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Amino Acid Signaling in Yeast: Casein Kinase I and the Ssy5 Endoprotease Are Key Determinants of Endoproteolytic Activation of the Membrane-Bound Stp1 Transcription Factor

Fadi Abdel-Sater, Mohamed El Bakkoury, Antonio Urrestarazu, Stephan Vissers, and Bruno André*

Laboratoire de Physiologie Moléculaire de la Cellule, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, Gosselies, Belgium

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Saccharomyces cerevisiae **cells possess a plasma membrane sensor able to detect the presence of extracellular amino acids and then to activate a signaling pathway leading to transcriptional induction of multiple genes, e.g., *AGP1*, encoding an amino acid permease. This sensing function requires the permease-like Ssy1 and associated Ptr3 and Ssy5 proteins, all essential to activation, by endoproteolytic processing, of the membrane-bound Stp1 transcription factor. The SCF^{Grr1} ubiquitin-ligase complex is also essential to *AGP1* induction, but its exact role in the amino acid signaling pathway remains unclear. Here we show that Stp1 undergoes casein kinase I-dependent phosphorylation. In the *yck* mutant lacking this kinase, Stp1 is not cleaved and *AGP1* is not induced in response to amino acids. Furthermore, we provide evidence that Ssy5 is the endoprotease responsible for Stp1 processing. Ssy5 is significantly similar to serine proteases, its self-processing is a prerequisite for Stp1 cleavage, and its overexpression causes inducer-independent Stp1 cleavage and high-level *AGP1* transcription. We further show that Stp1 processing also requires the SCF^{Grr1} complex but is insensitive to proteasome inhibition. However, Stp1 processing does not require SCF^{Grr1}, Ssy1, or Ptr3 when Ssy5 is overproduced. Finally, we describe the properties of a particular *ptr3* mutant that suggest that Ptr3 acts with Ssy1 in amino acid detection and signal initiation. We propose that Ssy1 and Ptr3 form the core components of the amino acid sensor. Upon detection of external amino acids, Ssy1-Ptr3 likely allows—in a manner dependent on SCF^{Grr1}—the Ssy5 endoprotease to gain access to and to cleave Stp1, this requiring prior phosphorylation of Stp1 by casein kinase I.**

Saccharomyces cerevisiae can detect the presence of amino acids in its extracellular environment and then induce transcription of a set of genes encoding permeases that mediate the uptake of these amino acids into the cell (12, 24). One such gene is *AGP1*, encoding a broad-specificity amino acid permease (31). Several proteins play an essential role in this nutritional signaling pathway. One is Ssy1, an amino acid permease homologue apparently devoid of transport activity and likely involved in recognition of external amino acids. Ssy1 differs from classical amino acid permeases by the presence of an unusually large cytosolic N-terminal domain that plays an important role in signaling (19, 31, 33, 35). Consistent with a direct role of Ssy1 in amino acid detection, a mutant form of Ssy1 (encoded by *ssy1-23*) has selectively lost the ability to respond to several amino acids (7). Furthermore, a constitutive mutant form of Ssy1 (encoded by $SSY1^{382K}$) has recently been described (25). Ptr3 and Ssy5, two peripheral membrane proteins without any predicted transmembrane domain, are also essential to induction of amino acid permeases in response to external amino acids (7, 33, 35). Although these proteins have been shown to interact in two-hybrid assays (7), their exact biochemical role in amino acid signaling remains unclear. Several lines of evidence indicate that the Ssy1, Ptr3, and Ssy5 proteins are associated in a plasma membrane-associated complex named SPS (23). Induction of *AGP1* depends on the synergistic action of two transcription factors, Stp1 and Uga35/Dal81 (1, 3, 31, 34). In the absence of amino acids, Stp1 is located at the cell surface. When amino acids are present, Stp1 undergoes Ssy1-, Ptr3-, and Ssy5-dependent endoproteolytic processing (3). The released C-terminal domain then translocates into the nucleus (3) and acts, together with Uga35/Dal81, through a common GC-rich upstream sequence named UAS_{AA}, to activate *AGP1* transcription (1). Similar UAS_{AA} elements promote SPS-dependent induction, by amino acids, of other amino acid permease genes, e.g., *BAP2* and *BAP3* (16, 17, 45). Recent work indicates that Uga35/Dal81 is also activated in response to amino acids, but the mechanism involved remains unknown (1). Finally, transcriptional induction of *AGP1* mediated by processed Stp1 and Uga35/Dal81 may be amplified severalfold by the Gln3 protein (1). This GATA family transcription factor acts through 5′-GATA-3′ core sequences and is specifically active in cells grown under limiting nitrogen supply conditions (15). Other key components of the external amino acid signaling pathway are ubiquitin and the SCF^{Grr1} ubiquitin-ligase complex (7, 8, 31). Transcription of *AGP1* is largely defective in the *doa4*/*npi2* mutant, where the internal pool of ubiquitin is severely reduced. This deficiency

* Corresponding author. Mailing address: Physiologie Moléculaire de la Cellule (CP300), Université Libre de Bruxelles, 12 rue des Pr. Jeener et Brachet, 6041 Gosselies, Belgium. Phone: 32-2-6509958. Fax: 32-2-6509950. E-mail: bran@ulb.ac.be.

TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
23344c	*MATα ura3*	M. Grenson
30629c	*MAT***a** *gap1*Δ::*kanMX2 ura3*	31
34065b	*MATα gap1*Δ::*kanMX2 ssy1*Δ::*kanMX2 ura3*	7
JA115	*MATα grr1*Δ::*kanMX2 ura3*	31
FB94	*MAT***a** *gap1*Δ *ptr3*Δ *ura3*	7
FB92	*MAT***a** *gap1*Δ *ssy5*Δ *ura3*	7
FB35	*MATα gap1*Δ::*kanMX2 ptr3-35 ura3*	This study
JA547	*MATα pdr5*Δ::*kanMX2 ura3*	This study
FA08	*MAT***a** *gap1*Δ *grr1*Δ *ura3*	This study
FA10	*MAT***a** *gap1*Δ *ssy1*Δ *ura3*	This study
FA44	*MATα GAL1-HA$_3$-SSY5 ura3*	This study
FA38	*MAT***a** *gap1*Δ *GAL1-HA$_3$-SSY5 ura3*	This study
FA20	*MAT***a** *gap1*Δ *ptr3*Δ *GAL1-HA$_3$-SSY5 ura3*	This study
38016c	*MAT***a** *gap1*Δ *grr1*Δ *GAL1-HA$_3$-SSY5 ura3*	This study
FA39	*MATα gap1*Δ *ssy1*Δ *GAL1-HA$_3$-SSY5 ura3*	This study
FA24	*MAT***a** *gap1*Δ *GAL1:HA$_3$-PTR3 ura3*	This study
FA26	*MAT***a** *gap1*Δ *ssy5*Δ *GAL1-HA$_3$-PTR3 ura3*	This study
FA33	*MATα gap1*Δ *grr1*Δ *GAL1-HA$_3$-PTR3 ura3*	This study
FA37	*MATα gap1*Δ *ssy1*Δ *GAL1-HA$_3$-PTR3 ura3*	This study
LRB341	*MAT***a** *his3 leu2 ura3-52*	50
LRB346	*MAT***a** *his3 leu2 ura3-52 yck1-*Δ*1 yck2-2^{ts}*	50
W303	*MAT***a** *his3-11 leu2-3 ura3-1 ade2-1 trp1-1 can1-100*	M. Tyers
MT670	*MAT***a** *his3-11 leu2-3 ura3-1 ade2-1 trp1-1 can1-100 cdc34-1*	M. Tyers 58
MT668	*MAT***a** *his 3-11 leu2-3 ura3-1 ade2-1 trp1-1 can1-100 cdc4-1*	M. Tyers 58
27071b	*MATα trp1 npi2/doa4 ura3*	54
37053a	*MATα gap1*Δ::*kanMX2 stp1*Δ::*kanMX2 ura3*	This study
38003a	*MAT***a** *gap1*Δ::*kanMX2 stp1*Δ::*kanMX2 GAL1-HA$_3$-SSY5 ura3*	This study
34304b	*MATα gap1*Δ::*kanMX2 uga35*Δ *ura3*	1
37082b	*MAT***a** *gap1*Δ::*kanMX2 uga35*Δ *GAL1-HA$_3$-SSY5 ura3*	This study
34686b	*MATα gap1*Δ *ssy5*Δ::*kanMX2 STP1-HA-kanMX2 ura3*	This study
34692c	*MAT***a** *ssy5*Δ::*kanMX2 agp1:lacZ kanMX2 ura3*	This study
FA101	*MAT***a** *his3 leu2 ura3-52 GAL1-HA$_3$-SSY5 yck1-*Δ*1 yck2-2^{ts}*	This study
JA495	*MAT***a** *gap1*Δ::*kanMX2 ptr3*Δ::*kanMX AGP1 ura3* *MATα gap1*Δ::*kanMX2 ptr3*Δ::*kanMX agp1*::*lacZ ura3*	This study

can be compensated by overproduction of ubiquitin. Furthermore, when the F-box protein Grr1 is absent, induction of *AGP1* transcription is totally abolished. Thermosensitive mutations affecting other components of the SCF complex also markedly reduce *AGP1* transcription at the nonpermissive temperature (7, 8, 31).

Here we report that casein kinase I (CKI) is involved in Stp1 phosphorylation and that this modification is a prerequisite to Stp1 activation by endoproteolytic processing. We further provide evidence that Ssy5 is the endoprotease that mediates Stp1 processing. Ssy5 indeed shares sequence similarity with several serine proteases and is self-processed. Its overproduction triggers Stp1 cleavage even when amino acids are not available and irrespective of whether the Ssy1, Ptr3, and SCF^{Grr1} elements are functional. We also describe a particular *ptr3* mutant which has lost the ability to respond to some but not all amino acids, a phenotype consistent with Ptr3 acting with Ssy1 in amino acid detection and signal initiation. A model integrating these novel data is discussed.

MATERIALS AND METHODS

Genetic background and growth conditions. Most *S. cerevisiae* strains used in this study derive from the wild-type Σ1278b strain (9), except for the *yck^{ts}*, *cdc4-1*, and *cdc34-1* mutants, for which the corresponding wild types were used (Table 1). Cells were grown in a minimal buffered (pH 6.1) medium with 3% glucose, raffinose, or galactose (when mentioned) as the carbon source. To this medium, urea (5 mM), $(NH_4)_2SO_4$ (10 mM), proline (5 mM), another amino acid (1 to 10 mM), or combinations of these compounds were added as a source(s) of nitrogen. Assays for resistance to the toxic amino acid analogue D,L-ethionine (20 μg/ml) were carried out on plates with $(NH_4)_2SO_4$ as the sole nitrogen source.

