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Amino Acid Signaling in Yeast: Post-genome Duplication Divergence of the Stp1 and Stp2 Transcription Factors*[S]

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When yeast cells detect external amino acids via their permease-like Ssy1 sensor, the cytosolic precursor forms of Stp1 and Stp2 transcription factors are activated by endoproteolytic removal of their N-terminal domains, a reaction catalyzed by the Ssy5 endoprotease. The processed Stp factors then migrate into the nucleus, where they activate transcription of several amino acid permease genes including *AGP1*. We report here that the *STP1* and *STP2* genes most likely derive from the whole genome duplication that occurred in a yeast ancestor. Although Stp1 and Stp2 have been considered redundant, we provide evidence that they functionally diverged during evolution. Stp2 is the only factor processed when amino acids are present at low concentration, and the transcriptional activation of *AGP1* promoted by Stp2 is moderate. Furthermore, only Stp2 can sustain Agp1-dependent utilization of amino acids at low concentration. In contrast, Stp1 is only processed when amino acids are present at high concentration, and it promotes higher level transcriptional activation of *AGP1*. Domain swapping experiments show that the N-terminal domains of Stp1 and Stp2 are responsible for these proteins being cleaved at different amino acid concentrations. Last, induction of the *DIP5* permease gene by amino acids depends on Stp2 but not Stp1. We propose that post-whole genome duplication co-conservation of the *STP1* and *STP2* genes was favored by functional divergence of their products, likely conferring to cells an increased ability to adapt to various amino acid supply conditions.

Yeast cells possess a signaling pathway activated by external amino acids. This activation leads to transcriptional induction of a set of amino acid permease genes, including *AGP1*, *GNP1*, *BAP2*, *BAP3*, and *DIP5* (1, 2). At the start of this pathway is Ssy1, a protein member of the amino acid permease family (3). Ssy1 is apparently devoid of transport activity and also differs from classical amino acid permeases by a much larger N-terminal cytosolic domain and two larger external loops between transmembrane domains (4–6). According to a recent model, Ssy1 would bind directly to external amino acids, thus stabilizing a signaling, outward-facing conformation (7). In response to this binding, the cytosolic precursor forms of the Stp1 and Stp2 transcription factors are cleaved by the Ssy5 endoprotease (8, 9). The released C-terminal domains of the Stp factors are then translocated into the nucleus (8), where they activate transcription of amino acid permease genes via an upstream UAS_{AA} sequence (10, 11). Ssy1-dependent activation of the Ssy5 protease in response to amino acids requires several intermediary factors, *i.e.* Ptr3, casein kinase I, and the SCF^{Grr1} ubiquitin ligase complex (1, 2). Ptr3 is a peripheral membrane protein associated with Ssy1 (3, 6, 12). It is hyperphosphorylated in response to amino acids (13). This phosphorylation depends on casein kinase I (13), known to play a crucial role in the amino acid signaling pathway (9, 13). The positive action of casein kinase I appears to be counteracted by a CKI phosphatase complex containing Rts1 as a regulatory subunit (14). The SCF^{Grr1} complex also plays an essential role in the pathway, but its exact role remains unclear (15). The mechanism by which the Ssy1-Ptr3-casein kinase I-SCF^{Grr1} factors activate the Ssy5 endoprotease remains unknown. Experiments suggest that Ssy5 activation involves relief from a negative control exerted by the N terminus of the enzyme on its C-terminal catalytic domain (16).

Here we report that the Stp1 and Stp2 factors most likely originate from the whole genome duplication (WGD)[4] event that occurred in the ancestor of the *Saccharomyces* and related yeast lineages (17, 18). We also show that Stp1 and Stp2 have diverged functionally, and we discuss possible scenarios and advantages of this functional divergence.

EXPERIMENTAL PROCEDURES

Strains and Media—The yeast strains used in this study are all isogenic with wild-type Σ1278b except for the mutations mentioned (Table 1). Cells were grown at 29 °C in minimal buffered medium (pH 6.1) (19) with 3% glucose as the carbon source. To this medium proline (10 mM) was added as the sole nitrogen source. Where indicated, other amino acids were also added. The DNA primers used to generate PCR fragments used in yeast strain construction (20) are listed in supplemental Table 1.

Plasmids—The plasmids used in this study are listed in Table 1. *STP* plasmids were constructed by *in vivo* recombination

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1 and S2.

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[4] The abbreviations used are: WGD, whole genome duplication; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HA, hemagglutinin; qPCR, quantitative PCR.

