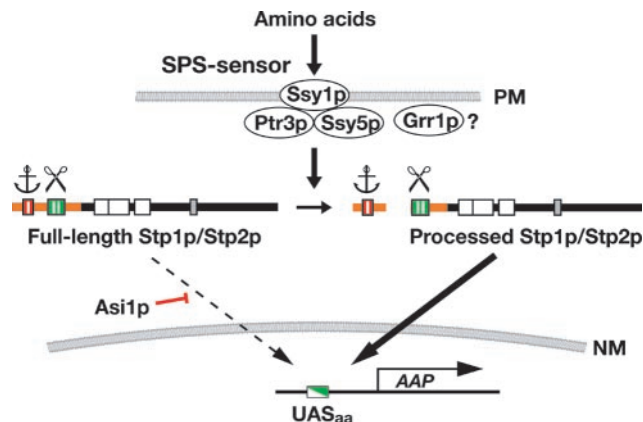


FIG. 7. Model of the amino acid-induced SPS-sensing pathway. The latent nature of Stp1p/Stp2p is determined by conserved sequence motifs region I (red) and region II (green) present in their N-terminal domains (orange). Region I appears to function as a cytoplasmic retention signal (anchor) that prevents the unprocessed full-length forms from efficiently entering the nucleus at rates sufficient to partially derepress amino acid permease (*AAP*) gene expression. Extracellular amino acids induce the proteolytic processing of Stp1p/Stp2p in an SPS sensor- and Grr1p-dependent manner. The precise function of Grr1p remains to be defined (?); Grr1p either functions concomitantly with the previously characterized SPS sensor components (Ssy1p-Ptr3p-Ssy5p) or is required for SPS sensor assembly. The processed shorter forms of Stp1p and Stp2p containing DNA-binding domains (white boxes) accumulate in the nucleus, where they bind UAS present within the promoters of amino acid permease genes (UAS_{aa}). PM, plasma membrane; NM, nuclear membrane.

Stp2p. Asi1p is a polytopic membrane protein with five membrane-spanning segments and a RING-like domain at its extreme C terminus (A. Zargari, C. Andréasson, and P. O. Ljungdahl, unpublished data). We anticipate that knowledge regarding the precise intracellular localization of Asi1p will dramatically influence the basis of the understanding of how this novel protein functions to modulate the activity of Stp1p/Stp2p.

The processing defect exhibited by *grr1* mutant strains indicates that *GRR1* encodes a component that functions in signaling events leading to Stp1p/Stp2p processing. This assignment is supported by our analysis of epistasis relationships. The constitutive activating form of Stp1p, encoded by the dominant *STP1Δ131* allele, and mutations in *ASII* were found to efficiently suppress *grr1Δ* mutant phenotypes. However, the actual mechanistic role of Grr1p in the SPS sensor signal transduction pathway is difficult to predict based on these newly established genetic relationships. We are currently considering two possibilities; Grr1p either is directly involved in the signal transduction events within the SPS sensor or is a factor required for the activity of one of the SPS sensor components. The latter possibility defines a role for Grr1p without direct involvement in a dynamic signaling process. Based on the previously characterized functions of Grr1p in glucose sensing and cell cycle control (6, 15, 31, 32, 36), it is compelling to imagine a direct role for ubiquitin in the SPS sensor pathway, as previously suggested (7).

The modular nature of the N-terminal region of Stp1p mo-

tivated us to examine whether it could be transferred to the well characterized synthetic transactivator LexA-AD. The presence of the N terminus of Stp1p converted this constitutively nucleus-localized factor into a latent fully SPS sensor-regulated factor. This conclusion is based on several observations. First, immunoblot analysis demonstrated that Stp1₍₁₋₁₂₅₎-LexA-AD is endoproteolytically processed in a manner similar to that of full-length Stp1p/Stp2p (Fig. 6A). Furthermore, *lexA* operator-driven promoter activation was shown to depend on amino acid induction and a functional SPS sensor (Fig. 6B). The *OP_{lexA}-lacZ* reporter gene was fully repressed in strains carrying mutations in the upstream components of the SPS sensor pathway (*ssy1Δ*, *ptr3Δ*, *ssy5Δ*, and *grr1Δ*). Finally, constitutive derepression of the *OP_{lexA}-lacZ* reporter construct was observed in *asi1Δ* cells. These data indicate that Stp1₍₁₋₁₂₅₎ is able to override the intrinsic NLS within LexA and to confer full SPS sensor pathway control.

The ability of the N-terminal domain of Stp1p to confer the entire known spectrum of SPS sensor pathway control to an unrelated protein supports the notion that all of the regulatory circuits converge on the N-terminal domain. This finding limits the number of feasible mechanisms that may regulate the latency of Stp1p/Stp2p. For example, it is unlikely that the N terminus masks or blocks an intrinsic NLS within Stp1p/Stp2p. Instead, the autonomous function of the N-terminal domain suggests that the inhibitory motif (region I) interacts with a protein that functions as a nuclear exclusion determinant, e.g., a cytoplasmic protein or a component of the nuclear export machinery. If this model proves correct, then the regulatory mechanism exhibits striking similarity with NF-κB/Rel signaling; IF-κB sequesters NF-κB in the cytoplasm and prevents nuclear translocation by binding the actin cytoskeleton via ankyrin repeats (9, 20).

Finally, it has not escaped our attention that the components described here comprise the basic building blocks of a highly specific system for the regulated expression of genes in yeast cells. The exploitation of such a system has some obvious advantages over existing expression systems. Presumably, the activities of other transcription factors (either heterologous, homologous, or completely artificial) can be placed under the control of the SPS sensor by appending the N-terminal domain of Stp1p. Consequently, amino acid-induced gene expression can be restricted to a single synthetic promoter, as we have accomplished with *OP_{lexA}-lacZ*. Additionally, the transferability of the N-terminal inhibitory domain of Stp1p suggests the possibility of specifically restricting the access of individual proteins to the nucleus in a manner that is readily reversible. This scenario may be useful for testing the physiologic consequences of redirecting and sequestering presumed nuclear factors in the cytoplasm.

ACKNOWLEDGMENTS

We thank the other members of the laboratory of P. O. Ljungdahl for constructive comments throughout the course of this work and Morten Kielland-Brandt for the kind gift of the plasmid carrying the dominant activated *SSY1-102* allele.

This research was supported by the Ludwig Institute for Cancer Research.

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