Plasmids and DNA methods. All procedures for manipulating DNA were standard ones (4, 52). The *Escherichia coli* strain used was JM109. Plasmid pCA047 bearing Stp1-hemagglutinin (HA) was a generous gift of P. Ljungdahl (3). Plasmid YCp*AGP1-lacZ* has been described elsewhere (31). The YCp-*ptr3-35* plasmid was isolated by inserting into BamH1-EcoRI-cleaved plasmid pFL38 a 3,288-bp fragment bearing the *ptr3-35* allele. This fragment was obtained by PCR amplification, using genomic DNA of strain FB35 as template and primers 5-PTR3-C and 3-PTR3-C (Table 2). Plasmid pFA153 is a centromere-based *URA3* vector bearing the *SSY5* gene (with 954 bp of upstream and 2,364 bp of downstream sequences) cloned from strain Σ1278b. The pFA138*SSY5* plasmid was constructed by cotransforming cells of strain 23344c with the SacI-cleaved pFA153 plasmid (SacI cuts upstream from the insertion site of *SSY5* sequences) and a 641-bp DNA fragment made of the *GAL1* gene promoter followed by the triple HA tag-coding DNA. This DNA fragment (flanked by appropriate 40-bp recombination sequences) was obtained by PCR with plasmid pFA6a-KanMX6-*PGAL1-3HA* as template (57) and primers 5GALHASSY5 and 3SSY5HA (Table 2). Plasmid pFA150 is the same as pFA153 except that the encoded Ssy5 protein is tagged at its N terminus by the triple HA tag. It was constructed by cotransforming cells of the 23344c strain with Pme1-cleaved pFA153 plasmid (Pme1 cuts just downstream from the ATG initiation codon of *SSY5*) and a 100-bp DNA fragment made of the triple HA tag-coding DNA. This DNA fragment (flanked by appropriate 40-bp recombination sequences) was obtained by PCR using plasmid pFA6a-KanMX6-*PGAL1-3HA* as template (57) and primers 5SSY5NtHA and 3SSY5HA (Table 2). Plasmid pFA154 is the same as pFA153 and plasmid pFA144 is the same as pFA138, except that the codon corresponding to the serine at position 640 has been replaced by the GCA triplet corresponding to an alanine (Ssy5$^{S\rightarrow A}$). Both plasmids were constructed with the

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
5D-SSY1	5′-TATGTACATAGACATATAAAGGGCATGTGGTAATTATGACGCGGCCGCCAGCTGAAGCTT-3′
3D-SSY1	5′-CCAATATGATACCCTTATTTTTATCCCGTGTTATATTTAAGCGGCCGCATAGGCCACTAG-3′
5D-GRR1	5′-AAGAGAGCAAAAAGTTGGGGAAGTAAGGAAATCCATTATCGCGGCCGCCAGCTGAAGCTT-3′
3D-GRR1	5′-GGTTAATTCAGGTATTATTTTTATGGCGGTTTCAGGTATCGCGGCCGCATAGGCCACTAG-3′
5D-PDR5	5′-AAGTTTTCGTATCCGCTCGTTCGAAAGACTTTAGACAAAAGCGGCCGCCAGCTGAAGCTT-3′
3D-PDR5	5′-CTTATTATTACGCACCTATATGTAGTGATTATGTATGTTTCGGCCGCATAGGCCACTAG-3′
5-PTR3-C	5′-GCCGAATTCTCGAGGTATACACTCCTACG-3′
3-PTR3-C	5′-CCGCCATCCCAAACTCAACTGGCATCTCG-3′
3-PTR3-HA	5′-TATCCGTCTGGCGGCCCCACTTTTGTCTATGAGAGTGCATCATGCACTGAGCAGCGTAATCTG-3′
F4-GAL1-PTR3	5′-CATCAATGATTACCTTATCAGAACTAATTTAAGTGAAATGAATTCGAGCTCGTTTAAAC-3′
3-SSY5-HA	5′-CTTCGTTCTTTTTCTTGTTTAAACCAAAAAATCTGACCATGCACTGAGCAGCGTAATCTG-3′
F4-GAL1-SSY5	5′-ATTGTATGTAATTATTACCCAAACTAATTTAAGTGAAATGAATTCGAGCTCGTTTAAAC-3′
5-SSY5-mutS-A	5′-GCTAGTGCGGGGGATGCAGGCGCATGGATC-3′
3-SSY5-mutS-A	5′-GATCCATGCGCCTGCATCCCCCGCACTAGC-3′
5-SSY5ΔPISML	5′-GCAGTTTCAGAGAATCCACTTGAATTGTCCACTGAGACAAGCGCTAAAATTACAGG-3′
3-SSY5ΔPISML	5′-CCTGTAATTTTAGCGCTTGTCTCAGTGGACAATTCAAGTGGATTCTCTGAAACTGC-3′
5SSY5NtHA	5′-TTTTGCATGTACATAGTACTGGTGTAAACTCGATATACCGATGTACCCATACGATGTTCCTGACTATG-3′
5GALHASSY5	5′-TTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCTAAAGAGCCCCATTATCTTA-3′
3SSY5HA	5′-GCAAGTCTGTATTTTCCTTTTCTTCGTTCTTTTTCTTGTTTAAACCAAAAAATCTGACCAT-3′

QuikChange site-directed mutagenesis kit (Stratagene) and primers 5-SSY5-mutS-A and 3-SSY5-mutS-A (Table 2). Plasmid pFA148 is a derivate of pFA138 in which the DNA region spanning positions 1560 to 1578 relative from the ATG initiation codon was deleted using primers 5-SSY5ΔPISML and 3-SSY5ΔPISML (Table 2). The accuracy of each mutagenized plasmid was checked by sequencing.

Construction of yeast strains. The *gap1*Δ *ssy1*Δ, *gap1*Δ *grr1*Δ, and *pdr5*Δ strains were constructed by the PCR-based gene deletion method (57). The DNA segments used to introduce these mutations were generated by PCR using the *kanMX2* gene from plasmid pFA6a-*kanMX2* as a template and the following PCR primers (Table 2): *ssy1*Δ:*kanMX2*, 5D-SSY1 and 3D-SSY1; *grr1*Δ:*kanMX2*, 5D-GRR1 and 3D-GRR1; *pdr5*Δ:*kanMX2*, 5D-PDR5 and 3D-PDR5. The yeast strains EK007 (*ura3 gap1*Δ) and 23344c (*ura3*) were transformed with the PCR fragments by the lithium method as previously described (26). Transformants were selected on complete medium containing 200 μg of Geneticin (Gibco-BRL)/ml. A similar transformation and selection procedure was used to construct strains *GAL1-HA$_3$–SSY5 gap1*Δ, *GAL1-HA$_3$–SSY5 gap1*Δ *ssy1*Δ, *GAL1-HA$_3$–SSY5 gap1*Δ *ptr3*Δ, *GAL1-HA$_3$–PTR3 gap1*Δ, *GAL1-HA$_3$–PTR3 gap1*Δ *ssy1*Δ, *GAL1-HA$_3$–PTR3 gap1*Δ *ssy5*Δ, *GAL1-HA$_3$–PTR3 gap1*Δ *grr1*Δ, and *GAL1-HA$_3$-SSY5 yck1-*Δ*1 yck2-2^{ts}*. For these strains, insertion DNA cassettes made of the *GAL1* promoter, the sequence encoding the triple HA tag, and the first codons of the *SSY5* or *PTR3* gene were generated by PCR with plasmid pFA6a-kanMX6-PGAL1-3HA (57) as template DNA and with the following PCR primers: 3-SSY5-HA and F4-GAL1-SSY5 or 3-PTR3-HA and F4-GAL1-PTR3 (Table 2).

β-Galactosidase assays. All β-galactosidase assays were performed on cells that had reached the state of balanced growth. The β-galactosidase assays were performed as described earlier (2), and activities are expressed in nanomoles of *o*-nitrophenol formed per minute per milligram of protein. Protein concentrations were measured with the Folin reagent, bovine serum albumin being used as a standard (41).

Yeast cell extracts and immunoblotting. Crude cell extracts were prepared as previously described (28). For Western blot analysis, equal quantities of proteins were loaded onto an sodium dodecyl sulfate–8% polyacrylamide gel in a Tricine system. After transfer to a nitrocellulose membrane (Shleicher & Schüell), the proteins were probed with anti-HA monoclonal antibodies 12CA5 (1:10,000; Roche Diagnostics), with polyclonal antibodies raised against the N-terminal tail of Agp1 (1:5,000) (1), or with polyclonal antibodies raised against Gic2 (1:2,500) (13). Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G secondary antibody (Amersham Pharmacia Biotech).

Alkaline phosphatase treatment of protein extracts. Crude cell extracts were prepared as previously described (28) except that the final pellets were dissolved in 4% sodium dodecyl sulfate–0.1 M Tris-HCl (pH 6.8)–20% glycerol–2% 2-mercaptoethanol. Alkaline phosphatase (20 U), 20 μl of dephosphorylation buffer (Roche Diagnostics), and 20 μl of 0.5 M Tris (pH 8.0) were added to 20 μl of extract, and the mixture was then incubated for 2 h at 37°C.

RESULTS

Essential role of CKI in amino acid signaling. Previous work has shown that components of the SCF^{Grr1} ubiquitin-ligase complex are essential to transcriptional induction of the *AGP1* gene in response to external amino acids (7, 8). Although SCF ubiquitin ligases most often catalyze ubiquitylation of protein substrates only once these have been phosphorylated (18), previous genetic screens failed to identify any protein kinase involved in amino acid signaling in yeast (7, 23, 33, 35). The SCF^{Grr1} complex is also essential to transmission of the glucose signal initiated by Snf3 and Rgt2, the transporter-like sensors of external glucose (22, 37). Recent work has shown that the yeast membrane-bound CKI is an essential component of Rgt2-mediated glucose signaling. Its role is to phosphorylate Std1 and Mth1, these modifications being essential to both proteins' Grr1-dependent degradation and to subsequent *HXT* gene induction in response to glucose (44). In *Kluyveromyces lactis*, the Snf3/Rgt2 homologue encoded by the *RAG4* gene is essential to transcriptional induction of the *RAG1* gene encoding a low-affinity glucose transporter (10). Interestingly, the *K. lactis* equivalent of CKI (Rag8) is also required for transcriptional induction of the *RAG1* gene (11). Hence, CKI appears to play an important role in glucose signaling in both *S. cerevisiae* and *K. lactis*. These data prompted us to test whether CKI is also involved in amino acid signaling. For this, we used a strain with the *YCK1* gene deleted and containing a thermosensitive *yck2^{ts}* allele. This mutant grows normally at 24°C but fails to grow at the restrictive temperature of 37°C (50). We transformed the mutant and the congenic wild type with a *lacZ* reporter gene under the control of the *AGP1* upstream region. Cells were grown at the permissive temperature of 24°C and then placed for 20 min at 37°C. Phenylalanine, a potent inducer of *AGP1*, was then added to the medium, and the culture was incubated for 2 h. The results (Fig. 1) showed that transcription of the *AGP1-lacZ* reporter gene was induced by phenylalanine in the wild type transferred to 37°C, but that this induction was totally abolished in the *yck^{ts}* mutant. At the permissive temperature of 24°C, *AGP1-lacZ* expression was

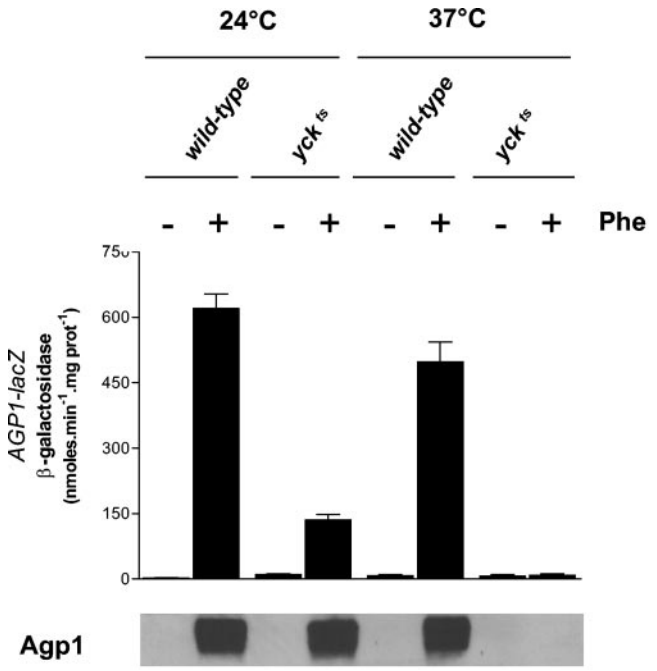

FIG. 1. CKI is essential to *AGP1* induction in response to amino acids. The wild type (LRB341) and the *yck1*Δ*-1 yck2-2*^{ts} (LRB346, *yck*^{ts}) strain were transformed with the YCp*AGP1-lacZ* plasmid (*URA3*) and the YCpJYS-20 plasmid bearing the *HIS3* and *LEU2* genes for complementation of auxotrophies. The strains were grown at 24°C on minimal medium containing urea as sole nitrogen source. Half of the cultures were transferred to 37°C for 20 min. Phenylalanine was then added (+) or not (−), and the cultures were incubated for 2 h. Then, β-galactosidase activity was measured as described in Materials and Methods. Total cellular extracts were also analyzed in a Western blotting experiment using anti-Agp1 antibodies.

induced in the *yck*^{ts} strain, but to a level significantly lower than in the wild type. Immunodetection of the Agp1 permease in extracts of wild-type and *yck*^{ts} cells confirmed that induction of Agp1 synthesis was totally abolished in the *yck*^{ts} strain transferred to 37°C (Fig. 1). These data clearly show that CKI plays an essential role in transcriptional induction of the *AGP1* gene in response to amino acids.