TABLE 1
Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference
Strain		
23344c	*ura3*	Laboratory collection
KW018	*stp1Δ ura3*	This study
KW021	*stp2Δ ura3*	This study
KW023	*stp1Δ stp2Δ ura3*	This study
KW097	*uga35Δ ura3*	This study
KW098	*uga35Δ stp1Δ ura3*	This study
KW052	*uga35Δ stp2Δ ura3*	This study
30629c	*gap1Δ ura3*	5
37053a	*gap1Δ stp1Δ ura3*	11
KW034	*gap1Δ stp2Δ ura3*	This study
34034b	*gap1Δ uga35Δ ura3*	11
38009a	gap1Δ *uga35Δ stp1Δ ura3*	11
KW068	*gap1Δ uga35Δ stp2Δ ura3*	This study
KW037	*gap1Δ stp1Δ stp2Δ ura3*	This study
30633c	*gap1Δ agp1Δ ura3*	5
CA045	*STP1-HA$_3$ ura3*	This study
KW057	*STP1-HA$_3$ stp2Δ ura3*	This study
JA827	*STP2-(GA)$_5$-HA$_3$ ura3*	This study
KW059	*stp1Δ STP2-(GA)$_5$-HA$_3$ ura3*	This study
AA01	*AGP1-lacZ ura3*	Laboratory collection
KW067	*AGP1-lacZ ptr3Δ ura3*	This study
KW070	*AGP1-lacZ ptr3Δ stp1Δ ura3*	This study
KW071	*AGP1-lacZ ptr3Δ stp2Δ ura3*	This study
CA006	*rts1Δ ura3*	This study
CA099	*rts1Δ stp1Δ ura3*	This study
CA117	*rts1Δ stp2Δ ura3*	This study
CA119	*rts1Δ stp1Δ stp2Δ ura3*	This study
KW109	*AGP1-lacZ stp1Δ stp2Δ ura3*	This study
FA064	*stp2 ura3*	11
Plasmid		
YCpAGP1-lacZ	*CEN-ARS URA3 AGP1-lacZ*	5
YCpBAP2-lacZ	*CEN-ARS URA3 BAP2-lacZ*	This study
YCpDIP5-lacZ	*CEN-ARS URA3 DIP5-lacZ*	This study
YEp-ptr3–35	*2μ URA3 ptr3-35*	9
pKW010	*CEN-ARS URA3 STP1-HA$_3$*	This study
pKW012	*CEN-ARS URA3 STP1-HA$_3^{\Delta Nt}$*	This study
pKW014	*CEN-ARS URA3 STP1-HA$_3^{STP2prom}$*	This study
pKW016	*CEN-ARS URA3 STP1-HA$_3^{STP1Nt}$*	This study
pKW018	*CEN-ARS URA3 STP2-(GA)5-HA$_3$*	This study
pKW021	*CEN-ARS URA3 STP2-(GA)5-HA$_3^{\Delta Nt}$*	This study
pKW022	*CEN-ARS URA3 STP2-(GA)5-HA$_3^{STP1prom}$*	This study
pKW024	*CEN-ARS URA3 STP2-(GA)5-HA$_3^{STP1Nt}$*	This study

between the CEN-based pFL38 vector and one, two, or three DNA fragments amplified by PCR using the genomic DNA of strains CA045 (*STP1-HA$_3$ ura3*) or JA827 (*STP2-(GA)$_5$-HA$_3$ ura3*) as a template. The oligonucleotides used to generate the PCR fragments are listed in supplemental Table 1. All plasmids were verified by sequencing.

Yeast Cell Extracts and Immunoblotting—Total protein extracts were prepared as described previously (21). Proteins were resuspended in 200 μl of sample buffer and incubated for 10 min at 95 °C. For immunoblot analysis, equal quantities of proteins were loaded onto an 8% SDS-polyacrylamide gel in a Tricine system. After transfer to a nitrocellulose membrane (PerkinElmer Life Sciences), the Stp1-HA$_3$ and Stp2-(GA)$_5$-HA$_3$ proteins were detected with monoclonal antibodies raised against HA (1:10000; Roche Diagnostics). Phosphoglycerate kinase, used as a loading control, was detected with monoclonal antibodies (1:5000; Invitrogen). Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (GE Healthcare) followed by enhanced chemiluminescence (Roche Diagnostics).

β-Galactosidase Assays—β-Galactosidase activities were measured as described previously (22) and are expressed in nmol of *o*-nitrophenol/min/mg of protein. Protein concentrations were measured with the Folin reagent, and the standard used was bovine serum albumin. For each β-galactosidase assay, the indicated values correspond to the means of at least two independent sets of cultures and experiments, and the error bars correspond to S.D.

Growth Yield Comparison Assays—Flasks of minimal medium containing increasing concentrations of phenylalanine as the sole nitrogen source were inoculated with identical amounts of cells and incubated for 2 days. The optical density at 660 nm reached by each culture was measured, and cultures without phenylalanine served as blanks.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed essentially as described previously (23). All experiments were performed in duplicate using independent cultures. Stp1-HA$_3$ and Stp2-(GA)$_5$-HA$_3$ were immunoprecipitated with Pan-Mouse IgG Dynabeads (Invitrogen) using an anti-HA antibody (12CA5). Immunoprecipitated DNA was analyzed by quantitative real-time PCR on a StepOne Plus machine (Applied Biosystems) with the Platinium SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen). The occupancy level for a specific fragment was defined as the ratio of immunoprecipitated to total DNA. The *GAL1* ORF region was used as a non-transcribed control. The value 1.0 was arbitrarily given to the reference signal provided by amplifying the *GAL1* gene. The lengths of the qPCR products were 127 bp (AGP1-0), 120 bp (AGP1-4), and 170 bp (AGP1-8). The positions of the qPCR products are depicted in Fig. 7. The oligonucleotide pairs used are listed in supplemental Table 1. The specificity of the qPCR amplification was checked for each oligonucleotide pair by melting curve analysis at the end of each qPCR run. Negative controls where the template DNA was replaced with sterile water revealed that the primers used did not form dimers.