CKI is required for phosphorylation and endoproteolytic processing of Stp1. An important event in the signaling pathway leading to *AGP1* transcriptional induction is endoproteolytic processing of the membrane-bound Stp1 transcription factor (3). After cleavage, Stp1 translocates into the nucleus and acts together with the Uga35/Dal81 transcription factor, via the UAS_{AA} upstream element, to activate *AGP1* transcription (1). To gain further insight into the role of CKI in amino acid signaling, we transformed the *yck*^{ts} mutant and the congenic wild type with a low-copy-number plasmid expressing an HA-tagged Stp1 protein under the control of the natural *STP1* gene promoter. The cells were grown at 24°C and transferred for 20 min to 37°C, and then phenylalanine was added to the culture (Fig. 2A). Western blot analysis of wild-type cell extracts revealed that Stp1 is normally cleaved at 37°C in response to phenylalanine and that this correlates with immunodetection of a high-intensity Agp1 signal. In the *yck*^{ts} mutant, cleavage was totally abolished and Agp1 was not induced. Further examination of the immunoblots revealed faster migration of the full-length Stp1 signal in extracts of the *yck*^{ts} mutant than in extracts of wild-type cells (Fig. 2B). This suggests that Stp1 is phosphorylated in a CKI-dependent manner and that this modification occurs even when amino acids are not available in the medium. To test this, we examined the

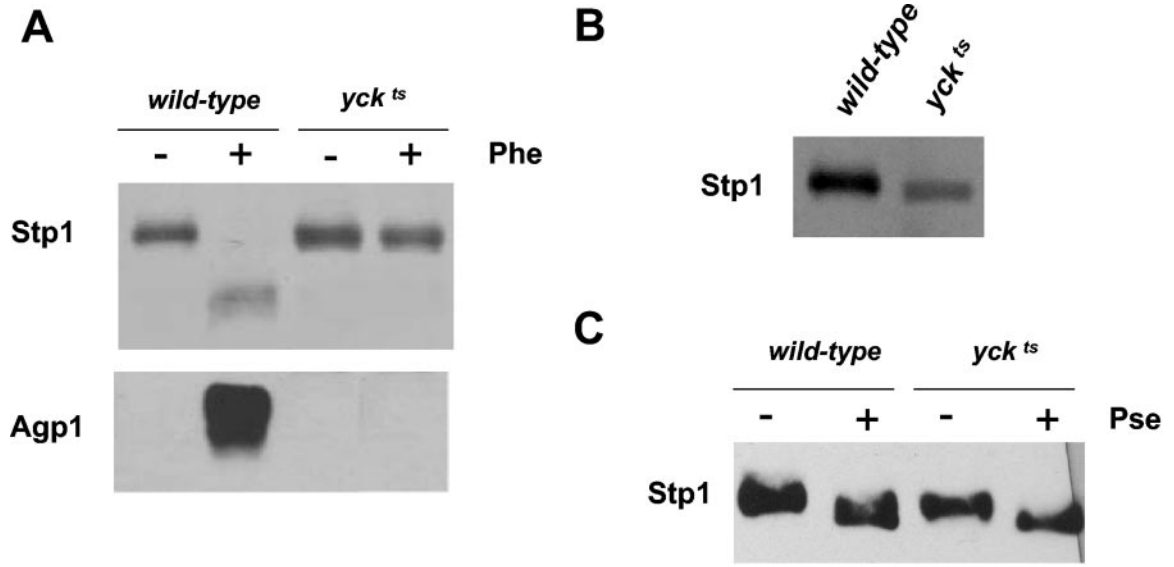

FIG. 2. Role of CKI in phosphorylation and endoproteolytic processing of Stp1. The wild-type (LRB341) and *yck1*Δ *yck2-2*^{ts} (LRB346, *yck*^{ts}) strains were transformed with plasmid pCA047 (*URA3*) bearing the *STP1-HA* gene and plasmid YCpJYS-20 bearing the *HIS3* and *LEU2* genes for complementation of auxotrophies. (A) The strains were grown at 24°C on minimal medium containing urea as sole nitrogen source. Cultures were then transferred to 37°C for 20 min. Phenylalanine was then added (+) or not (−) to the cultures, which were then incubated for 2 h before immunoblot analysis of total cell extracts with anti-HA antibodies. (B) The strains were grown at 24°C on urea medium, and total cell extracts were analyzed by immunoblotting with anti-HA antibodies. (C) The strains were grown at 24°C on urea medium. Cultures were then transferred to 37°C for 20 min, and total cell extracts were incubated (+) or not (−) in the presence of alkaline phosphatase (Pse) for 1 h. After migration, immunoblotting was carried out with anti-HA antibodies.

migration pattern of Stp1 immunodetected in wild-type and *yck*^{ts} cell extracts treated or not with alkaline phosphatase (Fig. 2C). Stp1 migrated faster after treatment of wild-type extracts with alkaline phosphatase, confirming that Stp1 is phosphorylated. In extracts of the *yck*^{ts} strain, Stp1 migrated faster compared to the situation with untreated wild-type extracts but slower compared to the same extracts treated with alkaline phosphatase. Hence, CKI is clearly involved in Stp1 phosphorylation, and other protein kinases likely contribute to generate fully phosphorylated Stp1. The CKI-dependent phosphorylation occurs in media devoid of amino acids and seems essential to Stp1 endoproteolytic processing in response to amino acids.

Components of the SCF^{Grr1} ubiquitin-ligase complex are also essential to endoproteolytic processing of Stp1. Endoproteolytic processing of transcription factors often requires their prior ubiquitylation. For instance, yeast Stp23 and Mga2 are initially synthesized as inactive precursors anchored to the endoplasmic reticulum through their C-terminal tails. Activation of these factors involves their ubiquitylation and proteasome-mediated degradation of their C-terminal domains, whereas the N-terminal domains are left intact and migrate into the nucleus to activate gene transcription (30). The exact mechanism of Stp1 endoproteolytic processing remains unknown. Andreasson and Ljungdahl (3) reported that cleavage of Stp1 is normal in mutant cells lacking the F-box protein Grr1. They suggested that the SCF^{Grr1} ubiquitin-ligase complex is involved in later steps of the amino acid signaling pathway. In the course of our work, we repeated this experiment and obtained a completely different result: upon addition of phenylalanine to the growth medium, Stp1 was cleaved in the wild type but not in the *grr1*Δ mutant (Fig. 3A). This result was observed in several independent experiments involving longer incubation times in the presence of diverse amino acids (data not shown). It has since also been observed by the group of P. Ljungdahl (personal communication). To determine if Grr1 were required for Stp1 cleavage as part of the SCF complex, we tested whether Stp1 processing occurs or not in the thermosensitive *cdc34*^{ts} mutant strain (Fig. 3B). *CDC34* encodes the E2 ubiquitin-conjugating enzyme, which is normally part of SCF complexes and directly involved in transfer of ubiquitin to the target protein (18). The *cdc34*^{ts} mutant and the congenic wild type were grown at 30°C without any amino acid, after which they were transferred to the restrictive temperature of 37°C and incubated for 60 min. Then, phenylalanine was added to the medium and the culture was incubated for 15 more minutes. The data presented in Fig. 3B clearly show that Stp1 undergoes normal endoproteolytic processing in the wild type at 37°C. Processing, however, was totally abolished in the *cdc34*^{ts} strain. This lack of cleavage is not an indirect consequence of an effect of the *cdc34*^{ts} mutation on the cell cycle, since Stp1 was normally cleaved in the *cdc4-1* strain (also defective in cell cycle progression at 37°C). Stp1 cleavage was also totally abolished when transfer of cells to 37°C and addition of phenylalanine were simultaneous (data not shown), further suggesting that the effect of the *cdc34*^{ts} mutation is not the indirect consequence of some deficiency in another Cdc34-dependent cell function. In conclusion, our data show that at least two components of the SCF^{Grr1} ubiquitin-ligase complex, namely the F-box protein Grr1 and the E2 enzyme Cdc34, are essential to amino acid-induced endoproteolytic processing of transcription factor Stp1. These data corroborate previous results showing that transcriptional induction of *AGP1* is impaired in *grr1*Δ and *cdc34*^{ts} mutants as well as in strains with impaired function in other components of the SCF^{Grr1} complex (8).

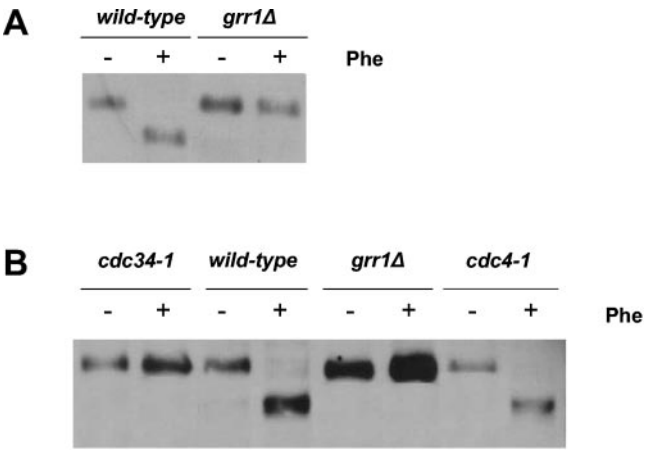

FIG. 3. Components of the SCF^{Grr1} ubiquitin-ligase complex are essential to amino acid-induced endoproteolytic processing of Stp1. (A) Wild-type (23344c) and *grr1*Δ (JA115) strains transformed with the pCA047 plasmid (*STP1-HA*) were grown on minimal medium with proline as sole nitrogen source. Phenylalanine (5 mM final concentration) was added (+) or not (−), and the culture was incubated for 20 min. Crude cell extracts were then prepared, and immunoblotting was carried out with anti-HA antibodies. (B) Wild-type (W303), *cdc34-1* (MT670), *cdc4-1* (MT668), and *grr1*Δ (JA115) strains transformed with the pCA047 plasmid (*STP1-HA*) were grown at 30°C on minimal medium with proline as sole nitrogen source. Cells were transferred to 37°C and incubated for 60 min. Then, phenylalanine (5 mM final concentration) was added (+) or not (−), and the cultures were incubated for an additional 15 min. Crude cell extracts were then prepared, and immunoblotting was carried out with anti-HA antibodies.

Role of the proteasome in Stp1 cleavage. Proteins ubiquitylated by SCF complexes generally undergo regional or complete degradation catalyzed by the proteasome (47). It has been reported, however, that the proteasome is not involved in Stp1 cleavage. During growth on synthetic complete medium, investigators readily detected the processed form of Stp1 in *pre* mutants impaired in three major activities of the proteasome (3). We sought to further investigate the role of the proteasome in Stp1 processing by using MG132, an inhibitor of the proteasome (36). As this toxic compound can be extruded from cells via the Pdr5 multidrug resistance pump (20), experiments were carried out in a *pdr5*Δ mutant. Cells were first grown in the absence of amino acids. MG132 was then added to the medium, and 2 h later, phenylalanine was added to induce Stp1 cleavage (Fig. 4). The results showed that Stp1 undergoes normal phenylalanine-induced cleavage regardless of whether MG132 is present in the medium (Fig. 4A). Although the mobility of Stp1 seems to change over time in cells not treated with MG132 (Fig. 4A), this was not reproduced in other experiments (data not shown). To confirm inhibition of proteasome function, we probed the same cell extracts with antibodies against Gic2, a protein previously reported to undergo SCF^{Grr1}-dependent ubiquitylation and to accumulate as a ladder of high-molecular-weight forms in mutants impaired in proteasome function (32). MG132 did induce the appearance

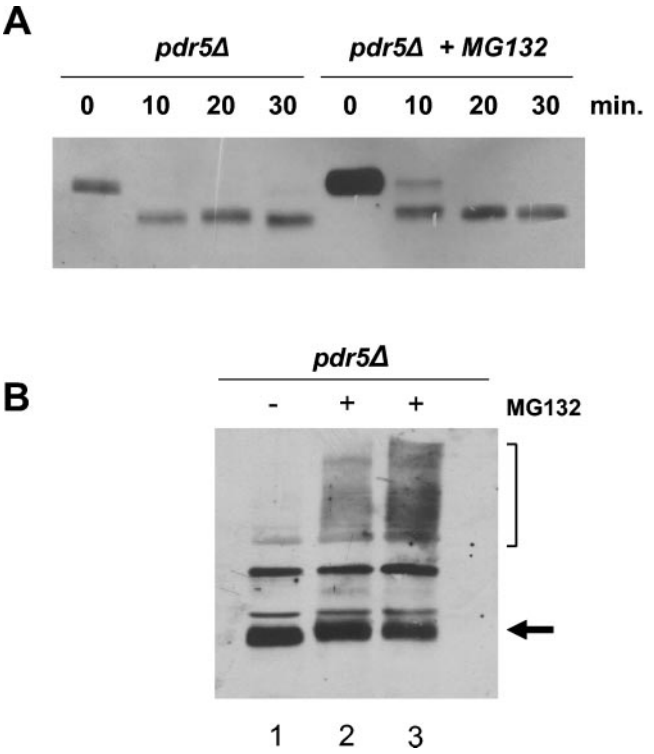

FIG. 4. Stp1 cleavage in response to amino acids is insensitive to proteasome inhibition. (A) Strain *pdr5*Δ (JA547) transformed with plasmid pCA047 (*STP1-HA*) was grown on minimal proline medium. The proteasome inhibitor MG132 (50 μM final concentration) was added (or not), and the cultures were incubated for 2 h before addition of phenylalanine (5 mM). Before and several times after addition of the amino acid, culture samples were collected and crude extracts were analyzed by immunoblotting with anti-HA antibodies. (B) The same extracts of cells grown without MG132 (−) or incubated with MG132 (+) for 90 min (lane 2) or 120 min (lane 3) were analyzed by immunoblotting with antibodies against Gic2.

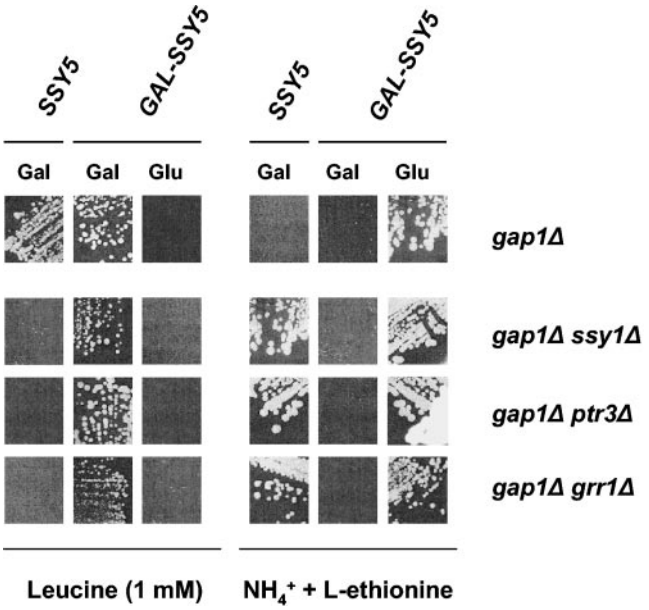

FIG. 5. Overexpression of *SSY5* suppresses the growth defects caused by the *grr1*Δ, *ssy1*Δ, and *ptr3*Δ mutations. Cells were spread on minimal medium with leucine (1 mM) as sole nitrogen source or on minimal NH_4^+ medium containing the toxic amino acid analogue L-ethionine (20 $\mu g \cdot ml^{-1}$). Glucose (Glu) or galactose (Gal) was added as sole carbon source. The strains were FA38 (*GAL1-HA–SSY5 gap1*Δ *ura3*), FA39 (*GAL1-HA–SSY5 gap1*Δ *ssy1*Δ *ura3*), FA20 (*GAL1-HA–SSY5 gap1*Δ *ptr3*Δ *ura3*), 38016c (*GAL1-HA–SSY5 gap1*Δ *grr1*Δ *ura3*), 30629c (*gap1*Δ *ura3*), 34065b (*gap1*Δ *ssy1*Δ *ura3*), FB94 (*gap1*Δ *ptr3*Δ *ura3*), and FA08 (*gap1*Δ *grr1*Δ *ura3*).

of high-molecular-weight forms of Gic2p, likely corresponding to ubiquitin-conjugated forms of the protein (Fig. 4B). Hence, our data corroborate previous conclusions based on the use of *pre* mutants (3), and they indicate that Stp1 endoproteolytic processing is largely independent of proteasome function.

In all reported cases of ubiquitin-dependent modification and activation of transcription factors, the N- or C-terminal domain of the protein is degraded by the proteasome. Even in cases where degradation is initiated by an internal cut within a polypeptide loop of the protein, subsequent processive degradation of the polypeptide chain is mediated by the proteasome (38, 47). These situations differ from that described for Stp1, since the released N-terminal domain is apparently left intact after endoproteolytic cleavage (3). This may account for the fact that the proteasome does not intervene in Stp1 processing. Our observations are thus consistent with the intervention of another endoprotease activity.

Overproduction of Ssy5 leads to inducer-independent Stp1 processing and *AGP1* transcription. One might predict that cells defective in the protease mediating Stp1 endoproteolytic processing will be unable to cleave Stp1 and induce *AGP1* transcription in response to amino acids. Such a phenotype has been described for strains functionally impaired in Ssy1, Ptr3, or Ssy5, three factors proposed to form a complex (SPS) at the plasma membrane (3). We reasoned that overproduction of the endoprotease involved in Stp1 processing might induce Stp1 cleavage and thus activate *AGP1* transcription even in a medium devoid of amino acids. We thus tested the effect of Ssy5 and Ptr3 overproduction on *AGP1* transcription.

To overproduce Ssy5, we placed the chromosomal copy of the *SSY5* gene under the control of the *GAL* promoter. The construct also introduced a triple HA tag at the extreme N terminus of the protein. This construct was introduced into various strains, including the *gap1*Δ mutant lacking the general amino acid permease. When the Ssy5 function is impaired in this strain, readily detectable growth phenotypes are produced (7). The HA-tagged Ssy5 protein proved functional, since the *gap1*Δ *GAL-SSY5* strain behaved like a *gap1*Δ *SSY5*$^+$ mutant when grown on galactose, i.e., cells could grow on leucine (1 mM) as sole nitrogen source and could not grow in the presence of the toxic amino acid analogue L-ethionine (Fig. 5). When grown on glucose, in contrast, the *gap1*Δ *GAL-SSY5* strain did not grow on leucine and was resistant to L-ethionine, phenotypes typically displayed by *gap1*Δ strains additionally defective in any component of the SPS complex (7) (Fig. 5).

The *gap1*Δ *GAL-SSY5* strain was grown on a minimal medium without any amino acid and with raffinose as a carbon source. At time zero of the experiment, galactose was added to the medium. Samples of the culture were withdrawn at time intervals, and crude cell extracts were prepared and probed with antibodies against HA (Fig. 6A). As expected, Ssy5 was

not detected in cells grown on raffinose. Thirty minutes after galactose addition, a single band migrating as an ~80-kDa protein was readily detected. This mobility corresponds with the calculated molecular mass of Ssy5 (76 kDa) tagged with a triple HA. After 120 min, remarkably, an additional band migrating as a ~50-kDa protein was also detected, indicating that the overproduced Ssy5 protein had undergone endoproteolytic cleavage (Fig. 6A). The same immunoblot was then probed with antibodies against Agp1 (Fig. 6A). As expected, no Agp1 signal was detected in cells growing on raffinose without any amino acid. The same was true 30 min after galactose addition, but 120 min after galactose induction a high-intensity Agp1 signal was readily detected. These results indicate that overproduction of Ssy5 leads to high-level synthesis of the Agp1 permease even if amino acids are not present on the medium. This conclusion was confirmed by assaying β-galactosidase activity in *GAL-SSY5* cells transformed with *lacZ* reporter genes under the control of the entire *AGP1* upstream region or the UAS_{AA} element alone (Table 3): growth of this strain on galactose coincided with β-galactosidase values at least as high as those measured in wild-type cells grown in the presence of amino acids. On glucose, however, no *lacZ* reporter gene was expressed, even when phenylalanine was present in the medium. These data clearly show that overproduction of Ssy5 leads to high-level constitutive activation of *AGP1* transcription.

To determine if high-level constitutive activation of *AGP1* transcription in Ssy5-overproducing cells correlates with constitutive Stp1 processing, we transformed the *GAL-SSY5* strain with a low-copy-number plasmid expressing the HA-tagged Stp1 protein. Cells were first grown without any amino acid and with raffinose as a carbon source. As expected, Stp1 was readily detected on immunoblots as its full-length form (Fig. 6B). Thirty minutes after addition of galactose, the ~80-kDa form of Ssy5 became detectable and Stp1 still migrated mainly as its full-length form. Ninety minutes after galactose addition, both forms of Ssy5 were readily detected and Stp1 migrated as its processed form. The cleaved form of Stp1 was still detected several hours after galactose addition. These data clearly show that overproduction of Ssy5 causes efficient inducer-independent endoproteolytic processing of Stp1.

The Ssy5 overproduction experiment was also performed in the CKI-deficient strain (Fig. 6C). Synthesized Ssy5 undergoes normal endoproteolytic cleavage both in the wild-type and *yck*^{ts} strains transferred to the restrictive temperature of 37°C. Stp1, however, was cleaved in the wild type but not in the *yck*^{ts} strains upon Ssy5 expression. This shows that CKI is essential to Stp1 cleavage even under conditions of Ssy5 overproduction. Furthermore, Ssy5 seems to undergo normal processing in CKI-deficient cells.

Finally, we tested whether endoproteolytic processing of the same HA-tagged Ssy5 protein was also detectable in cells where the unique *SSY5* gene was expressed under the control of its own promoter (Fig. 6D). In cells grown without amino acids, the same two bands were detected, but the one corresponding to the unprocessed form of Ssy5 was of much lower intensity compared to experiments in which Ssy5 had been expressed under the control of the *GAL* promoter. Furthermore, addition of phenylalanine did not alter this migration pattern of Ssy5. We conclude that Ssy5 is efficiently processed even when synthesized at normal levels and that this reaction occurs independently of the presence or absence of amino acids in the medium.