Bioinformatic Analysis of STP Orthologues—Orthologues of *STP* genes were retrieved from the Genolevures data base (24) and used in Blastp analyses (E threshold: 1e-40) against all proteins of several pre-WGD species (*Zygosaccharomyces rouxii*, *Saccharomyces kluyveri*, *Kluyveromyces thermotolerans*, *Kluyveromyces lactis*, *Eremothecium (Ashbya) gossypii*) and post-WGD species (*Candida glabrata*, *Kluyveromyces polysporus*) (supplemental Table 2) (24). The syntenic context of the *STP* genes was analyzed with the Yeast Gene Browser online tool (25).

RESULTS

STP1 and STP2 Likely Originate from the Whole Genome Duplication Event—It is established that an ancestor of *Saccharomyces cerevisiae* underwent a WGD after diverging from yeast lineages like *K. lactis*, *K. thermotolerans*, or *Kluyveromyces waltii* (17, 26). One member of most pairs of "twin" genes was subsequently lost from one of the chromosomes, and a minor fraction of these genes, about 10% in *S. cerevisiae*, subsisted as duplicates. Analysis of the *STP* gene orthologues in yeast lineages having diverged before or after the WGD (see "Experimental Procedures") strongly suggests that the *STP1* and *STP2* genes derive from the WGD. In support of this view, pre-WGD yeast lineages typically contain a single *STP* gene, whereas *C. glabrata* and *Saccharomyces castellii*, two post-WGD yeast species, contain two. Furthermore, the neighboring genes of the unique *STP* gene present in pre-WGD yeast species

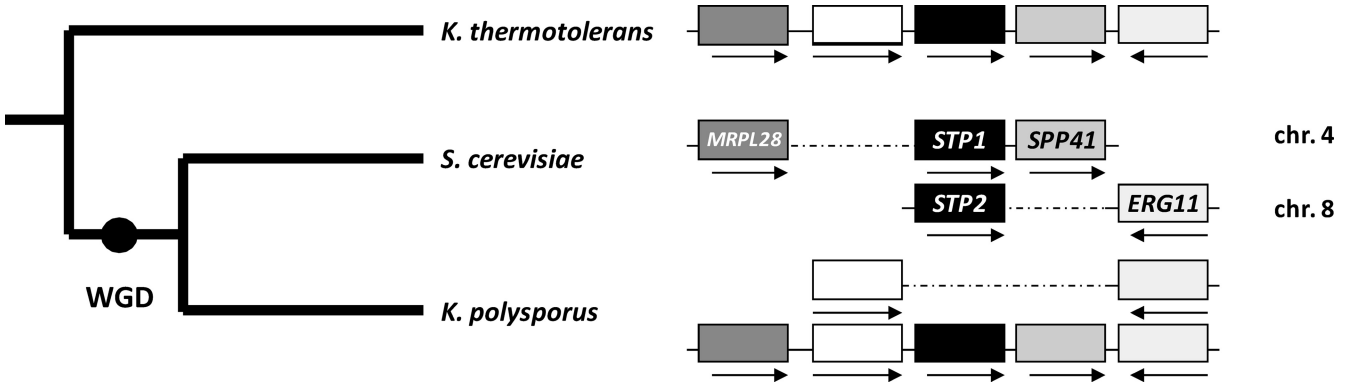


FIGURE 1. ***STP1* and *STP2* likely derive from the ancestral whole genome duplication event.** *Left*, shown are phylogenetic relationships between three yeast species whose lineages diverged before or after the WGD. *Right*, shown is a schematic representation of five consecutive genes of *K. thermotolerans* (chromosome (*chr.*) 7, genes G05060g , G05082g, G05104g, G05126g, and G05148g) and of their homologues present on chromosomes 4 and 8 of *S. cerevisiae* and on two chromosomes of *K. polysporus*. *Dotted lines* correspond to enlarged spacing between adjacent genes so as to arrange homologous genes in columns. Gene names are indicated only for *S. cerevisiae*.

like *K. thermotolerans* display syntenic conservation with genes close to either *STP1* or *STP2* of *S. cerevisiae* (Fig. 1). *K. polysporus*, a post-WGD species distantly related to *S. cerevisiae* (27), contains only one *STP* gene, suggesting that the other gene copy was lost during evolution. In contrast, some of the neighboring genes of this unique *STP* gene have been conserved as gene duplicates (Fig. 1).

Conservation of both *STP* gene copies in *S. cerevisiae* and other post-WGD lineages raises the interesting possibility that Stp1 and Stp2 may have diverged functionally during evolution. We have accordingly shown that induction of *AGP1* transcription by phenylalanine is reduced by more than 80% in a *stp1*Δ mutant but remains unaltered in a *stp2*Δ mutant (11). Yet other papers (8, 28, 29) suggest that Stp1 and Stp2 might be functionally redundant, at least when *AGP1* expression was induced by leucine; the *stp1*Δ and *stp2*Δ single mutations did not significantly alter this induction, whereas none was observed with the *stp1*Δ *stp2*Δ double mutant. We, thus, sought to determine whether the relative contributions of Stp1 and Stp2 to induction of *AGP1* expression vary according to the amino acid inducer. For this we isolated a set of mutant strains containing a *stp1*Δ mutation, a *stp2*Δ mutation, or both and used them to monitor induction of *AGP1* expression by various amino acids. We also analyzed the influence of a deletion mutation altering Uga35/Dal81, a pleiotropic transcription factor contributing importantly to induction of *AGP1* transcription (5, 11, 30).