Ssy1, Ptr3, and SCF^{Grr1} are not required for Stp1 activation in Ssy5-overproducing cells. We next sought to determine whether factors normally essential to amino acid-induced Stp1 processing and *AGP1* transcription are also involved in constitutive Stp1 cleavage and *AGP1* expression when Ssy5 is overproduced. The *SSY1*, *PTR3*, and *GRR1* genes were thus deleted individually in the *GAL-SSY5* strain. The resulting strains were then transformed with a plasmid bearing the *AGP1-lacZ* reporter gene. Table 4 shows the β-galactosidase activities measured in transformed cells grown on glucose (*SSY5* not expressed) or galactose (*SSY5* overexpressed) as a carbon source. The data show that neither Ssy1 nor Ptr3 nor Grr1 is required for constitutive *AGP1-lacZ* expression in Ssy5-overproducing cells. Consistent with this result, overproduction of Ssy5 also relieved the growth phenotypes caused by lack of Ssy1, Ptr3, or Grr1 in the *gap1*Δ strain grown on leucine (1 mM) or in the presence of L-ethionine (Fig. 5). That similar effects of Ssy5 overproduction were observed in wild-type, *ssy1*Δ, *ptr3*Δ, and *grr1*Δ mutant strains also tallies with the observation that the Ssy5 protein is overexpressed to similar levels and undergoes similar endoproteolytic processing in these strains (data not shown). Taken together, these data clearly show that once Ssy5 is overproduced in cells, Stp1 is cleaved and *AGP1* is transcribed to a high level, independently of the presence or absence of any external amino acid or functional Ssy1, Ptr3, or Grr1 factor.

Role of transcription factors Stp1 and Uga35/Dal81 in constitutive *AGP1* transcription in Ssy5-overproducing cells. In a previous study we showed that the Stp1 and Uga35/Dal81 transcription factors are activated in response to amino acids and that both contribute importantly to the high-level *AGP1* transcription observed in cells grown in the presence of external amino acids. Namely, in *stp1*Δ and *uga35*/*dal81*Δ single mutant strains, *AGP1* is induced, though weakly, but induction is abolished when both factors are lacking (1). The data presented in Table 4 show that in Ssy5-overproducing cells, Stp1 was absolutely essential to the high-level constitutive expression of *AGP1-lacZ* observed in these cells. When Uga35/Dal81 was lacking, this constitutive expression was reduced by ~85%. This shows that while Stp1 is essential, Uga35/Dal81 also contributes importantly to the high constitutive expression of *AGP1* caused by Ssy5 overproduction. It thus seems that Ssy5 overproduction specifically activates Stp1, which then acts together with Uga35/Dal81 to promote maximal constitutive activation of *AGP1* transcription. This situation contrasts with that observed when amino acids are present. In the latter case, Uga35/Dal81 can stimulate *AGP1* transcription to some extent even if Stp1 is not functional (1). Our results further show that when phenylalanine is added to the medium of *stp1*Δ mutant cells overexpressing *SSY5*, a significant level of *AGP1-lacZ* expression is observed. This activation is likely due to the Uga35/Dal81 factor, which under these conditions is activated by amino acids (1).

Ssy5 shares significant sequence similarity with serine proteases. The above experiments indicated that Ssy5 is a major limiting factor of Stp1 endoproteolytic processing. Ssy5 might thus be the endoprotease that catalyzes Stp1 cleavage, this

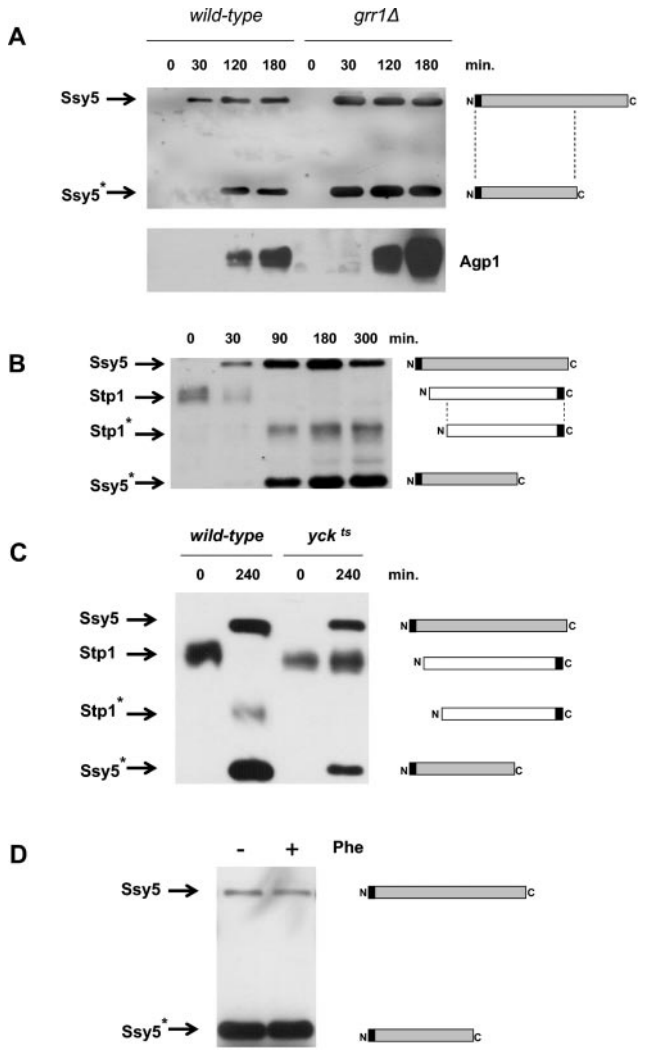

FIG. 6. Overproduction of Ssy5 is accompanied by its endoproteolytic processing and causes constitutive Stp1 cleavage and Agp1 synthesis. (A) Strains FA38 (*GAL-HA–SSY5*, noted as wild type) and 38016c (*GAL-HA–SSY5 grr1*Δ, noted as *grr1*Δ) were grown on raffinose medium with proline as the sole nitrogen source. At time zero, galactose was added to the medium. Crude extracts were prepared from culture samples collected before and at the indicated time intervals after galactose addition, and immunoblotting was carried out with either anti-HA (upper panel) or anti-Agp1 (lower panel) antibodies. (B) Strain 38003a (*GAL1-HA–SSY5*) transformed with the CEN-based plasmid pCA047 (*STP1-HA*) was grown on minimal raffinose medium with proline as the sole nitrogen source. Crude extracts were prepared from culture samples collected before (time zero) and at two time intervals after galactose addition, and immunoblotting was carried out with anti-HA antibodies. The immunodetected signals corresponding to the full-length (Ssy5, Stp1) and processed (Ssy5*, Stp1*) forms of Ssy5 and Stp1 are indicated. (C) Strains FA44 (*GAL1-HA-SSY5*, noted as wild type) and FA101 (*yck1*Δ*1 yck2-2*^{ts} *GAL1-HA–SSY5*, noted as *yck*^{ts}), both transformed with the YCpJYS-20 plasmid to complement auxotrophies and the pCA047 plasmid (*STP1-HA*), were grown at 24°C on raffinose medium with proline as the sole nitrogen source. Cultures were then transferred to 37°C for 20 min. At time zero, galactose was added to the medium. Crude extracts were prepared from culture samples collected before and at the indicated times after galactose addition and immunoblotted using anti-HA antibodies. (D) Strain 34692c (*ssy5*Δ) transformed with the pFA150 plasmid (*HA-SSY5*) was grown on minimal medium with proline as sole nitrogen source. Phenylalanine (5 mM final concentration) was added (+) or not (−), and the culture was incubated for 30 min. Crude cell extracts were then prepared, and immunoblotting was carried out with anti-HA antibodies.

TABLE 3. Overproduction of Ssy5 leads to high constitutive activation of *AGP1* transcription[a]

Strain	β-Galactosidase activity (nmol · min^{-1} · mg of protein^{-1})							
	AGP1-lacZ				UAS_{AA}-lacZ			
	Glucose		Galactose		Glucose		Galactose	
	−	Phe	−	Phe	−	Phe	−	Phe
Wild type	≤10	1,563	≤10	1,495	≤10	567	≤10	498
GAL-SSY5	≤10	≤10	1,976	2,019	≤10	≤10	645	703
GAL-PTR3	≤10	≤10	≤10	1,390	≤10	≤10	≤10	433

[a] Strains 23344c (*ura3*); FA44 (*GAL1-HA*$_3$*–SSY5 ura3*), and FA24 (*GAL1-HA*$_3$*–PTR3 gap1*Δ *ura3*) transformed with the CEN-based plasmid YCp*AGP1-lacZ* or the episomal plasmid pFA10 (UAS_{AA}-lacZ) were grown on minimal medium containing, as sole carbon source, glucose or galactose and as nitrogen source, proline alone (−) or proline plus the inducer phenylalanine (Phe) at 5 mM final concentration. The reported β-galactosidase activities are means of two to three independent experiments. Variations were less than 15%.

endoprotease function being dependent on endoproteolytic processing of Ssy5 itself (readily detected when Ssy5 is overproduced). Algorithms commonly used for sequence similarity searches (BLAST, PSI-BLAST) failed to reveal any significant resemblance between Ssy5 and any peptidase characterized to date or any other protein of known biochemical function (7, 23, 33, 35). The only proteins found to be similar to Ssy5 were in fact close Ssy5 orthologues of other fungal species. Furthermore, Ssy5 does not appear in the MEROPS database (49) (accessible at http://merops.sanger.ac.uk/), which inventories no less than 100 proteases in the proteome of *S. cerevisiae*, including 31 proteins of unknown function. We next turned to the InterProScan tool, which combines different protein signature recognition methods (59) to compare a query sequence

TABLE 4. Role of Ssy1, Ptr3, Grr1, Uga35/Dal81, and Stp1 in constitutive activation of *AGP1* transcription in Ssy5-overproducing cells[a]

Strain	β-Galactosidase activity (nmol · min^{-1} · mg of protein^{-1})			
	Glucose		Galactose	
	−	Phe	−	Phe
*gap1*Δ	≤10	1,693	≤10	1,876
*gap1*Δ *ssy1*Δ	≤10	≤10	≤10	≤10
*gap1*Δ *ptr3*Δ	≤10	≤10	≤10	≤10
*gap1*Δ *grr1*Δ	≤10	≤10	ND	ND
*gap1*Δ *stp1*Δ	≤10	319	ND	ND
*gap1*Δ *uga35/dal81*Δ	≤10	152	ND	ND
*GAL1-HA–SSY5 gap1*Δ	≤10	≤10	2,469	1,933
*GAL1-HA–SSY5 gap1*Δ *ssy1*Δ	≤10	≤10	1,945	1,703
*GAL1-HA–SSY5 gap1*Δ *ptr3*Δ	≤10	≤10	1,812	1,704
*GAL1-HA–SSY5 gap1*Δ *grr1*Δ	25	37	2,643	2,265
*GAL1-HA–SSY5 gap1*Δ *stp1*Δ	≤10	≤10	≤10	353
*GAL1-HA–SSY5 gap1*Δ *uga35*Δ	≤10	≤10	345	505

[a] Strains 30629c (*gap1*Δ *ura3*), FA10 (*gap1*Δ *ssy1*Δ *ura3*), FB94 (*gap1*Δ *ptr3*Δ *ura3*), FA08 (*gap1*Δ *grr1*Δ *ura3*), 37053a (*gap1*Δ *stp1*Δ *ura3*), 34304b (*gap1*Δ *uga35*Δ *ura3*), FA38 (*GAL1-HA-SSY5 gap1*Δ *ura3*), FA39 (*GAL1-HA–SSY5 gap1*Δ *ssy1*Δ *ura3*), FA20 (*GAL1-HA–SSY5 gap1*Δ *ptr3*Δ *ura3*), 38016c (*GAL1-HA*$_3$*–SSY5 gap1*Δ *grr1*Δ *ura3*), 38003a (*GAL1-HA–SSY5 gap1*Δ *stp1*Δ *ura3*), and 37082b (*GAL1-HA–SSY5 gap1*Δ *uga35*Δ *ura3*) transformed with the CEN-based plasmid YCp*AGP1-lacZ* were grown on minimal medium containing proline with (Phe) or without (−) the inducer phenylalanine at 5 mM final concentration and, as sole carbon source, glucose or galactose. The reported β-galactosidase activities are means of two to three independent experiments. Variations were less than 20%. ND, not determined.