The Relative Contributions of Stp1 and Stp2 to Induction of AGP1 Expression Depend on the Amino Acid Inducer—AGP1 expression was measured 2 h after the addition of phenylalanine, isoleucine, valine, tryptophan, or citrulline at high concentration (5 mM) to cells transformed with a low copy number plasmid bearing the *AGP1-lacZ* reporter construct (Fig. 2). In the wild type, the induction level varied according to the amino acid, Phe being the strongest and citrulline the weakest inducer. In the *stp1*Δ *stp2*Δ double-mutant strain, we observed no induction by any amino acid. Hence, the expression level observed in either single mutant should reflect the ability of the corresponding Stp protein to mediate induction. When Stp1 alone was present, the induction level varied strongly according to the amino acid and was often the same as, or close to that observed in the wild type. As a notable exception, citrulline promoted only weak Stp1-mediated induction. Stp2-mediated induction, on the other hand, varied much less according to the amino acid inducer, its level always remaining lower than the highest Stp1-mediated induction levels. Furthermore, Stp2 appeared largely responsible for induction of *AGP1* expression by citrulline, the weakest inducer. Thus, Stp1 and Stp2 do not respond similarly to external amino acids. Interestingly, induction by Leu was about the same in the wild-type, *stp1*Δ, and *stp2*Δ strains, indicating that, as previously observed (8, 28, 29), Stp1 and Stp2 are largely redundant under these conditions. With an inducer such as Val, in contrast, the contributions of Stp1 and Stp2 appeared largely additive.

In a previous work (11) we presented results suggesting that the residual induction of *AGP1* expression by Phe observed in the *stp1*Δ mutant was not mediated by Stp2. Our present results obtained with the *stp2*Δ and *stp1*Δ *stp2*Δ strains isolated in this study (Fig. 2) contradict this finding. We have, thus, further analyzed the *stp2*Δ strain used here and the one (strain FA064) in that earlier study. In the *stp2*Δ strains used previously, it appears that the antibiotic resistance gene used to delete the *STP2* coding region by gene replacement was integrated at the *STP2* locus but without deleting the *STP2* gene (supplemental Fig. S1). In the newly isolated *stp2*Δ mutants, *STP2* is appropriately deleted. The data obtained with these mutants (Fig. 2) are also fully consistent with those of previous studies showing complete loss of induction in the *stp1*Δ *stp2*Δ mutant (8, 28, 29).

Uga35/Dal81 Behaves as a Co-activator of Stp1- and Stp2-mediated Induction—Uga35/Dal81 is a pleiotropic transcription factor containing a $Zn(II)_2$-Cys_6 cluster-type DNA binding domain (31, 32). It is essential to full induction of *AGP1* expression (5, 11) and to expression of other nitrogen compound-inducible genes, such as those involved in γ-aminobutyric acid, allophanate, and allantoin utilization (31, 32). In each induction mechanism, Uga35/Dal81 acts in conjunction with an inducer-specific transcription factor, *e.g.* Uga3 for γ-aminobutyric acid (33) and Dal82/DurM for allophanate (34, 35). The results shown in Fig. 2*A* reveal that Uga35/Dal81 strongly potentiates Stp1-dependent induction, in keeping with previous results (11). A weak though significant induction nevertheless subsists in the absence of Stp2 and Uga35, suggesting that Stp1 alone can activate *AGP1* expression to some degree. In contrast,