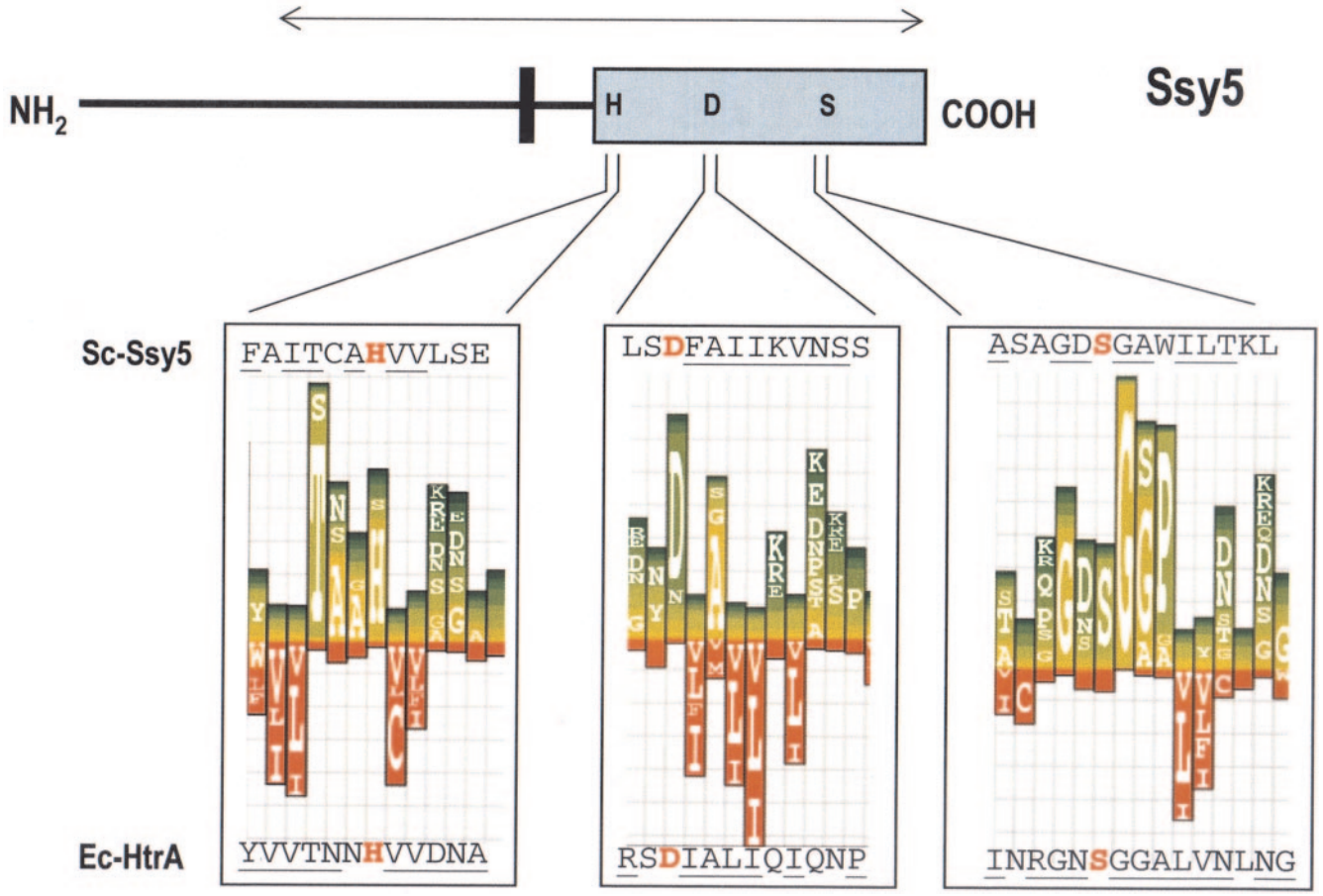

FIG. 7. The C-terminal region of Ssy5 shows similarity to S1-family serine proteases. (Top) Schematic representation of the domain structure of the Ssy5 protein. The C-terminal serine-protease-like domain (residues 459 to 687) defined by the Superfamily program is boxed. The approximate relative positions of the histidine (H), aspartate (D), and serine (S) residues corresponding to the probable catalytic endoprotease site of Ssy5 are indicated. The black box just upstream from the protease domain represents the position of the putative cleavage site of Ssy5 (see Fig. 8). The horizontal line with arrows delimits the Ssy5 region most highly conserved between Ssy5 proteins of various fungal species. (Bottom) Sequence logos taken from HMM 0011293 around residues of the catalytic triad (see text). The greater the height of the amino acid (single-letter code), the higher its conservation in the HMM alignment. Ssy5 sequences around the three amino acids (positions 465, 545, and 640) are also shown. The seed sequence used to initiate building of HMM 0011293 was the catalytic domain of the HtrA protease of *E. coli* (39). The sequence of the catalytic triad of HtrA is also shown.

against commonly used protein signature databases. Among these is Superfamily (27), a library of hidden Markov models (HMMs) constructed from the sequence alignments of 1,232 protein superfamilies inventoried in the Structural Classification of Proteins (SCOP) database (40). Remarkably, the InterProScan database (accessible at http://www.ebi.ac.uk/interpro/) assigned yeast Ssy5 as a possible member of the trypsin-like serine protease superfamily (superfamily code SSF50494). Further analysis using the Superfamily sequence search facility (http://supfam.org/SUPERFAMILY/) revealed that it is the C-terminal domain of Ssy5 (residues 459 to 687) that is significantly similar (E value of 2.5e−09) to HMMs corresponding to this serine protease superfamily. In particular, the triad of amino acids (His, Asp, and Ser) corresponding to the catalytic site of this protease superfamily, as well as surrounding residues, are highly conserved in the Ssy5 protein of *S. cerevisiae* (Fig. 7) and its orthologues in other fungal species. The analyses also suggest that the Ssy5 proteins are more closely related to different subfamilies included in the S1 family of serine proteases (49). Interestingly, only one yeast protein, the product of the *NMA111/YNL123W* gene, was assigned to the serine protease superfamily after analyses of the complete yeast proteome with sequences and tools of the Superfamily or MEROPS databases, and a recent study reported a role of this serine protease in apoptosis (21). We now propose that Ssy5 corresponds to another serine protease. However, contrary to Nma111 (a member of the Htra family of serine proteases) (14), Ssy5 is not highly similar to any previously described protease family. As described above, the Ssy5 protein undergoes endoproteolytic cleavage (Fig. 6). This tallies with the fact that many S1 proteases are expressed as inactive pro-forms characterized by an N-terminal inhibitory peptide and requiring proteolytic maturation for activation (48). The apparent molecular mass of the N-terminal peptide released after Ssy5 processing (~47 kDa) suggests that cleavage of Ssy5 takes place in the region just preceding the C-terminal S1 protease-like domain (Fig. 7).

These novel observations on the Ssy5 primary sequence, together with the data obtained with Ssy5-overproducing cells, strongly suggest that Ssy5 is the endoprotease catalyzing the

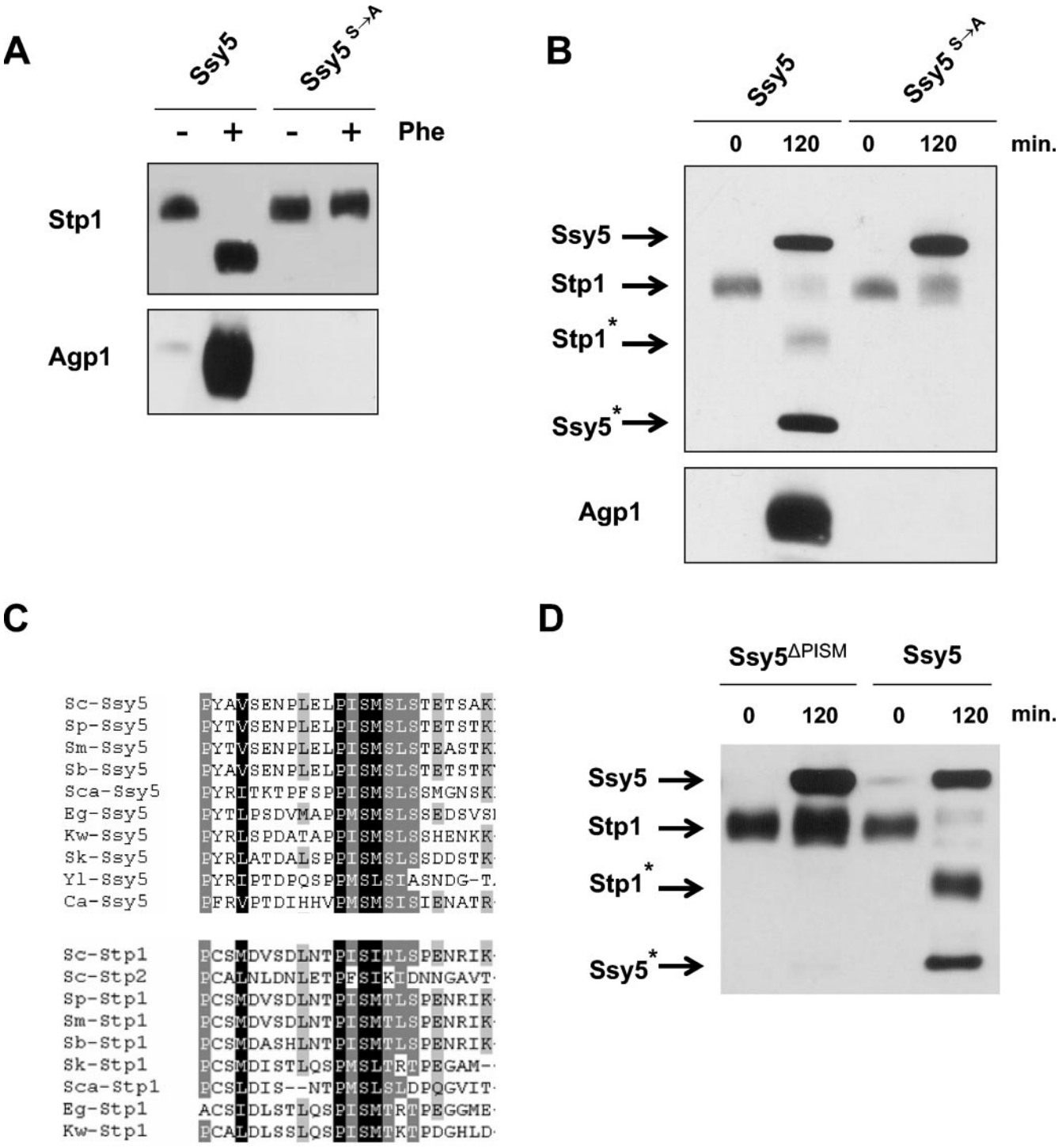

FIG. 8. Ssy5 self-processing is required for Stp1 cleavage and Agp1 synthesis. (A) A serine-to-alanine substitution in the predicted catalytic site of Ssy5 impairs amino acid-induced Stp1 cleavage and Agp1 synthesis. Strain 34686b (*ssy5*Δ, *STP1-HA*) transformed with the CEN-based plasmid pFA153 (*SSY5*) or pFA154 ($SSY5^{S\rightarrow A}$) was grown on minimal medium with proline as sole nitrogen source. Phenylalanine (5 mM final concentration) was added (+) or not (−), and the culture was incubated for 45 min. Crude cell extracts were then prepared, and immunoblotting was carried out with anti-HA (upper panel) or anti-Agp1 (lower panel) antibodies. (B) A serine-to-alanine substitution in the predicted catalytic site of Ssy5 impairs Ssy5 processing, Stp1 cleavage, and Agp1 synthesis under conditions of Ssy5 overproduction. Strain 34686b (*ssy5*Δ *STP1-HA*) transformed with the CEN-based plasmid pFA138 (*GAL1-HA–SSY5*) or pFA144 ($GAL1\text{-}HA\text{-}SSY5^{S\rightarrow A}$) was grown on minimal raffinose medium with proline as the sole nitrogen source. At time zero, galactose was added to the medium. Crude extracts were prepared from culture samples collected before and 120 min after galactose addition, and immunoblotting was carried out with either anti-HA (upper panel) or anti-Agp1 (lower panel) antibodies. (C) Conservation of a putative endoproteolytic cleavage site in the Ssy5 and Stp1 proteins. The alignment was generated with CLUSTAL and the following Ssy5-orthologous sequences: Sc-Ssy5, Sc-Stp1, and Sc-Stp2 (*S. cerevisiae*, Ssy5 positions 409 to 432, Stp1 positions 84 to 107, Stp2 positions 92 to 115), Sp-Ssy5 and Sp-Stp1 (*Saccharomyces paradoxus*), Sm-Ssy5 and Sm-Stp1 (*Saccharomyces mikatae*), Sb-Ssy5 and

endoproteolytic processing of membrane-bound Stp1 transcription factor in response to amino acids. Furthermore, as illustrated for many serine proteases (48), it is likely that endoproteolytic cleavage of the Ssy5 protein itself is required for its endoprotease function.

Mutations preventing autoprocessing of Ssy5 also impair Stp1 cleavage and Agp1 induction. We next introduced mutations in the *SSY5* gene and tested the influence of these mutations on Stp1 cleavage and Agp1 induction. The results in Fig. 8A show that phenylalanine-induced Stp1 cleavage and Agp1 synthesis were totally defective in cells expressing an Ssy5 protein in which the serine residue of the predicted catalytic triad had been replaced by an alanine ($Ssy5^{S\rightarrow A}$). The same mutation was introduced into the plasmid-borne *SSY5* gene expressed under the control of the strong *GAL* promoter. In this case, a triple HA tag at the N terminus allowed immunodetection of the $Ssy5^{S\rightarrow A}$ mutant protein upon induction by galactose (Fig. 8B). Remarkably, $Ssy5^{S\rightarrow A}$ failed to undergo endoproteolytic processing after 2 h of induced synthesis. Furthermore, Stp1 was not cleaved in response to overproduction of this unprocessed form of Ssy5 (Fig. 8B). The same result has been obtained when this $Ssy5^{S\rightarrow A}$ form is expressed under the control of the natural *SSY5* gene's promoter (data not shown). These results show that at least one of the residues of the predicted catalytic site of Ssy5 is essential to Ssy5 processing and Stp1 cleavage. These data are fully consistent with the above proposed model, i.e., Ssy5 is most likely the endoprotease responsible for Stp1 cleavage and, like many other proteases, this enzyme needs to be autoprocessed for being active. To immunodetect the C-terminal protease domain of Ssy5 released after processing, a triple HA tag was added to the C terminus of the protein. However, it turned out that this form of Ssy5 lost its ability to be autoprocessed and to mediate Stp1 cleavage (data not shown).

The apparent molecular mass of the N-terminal Ssy5 fragment released after processing suggests that Ssy5 cleavage takes place within a region lying around residue 420, i.e., just upstream from the protease homology region (Fig. 7). Remarkably, this Ssy5 putative cleavage region includes a short sequence highly conserved among Ssy5 orthologues in other yeast species (Fig. 8C). A deletion of five residues in this sequence (PISMS) led to an Ssy5 mutant form ($Ssy5^{\Delta PISMS}$) that proved defective in both self-cleavage and Stp1 cleavage (Fig. 8D). This strongly suggested that this conserved sequence corresponds to the Ssy5 self-cleavage site.

In another study (23), the Ssy5 protein tagged within its N terminus with the c-*myc* epitope and expressed under the natural *SSY5* gene promoter was detected as a single band with an apparent molecular mass of 67 kDa, whereas the protein migrated as a 76-kDa protein in extracts of the *ptr3* mutant. The authors concluded that Ssy5 is proteolytically modified in its C-terminal region in a Ptr3-dependent manner. These observations are not entirely consistent with ours, which suggest that Ssy5 cleavage takes place in a more-N-terminally located region (Fig. 7 and 8). However, in accord with our data (Fig. 6D), these investigators observed this modification in cells growing in a medium without any amino acid, i.e., Ssy5 processing is not induced in response to amino acids. Furthermore, this processing was not observed in a *ptr3* mutant (23), suggesting that autoprocessing of Ssy5 requires normal Ptr3 function. As indicated above, however, Ssy5 processing does not require Ptr3 if the endoprotease is overproduced in the cell.

Ptr3 acts with Ssy1 in early steps of amino acid signaling. Ptr3 (6) is yet another membrane-associated protein essential to Stp1 cleavage (3) and amino acid signaling (7, 33, 35). Furthermore, data from two-hybrid experiments indicate that Ptr3 interacts both with Ssy5 and with itself (7). We thus tested whether overproduction of Ptr3 also leads to Stp1 cleavage and *AGP1* transcription in the absence of any amino acid. Using the same procedure as applied to *SSY5*, we isolated cells expressing the *PTR3* gene under the *GAL* promoter. A triple HA tag was also inserted at the extreme N terminus of the protein. When the cells were grown on galactose, HA-tagged Ptr3 migrated as a protein of ~80 kDa (data not shown), a mobility consistent with the calculated molecular mass of the protein. Furthermore, analysis of extracts of *GAL-SSY5* and *GAL-PTR3* cells on the same immunoblot suggested that Ssy5 and Ptr3 were present at similar levels (data not shown). The *AGP1-lacZ* gene was not induced by phenylalanine in the *GAL-PTR3* strain when the cells were grown on glucose (Table 3). This was expected, since Ptr3 is not synthesized under these conditions. In cells grown on galactose, *AGP1-lacZ* was expressed only if an amino acid was added to the medium. These data clearly show that the HA-tagged Ptr3 protein is functional and that its overproduction, contrary to that of Ssy5, does not lead to constitutive activation of the *AGP1* gene. They further suggest that the biochemical function of Ptr3 in the amino acid signaling pathway differs from that of Ssy5.

Other data are consistent with a role of Ptr3 in early steps of the signaling pathway, namely, amino acid detection and/or signal initiation. In a previous study (7) our investigators performed a genetic screen aimed at isolating mutants defective in activation of the amino acid-sensing pathway. Most mutants fell into the *SSY1*, *PTR3*, and *SSY5* complementation classes. We showed that mutants lacking both the general amino acid permease (Gap1) and any one of these genes failed to grow on several amino acids (e.g., leucine, isoleucine, and phenylalanine) used as sole nitrogen source (1 mM) because the *AGP1* permease gene essential to growth under these conditions was not expressed. Among the many *ssy1* mutants isolated, the *ssy1-23* strain differed from the others by its ability to induce *AGP1* transcription in response to leucine but not to several

Sb-Stp1 (*Saccharomyces bayanus*), Sca-Ssy5 and Sca-Stp1 (*Saccharomyces castellii*), Eg-Ssy5 and Eg-Stp1 (*Eremothecium gossypii*), Kw-Ssy5 and Kw-Stp1 (*Kluyveromyces waltii*), Sk-Ssy5 and Sk-Stp1 (*Saccharomyces kluyveri*), Yl-Ssy5 (*Yarrowia lipolytica*), and Ca-Ssy5 (*Candida albicans*). All sequences were retrieved from the SGD database (http://www.yeastgenome.org/). (D) A 4-amino-acid deletion in the putative endoproteolytic cleavage site of Ssy5 impairs Ssy5 self-processing and Stp1 cleavage. Strain 34686b (*ssy5*Δ *STP1-HA*) transformed with the CEN-based plasmid pFA138 (*GAL1-HA–SSY5*) or pFA148 ($GAL1\text{-}HA\text{–}SSY5^{PISMSL\Delta}$) was grown on minimal raffinose medium with proline as the sole nitrogen source. At time zero, galactose was added to the medium. Crude extracts were prepared from culture samples collected before and 120 min after galactose addition, and immunoblotting was carried out with anti-HA antibodies.

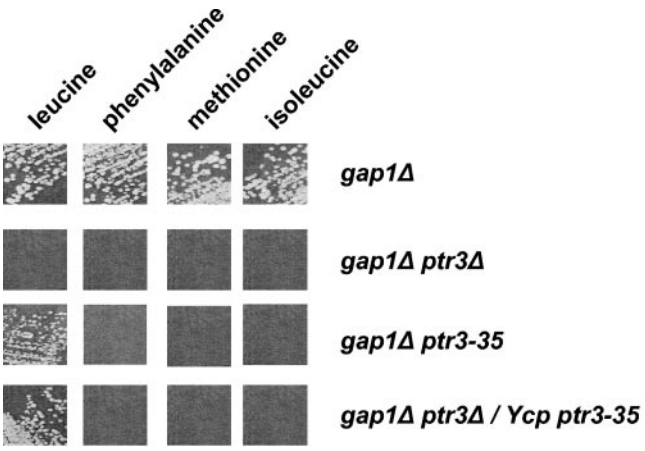

FIG. 9. The *ptr3-35* mutant responds to external leucine but not to other amino acids. Cells were spread on minimal medium with the indicated amino acid (1 mM) as sole nitrogen source. The strains were 30629c (*gap1*Δ *ura3*), FB94 (*gap1*Δ *ptr3*Δ *ura3*), FB35 (*gap1*Δ *ptr3-35 ura3*), and strain FB94 transformed by the CEN-based plasmid YCp-*ptr3-35.* Growth on these media is indicative of the expression of an active Agp1 permease (31).

other amino acids. These observations reinforced the view that the Ssy1 protein is directly involved in amino acid recognition (7). Interestingly, among the *ptr3* mutants isolated in the same screen, one (*ptr3-35*) behaved like the *ssy1-23* strain (Fig. 9). In other words, a *gap1*Δ *ptr3-35* mutant grew well on leucine at a low concentration used as sole nitrogen source but failed to grow on other amino acids like phenylalanine, methionine, or isoleucine. This phenotype differs from that of the *gap1*Δ *ptr3*Δ strain, which is unable to grow under any of these conditions. Furthermore, the *gap1*Δ *ptr3*Δ strain transformed with the cloned *ptr3-35* allele displayed normal growth on leucine but not on the other tested amino acids. It thus seems that the *ptr3-35* mutant has selectively lost the ability to induce *AGP1* transcription in response to several external amino acids, while retaining the ability to respond to leucine. Accordingly, β-galactosidase assays performed on cell extracts of this mutant, or on extracts of the *ptr3*Δ strain transformed with the cloned *ptr3-35* gene, showed high-level induction of *AGP1-lacZ* expression by leucine but no induction in response to phenylalanine, methionine, or isoleucine (Table 5). The apparently identical and specific phenotypes of the *ptr3-35* and *ssy1-23* mutants suggest that Ssy1 and Ptr3 contribute to the same molecular event required for the proper response of the cell to the presence of external amino acids. The *ptr3-35* allele contains a single G→A substitution that results in a glutamate→lysine substitution at position 521 in the protein. Interestingly, this residue is located in a region previously proposed to share significant sequence similarity with transcription factor Gcn4 (35). Furthermore, the region a few amino acids downstream is suggested to be conserved not only in Gcn4 but also in amino acid permeases of the AAP/YAT family (35). It has been proposed that this region shared by Ptr3 and amino acid permeases may be involved in amino acid recognition (24, 35).