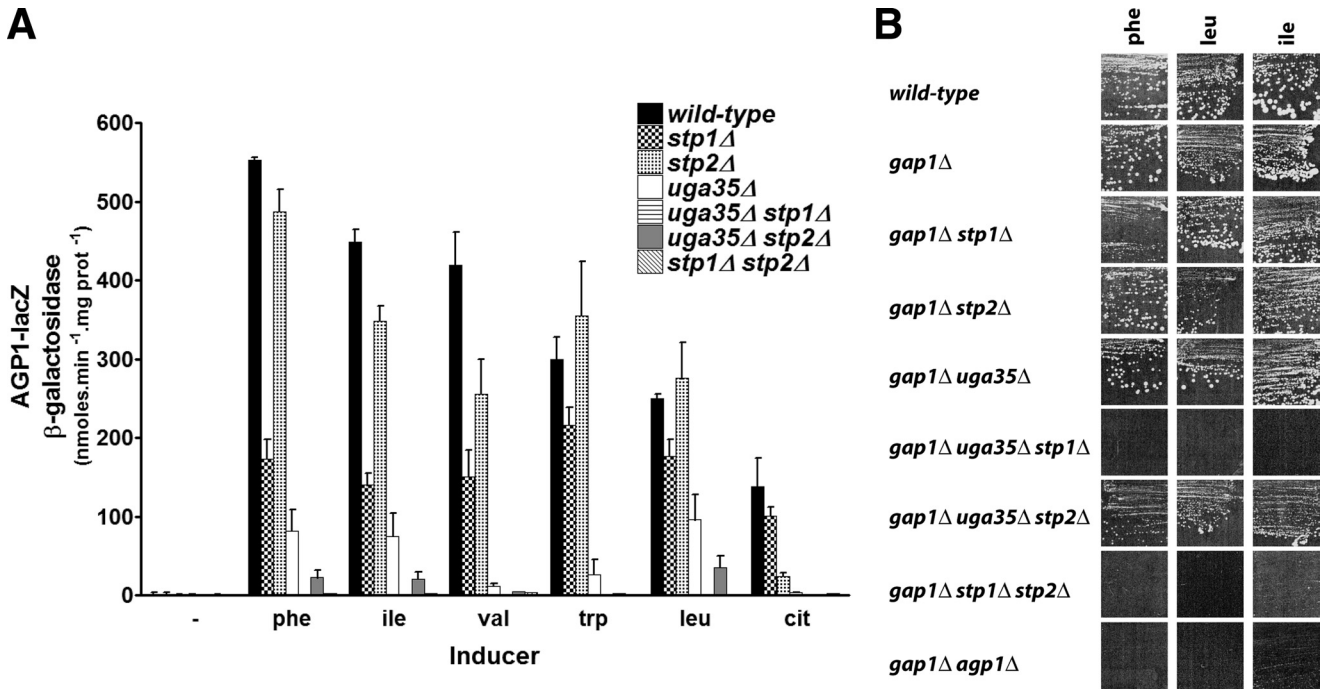


FIGURE 2. **Stp1 and Stp2 are not functionally redundant.** *A*, strains 23344c (*ura3*), KW018 (*stp1*Δ *ura3*), KW021 (*stp2*Δ *ura3*), KW097 (*uga35*Δ *ura3*), KW098 (*uga35*Δ *stp1*Δ *ura3*), KW052 (*uga35*Δ *stp2*Δ *ura3*), and KW023 (*stp1*Δ *stp2*Δ *ura3*) transformed with the CEN-based plasmid YCpAGP1-lacZ were grown on proline medium. Phe, Ile, Val, Trp, Leu, or citrulline (*cit*) (5 mM) was then added to the medium for 2 h before harvesting. The reported β-galactosidase activities are the means of at least two experiments. *B*, strains 23344c (*ura3*), 30629C (*gap1*Δ *ura3*), 37053a (*gap1*Δ *stp1*Δ *ura3*), KW034 (*gap1*Δ *stp2*Δ *ura3*), 34034b (*gap1*Δ *uga35*Δ *ura3*), 38009a (*gap1*Δ *uga35*Δ *stp1*Δ *ura3*), KW068 (*gap1*Δ *uga35*Δ *stp2*Δ *ura3*), KW037 (*gap1*Δ *stp1*Δ *stp2*Δ *ura3*), and 30633c (*gap1*Δ *agp1*Δ *ura3*) were spread on minimal medium with Phe, Leu, or Ile (1 mM) as the sole nitrogen source and incubated for 4 days at 29 °C.

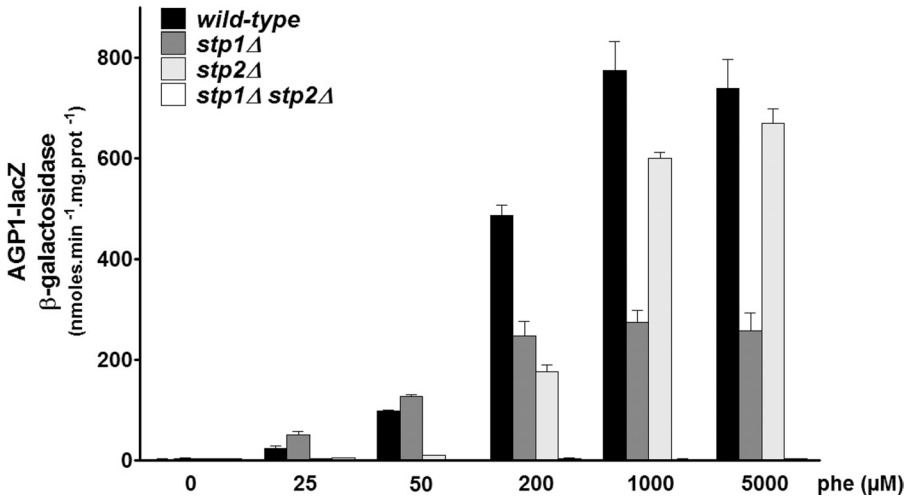


FIGURE 3. **The relative contributions of Stp1 and Stp2 to induction of *AGP1* expression differ according to the amino acid concentration.** Strains 23344c (*ura3*), KW018 (*stp1*Δ *ura3*), KW021 (*stp2*Δ *ura3*), and KW023 (*stp1*Δ *stp2*Δ *ura3*) transformed with the CEN-based plasmid YCpAGP1-lacZ were grown on proline medium. Phe was then added to the medium at various concentrations for 2 h.

Uga35/Dal81 is essential to Stp2-dependent induction, indicating that Stp2 alone cannot activate *AGP1* expression. These results were confirmed by growth tests on media containing a low concentration of Phe, Ile, or Leu as the sole nitrogen source (Fig. 2*B*). We previously reported that Agp1 is essential to growth on these media when the general amino acid permease, Gap1, is not functional (5). The results presented in Fig. 2*B* confirm that Agp1 is not functional when both Stp factors or Stp1 and Uga35 are defective, whereas it remains functional if Stp1 alone is functional.

The Stp2 Transcription Factor Responds with Higher Sensitivity to the Amino Acid Sensing System—The above results clearly show that Stp1 and Stp2 diverged functionally during evolution. The activating power of Stp1 appears higher, as revealed when induction of *AGP1* expression was assayed in response to the strongest inducers like Phe or Ile and by the ability of Stp1 to activate *AGP1* expression in the absence of the Uga35/Dal81 co-activator. The activation power of Stp2 is lower, and this might explain why Stp2 absolutely needs Uga35/Dal81 for significant transcriptional activation of *AGP1*. On the other hand, the stronger response of Stp2 to citrulline, the weakest inducer, raises the possibility that Stp2 might respond more efficiently than Stp1 to limited activation of the amino acid signaling pathway. To test this hypothesis, we examined the induction of *AGP1-lacZ* expression in response to increasing concentrations of Phe (Fig. 3). In response to low Phe concentrations (25 and 50 μM), *AGP1-lacZ* induction was weak and principally Stp2-dependent, but at the highest Phe concentrations (1 and 5 mM), the much higher induction of *AGP1-lacZ* appeared to be largely mediated by Stp1, as shown also in Fig. 2*A*. Interestingly, in the presence of intermediate

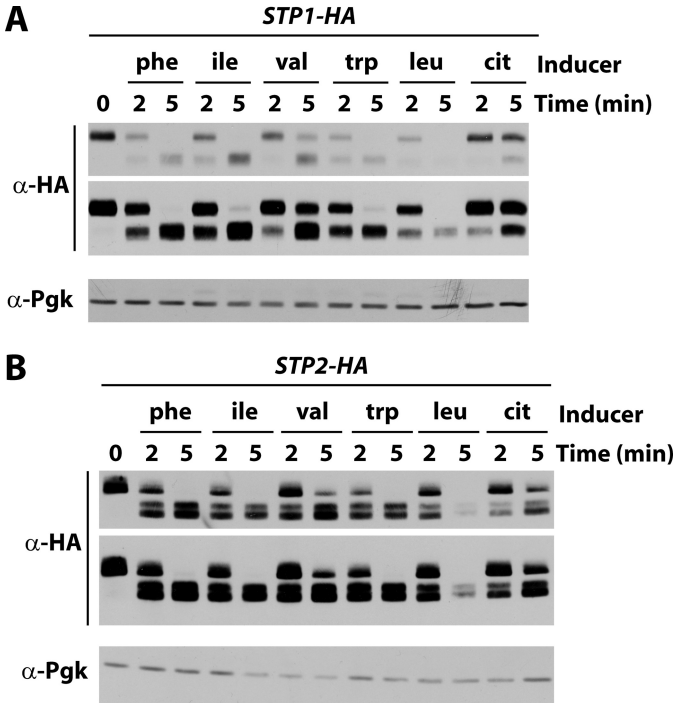


FIGURE 4. **Efficiencies of Stp1 and Stp2 processing vary according to the amino acid inducer.** Strains CA045 (*STP1-HA$_3$ ura3*) and JA827 (*STP2-(GA)$_5$-HA$_3$ ura3*) were grown on minimal medium with proline as the sole nitrogen source. Phe, Ile, Val, Trp, Leu, or citrulline (*cit*) (each at 5 mM) was then added or not to the medium for 2 or 5 min. Total cell extracts were prepared, and immunoblotting was carried out with anti-HA and anti-phosphoglycerate kinase antibodies. Two exposure times are presented for the anti-HA antibody.

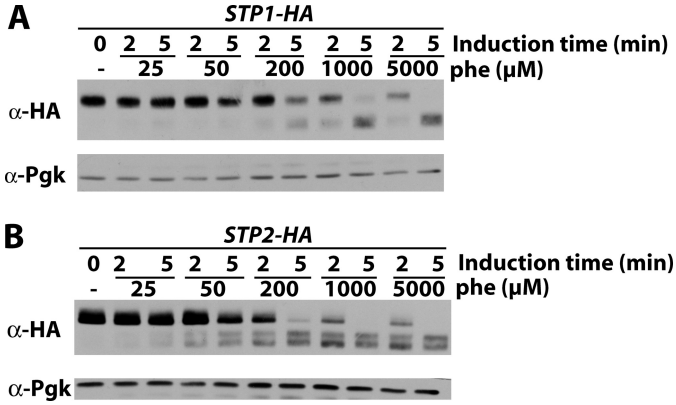


FIGURE 5. **Stp2 is more readily processed than Stp1 when amino acids are present at low concentration.** Strains CA045 (*STP1-HA$_3$ ura3*) and JA827 (*STP2-(GA)$_5$-HA$_3$ ura3*) were grown on minimal medium with proline as sole nitrogen source. Phenylalanine (25, 50, 200, 1000, or 5000 μM) was then added or not (-) to the medium for 2 or 5 min. Total cell extracts were then prepared, and immunoblotting was carried out with anti-HA and anti-phosphoglycerate kinase (*PgK*) antibodies.

Phe concentrations (200 μM), the contributions of Stp1 and Stp2 appeared similar and additive.

We next tested whether the apparent higher sensitivity of Stp2 to activation by the Ssy1 sensor correlates with a greater propensity to be cleaved by the Ssy5 endoprotease. Each Stp factor was tagged at its C terminus with three repeats of the HA epitope and tested for functionality (supplemental Fig. S2). In the case of Stp2, a linker consisting of glycine-alanine dipeptide repeats had to be inserted between the protein and the 3HA epitope to keep the protein active. In the absence of any amino acid inducer, Stp1 and Stp2 migrated in their uncleaved forms (Fig. 4). Two minutes after the addition of an amino acid, Stp1 and Stp2 appeared to have undergone partial processing. When Phe, Ile, Trp, or Leu was added to the medium, processing of both Stp factors was complete within 5 min. With Val and citrulline, cleavage of Stp1 and Stp2 remained partial even 5 min after the addition of the amino acid to the medium. Stp2 was more efficiently processed than Stp1 in the presence of citrulline, in keeping with the view that Stp2 contributes more importantly than Stp1 to activation of *AGP1* expression in response to this poor inducer. We next compared processing of Stp1 and Stp2 in response to increasing concentrations of Phe (Fig. 5). At low Phe concentrations (25, 50, and 200 μM), Stp2 was more efficiently cleaved than Stp1, whereas both Stp factors were processed with similar efficiency in response to higher Phe concentrations (1 and 5 mM).