TABLE 5. The *ptr3-35* mutant still responds to leucine[a]

Strain	β-Galactosidase activity (nmol · min^{-1} · mg of protein^{-1})				
	−	Leu	Phe	Met	Ile
*gap1*Δ/*YCpAGP1-lacZ*	21	1,634	1,565	1,098	1,356
*gap1*Δ *ptr3*Δ/*YCpAGP1-lacZ*	≤10	≤10			
*gap1*Δ *ptr3-35*/*YCpAGP1–lacZ*	≤10	612	≤10	≤10	≤10
$\frac{gap1\Delta ptr3\Delta AGP1}{gap1\Delta ptr3\Delta agp1::lacZ}$/*YCpptr3-35*	≤10	501	≤10	≤10	≤10

[a] Strains 30629c (*gap1*Δ *ura3*), FB94 (*gap1*Δ *ptr3*Δ *ura3*), and FB35 (*gap1*Δ *ptr3–35 ura3*) transformed with the CEN-based plasmid YCp*AGP1-lacZ* and the diploid strain JA495 (*gap1*Δ *ptr3*Δ *agp1::lacZ*/*gap1*Δ *ptr3*Δ *ura3*) transformed with the CEN-based plasmid YCp *ptr3–35* were grown on minimal medium containing proline with or without (−) leucine (Leu), phenylalanine (Phe), isoleucine (Ile), or methionine (Met) added at 5 mM final concentration. The reported β-galactosidase activities are means of two independent experiments. Variations were less than 15%.

DISCUSSION

In this study we have pursued our investigation of the mechanisms by which the yeast *AGP1* gene, encoding an amino acid permease, is transcriptionally induced in response to detection by the cell of external amino acids (12, 24). This induction is mediated by the concerted action of two transcription factors, Stp1 and Uga35/Dal81, acting via a short GC-rich upstream activating sequence named UAS_{AA} (1). In the absence of any external amino acid, Stp1 is present in an inactive pro-form somehow associated with the cell surface. In response to detection of external amino acids, Stp1 undergoes endoproteolytic processing in a region estimated to lie between residues 68 and 127 (3). The C-terminal domain of Stp1, containing a Kruppel-type zinc-finger domain, is thus released. It translocates into the nucleus where, in conjunction with Uga35/Dal81, it activates *AGP1* transcription.

Our data provide evidence that endoproteolytic processing of Stp1 depends on its prior phosphorylation, mediated by CKI. This CKI-dependent phosphorylation of Stp1 does not require the presence of external amino acids—it was observed also in media devoid of them. It thus seems that CKI is not activated by external amino acids. The role of CKI seems to be, rather, to phosphorylate Stp1 to make it amenable to endoproteolytic processing in response to amino acids. The Yck1 and Yck2 isoforms are peripheral plasma membrane-associated proteins (56). Studies of Yck2p have shown that this kinase undergoes palmitoylation on C-terminal Cys-Cys sequences by the palmitoyl transferase Akr1 (51) and is targeted to the cell surface via the classical secretory pathway (5). The Yck1 and Yck2 isoforms are involved in numerous cell functions, including bud morphogenesis (50), trafficking of plasma membrane transporters (42, 43) and pheromone receptors (29, 46), and cytokinesis (50). Our data show for the first time a role of yeast CKI in phosphorylation of a plasma membrane-bound transcription factor. Further experiments will be needed to determine which of the several potential target sequences for CKI found in Stp1 is indeed phosphorylated in a CKI-dependent manner. Further work will also be required to determine whether failure to process Stp1 is alone responsible for the complete lack of *AGP1* induction in the *yck^{ts}* mutant. For instance, we have previously reported that Uga35/Dal81 alone can, to some degree, activate transcription of *AGP1* in response to amino acids (1). Our observation that *AGP1* induction is totally abolished in the *yck^{ts}* strain at the nonpermissive temperature raises the possibility that activation of the Uga35/

Dal81 branch of the amino acid signaling pathway might also be CKI dependent. In a recent paper, Moriya and Johnston (44) reported that CKI is also involved in glucose signaling. Their data support a model in which glucose binding to the Snf3 and Rgt2 transporter-like glucose sensors activates CKI, which then phosphorylates the Mth1 and Std1 transduction factors bound to the sensors. Phosphorylation of Std1 and Mth1 apparently leads to their Grr1-dependent degradation, a step required for relieving repression of *HXT* gene expression by the Rgt1 repressor (44). CKI thus appears to play a central role in nutrient signaling in yeast. Our data, however, do not support a model in which CKI would be activated directly by the permease-like Ssy1 protein in response to amino acids, since Stp1 was found to be phosphorylated also in the absence of amino acids.

In the present work we have also focused on Ssy5, a protein reported to be essential to *AGP1* induction (7, 23, 33) and Stp1 processing (3) but whose exact biochemical role in the signaling pathway has remained obscure thus far. We provide evidence that Ssy5 is the endoprotease catalyzing cleavage of Stp1 in response to amino acids. First, we showed that overexpression of Ssy5 results in efficient Stp1 cleavage and high-level *AGP1* transcription in a medium without any amino acid. Second, we have conducted sequence comparisons revealing, in the C-terminal region of Ssy5, the protein signature typical of serine proteases, including the amino acid triad (H, D, S) constituting the catalytic site of this class of proteases. Third, we showed that the Ssy5 protein itself undergoes endoproteolytic processing, a property shared by many if not all serine proteases (48). Fourth, a single amino acid substitution in the predicted Ssy5 catalytic triad prevented Ssy5 processing, as well as Stp1 cleavage and *AGP1* transcription, indicating that Ssy5 is self-processed and that this reaction is essential to the endoprotease function of Ssy5. A previous study reported that Ssy5 is processed and that this processing is dependent on Ptr3 (23). On the other hand, Ptr3 was shown by two-hybrid experiments to interact with itself and with Ssy5 (7). Perhaps the Ssy5 pro-form interacts with a cell-surface Ptr3 dimer, this interaction favoring Ssy5-Ssy5 contacts and thus self-processing of Ssy5. We showed in this work that under conditions of Ssy5 overproduction, self-processing occurs independently of Ptr3, Ssy1, and Grr1. The high dosage of Ssy5 under these conditions might favor Ssy5-Ssy5 contacts and thus self-processing in a Ptr3-independent manner. A sequence in the Ssy5 region where self-cleavage occurs (just upstream from the protease domain [Fig. 7]) is particularly well conserved in fungal Ssy5 orthologues, and we showed that the integrity of this sequence is essential to Ssy5 self-processing and activity. Interestingly, a similar sequence is also conserved in Stp1 (Fig. 8C). In the latter, the sequence is also located in a region that likely includes the protein's endoproteolytic processing site (between residues 68 and 127 [3]). Furthermore, Stp1 orthologues in other yeast species also show high conservation in the corresponding regions, and this sequence is also conserved in the Stp2 transcription factor reported to undergo amino acid-induced endoproteolytic processing (Fig. 8C) (3). It is thus tempting to propose that Ssy5 self-processing and Ssy5-mediated Stp1 and Stp2 cleavage take place within these conserved sequences (Fig. 8C). Further experiments based on saturating site-directed mutagenesis experiments will be required to test the validity of these predictions and to further delineate the Ssy5 and Stp1 cleavage sites.

Our data thus imply that in response to detection by the cell of external amino acids, the Ssy5 endoprotease catalyzes endoproteolytic processing of Stp1. This suggests that Ssy5 itself is activated by external amino acids. As mentioned above, many serine proteases are synthesized as inactive pro-forms characterized by an N-terminal inhibitory peptide and thus require proteolytic maturation for activation (48). In the case of Ssy5, however, we showed that processing occurs independently of the presence or absence of amino acids in the medium. Hence, some other mechanisms must account for Stp1 cleavage—catalyzed by the processed Ssy5 form—to occur specifically in response to amino acids. Our group's previous genetic data provided evidence that Ssy1 and Ptr3 act upstream from Ssy5 in the amino acid signaling pathway (7). In keeping with this model, we showed in this study that Ssy1 and Ptr3 are entirely dispensable for Stp1 cleavage and *AGP1* transcription if Ssy5 is overproduced. Also in support of the model that Ptr3 acts early in the signaling cascade in tight conjunction with Ssy1, we described in this work a mutant form of Ptr3 (*ptr3-35*) that fails to respond to several amino acids but still responds efficiently to leucine. A similar phenotype has previously been described for the *ssy1-23* mutant (7). Previous work has also shown that leucine is the amino acid to which cells appear able to respond with the highest affinity (25). It is thus probable that both the *ssy1-23* and the *ptr3-35* mutants respond to external amino acids with a globally reduced affinity. That the same partial phenotypes may be caused by particular mutations in the *SSY1* and *PTR3* genes strongly suggests that Ssy1 and Ptr3 conjunctly act in the same molecular event early in the amino acid signaling pathway, i.e., amino acid detection and signal initiation. Furthermore, a systematic two-hybrid search for protein-protein interactions has revealed that Ssy1 interacts with Ptr3 (55). We thus propose that the sensor for external amino acids mainly consists of a complex made of Ssy1 and Ptr3. The Ssy5 endoprotease would associate with Ptr3, and this interaction likely permits Ssy5 self-processing. Upon detection of amino acids, the Ssy1-Ptr3 proteins would undergo some kind of modification allowing the associated Ssy5 endoprotease to gain access to and to cleave Stp1. The role of Ssy1-Ptr3 in Stp1 cleavage, however, becomes entirely dispensable if Ssy5 is overproduced.

A still-unresolved question is: what is the precise role of the SCF^{Grr1} ubiquitin-ligase complex in the amino acid signaling pathway? In this work we have shown that two key components of this complex, namely the F-box protein Grr1 and the ubiquitin-conjugating enzyme Cdc34, are essential to amino acid-induced Stp1 processing. Yet Grr1 is no longer essential to Stp1 cleavage if Ssy5 is overproduced. These data support the view that SCF^{Grr1} acts upstream from Ssy5 in the pathway, i.e., at the level of the Ssy1-Ptr3 sensor complex. Other observations are consistent with this model. For instance, overproduction of the cytosolic N-terminal tail of Ssy1 overcomes the amino acid utilization defects of the *grr1*Δ mutant (unpublished data). Furthermore, it has been reported previously that the migration pattern of Ptr3 is altered upon addition of amino acids to the medium and that this modification depends on Ssy1 but not on Ssy5 (23). This amino acid-induced modification of the Ptr3 migration profile is also Grr1 dependent (un-

published data). Hence, upon recognition of external amino acids by the Ssy1-Ptr3 complex, the latter might undergo some SCF^{Grr1}-dependent modification essential to subsequent activation of the Ssy5-dependent Stp1 cleavage reaction. Interestingly, the intervention of the SCF^{Grr1} ubiquitin-ligase complex does not seem to involve proteasome-catalyzed protein degradation: Stp1 cleavage is not deficient in *pre* mutants impaired in proteasome function (3) and, as shown here, it is insensitive to a proteasome inhibitor. It is known that the proteasome specifically mediates the degradation of proteins to which polyubiquitin chains linked through lysine-48 of ubiquitin have been attached. Perhaps SCF^{Grr1} catalyzes monoubiquitylation of one factor of the amino acid signaling pathway, this modification being essential to subsequent Ssy5 activation and Stp1 processing. There is indeed growing evidence that monoubiquitin may signal changes in protein location, activity, and interaction with binding partners (53). Our ongoing experiments thus aim to elucidate the protein targets(s) of SCF^{Grr1} in amino acid signaling and to better understand the molecular cascade from amino acid detection to Stp1 cleavage. We also are attempting to decipher how Uga35/Dal81, the other transcription factor responsible for high-level *AGP1* transcription, is activated in response to amino acids.

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