These results indicate that Stp2 has a greater propensity than Stp1 to be processed by the Ssy5 protease when the external amino acid-sensing system is weakly stimulated, *i.e.* when a good amino acid inducer (like Phe) is present at low concentration or when the sole amino acid present is a weak inducer (like citrulline). Under such conditions, *AGP1* induction depends mainly on Stp2. Stp1, in contrast, is efficiently processed only when the amino acid-sensing system is strongly stimulated, *i.e.* when a good amino acid inducer is present at sufficiently high concentrations. This suggests that Stp1 is less sensitive than Stp2 to cleavage by the Ssy5 endoprotease.

Limited Activation of AGP1 Transcription in the ptr3-35 and rts1Δ Mutants Depends Mainly on Stp2—The Ptr3 factor is associated with Ssy1 and is essential to activation of the Ssy5 endoprotease, with which it interacts (6, 13, 36). In the *ptr3-35* mutant, transmission of the amino acid signal is largely impaired, as *AGP1* transcription is activable only by leucine (9), the amino acid toward which Ssy1 displays the highest affinity (37). Furthermore, this induction is lower than in the wild type. The limited induction of *AGP1* by leucine in the *ptr3-35* mutant is, thus, equivalent to weak stimulation of the amino acid sensing system in the wild type and should, thus, depend largely on Stp2. To test this we combined the *ptr3-35* mutation with the *stp1*Δ or *stp2*Δ mutation. Induction of *AGP1* in the *ptr3-35* mutant was reduced 2-fold as compared with the wild type, and this residual induction was indeed largely dependent on Stp2 (Fig. 6*A*). This contrasts with the situation in the wild type, where both Stp1 and Stp2 can mediate maximal induction of *AGP1* in response to leucine (Fig. 2).

We also analyzed the respective contributions of Stp1 and Stp2 to induction of *AGP1* expression in the *rts1*Δ mutant. Rts1 is a regulatory subunit of protein phosphatase 2A, a phosphatase complex that probably counteracts the positive action of casein kinase I in the amino acid signaling pathway (14). In the *rts1*Δ mutant, *AGP1* is strongly expressed in the absence of any external amino acid (14). As this constitutive expression does not reach the maximal induction level observed in the wild type, we reasoned that the signaling pathway is only partially activated in the *rts1*Δ mutant and that the constitutive expression of *AGP1* observed in this strain might depend mainly on Stp2. We, thus, assayed *AGP1* expression in the *rts1*Δ strain deleted

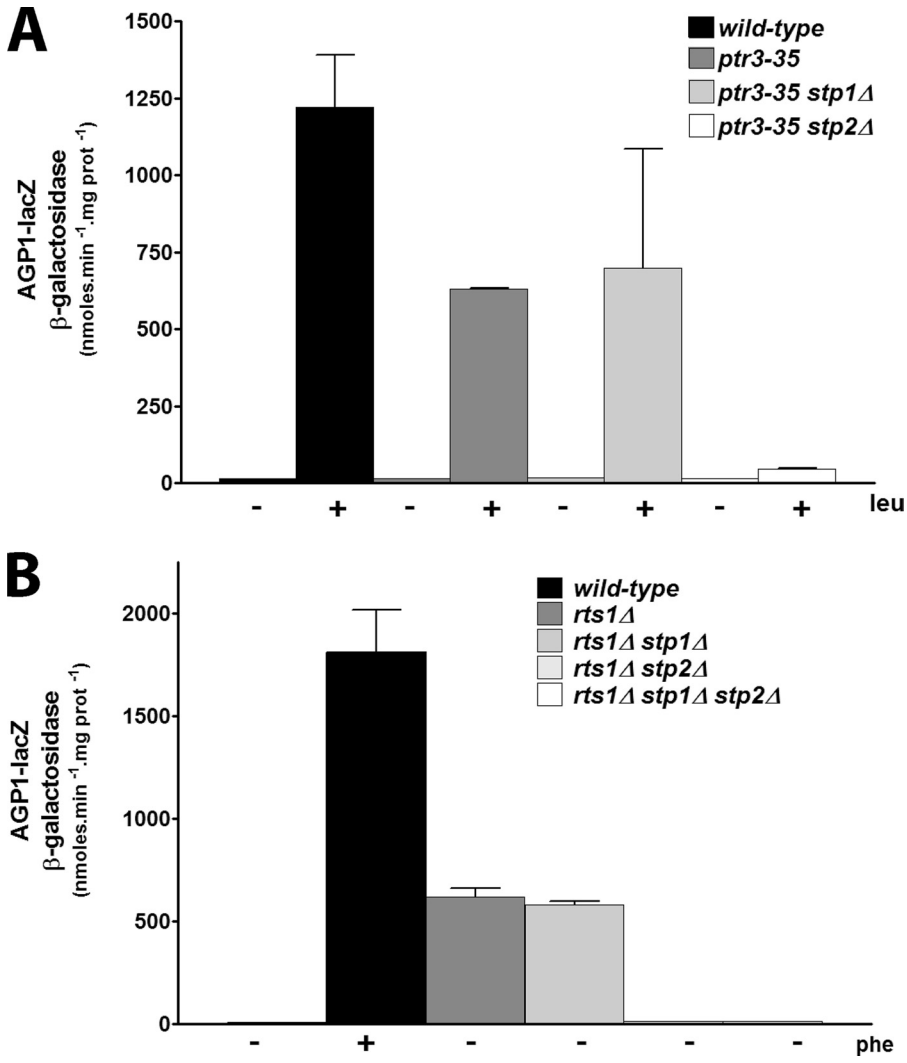


FIGURE 6. **Limited expression of *AGP1* in *rts1*Δ and *ptr3-35* mutants is mediated mainly by Stp2.** *A*, strain AA01 (*AGP1-lacZ ura3*) and strains KW067 (*AGP1-lacZ ptr3*Δ *ura3*), KW070 (*AGP1-lacZ ptr3*Δ *stp1*Δ *ura3*), and KW071 (*AGP1-lacZ ptr3*Δ *stp2*Δ *ura3*) transformed with the YEp-*ptr3-35* plasmid were grown on proline minimal medium, and leucine was added (+) or not (-) at 5 mM concentration to the medium for 2 h before cell harvesting. *B*, strains 23344c (*ura3*), CA006 (*rts1*Δ *ura3*), CA099 (*rts1*Δ *stp1*Δ *ura3*), CA117 (*rts1*Δ *stp2*Δ *ura3*), and CA119 (*rts1*Δ *stp1*Δ *stp2*Δ *ura3*) transformed with the YCpAGP1-lacZ plasmid were grown on proline minimal medium, and phenylalanine was added (+) or not (-) at 5 mM concentration to the medium for 2 h before cell harvesting.

of the *STP1* or *STP2* gene (Fig. 6*B*). The results clearly show that constitutive expression of *AGP1* in the *rts1*Δ mutant depends mainly on Stp2.

In conclusion, limited stimulation of the amino acid signaling pathway in the *ptr3-35* and *rst1*Δ mutants leads to specific activation of Stp2, *i.e.* the contribution of Stp1 to *AGP1* expression is negligible in these mutants. This further shows that Stp2 is more sensitive than Stp1 to activation by the amino acid sensing system.

Stp2 Is Essential to the Utilization of Amino Acids at Low Concentration—As Stp2 is more readily activated than Stp1 when the amino acid sensing system is moderately stimulated, we examined whether this factor might also play a greater role in the utilization of external amino acids present at low concentration. We first compared the growth of the *stp1*Δ and *stp2*Δ mutant strains on solid media containing increasing concentrations of Phe, Ile, or Leu as the sole nitrogen source (Fig. 7*A*). The strains were also deleted of the *GAP1* gene, so that amino acid uptake by the mutants was mainly dependent on Agp1 (5). The results clearly show that Stp2 is essential to growth on the lowest concentrations of amino acids. At higher concentrations, both Stp1 and Stp2 can sustain growth. This means that Stp2, but

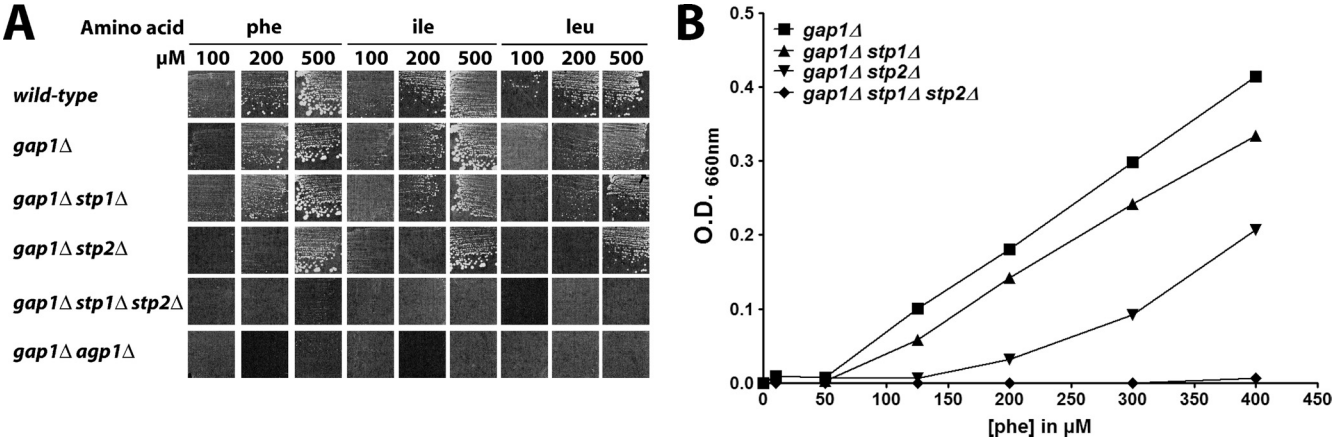


FIGURE 7. **Stp2 is the only factor able to sustain Agp1-dependent utilization of amino acids at low concentration.** *A*, strains 23344c (*ura3*), 30629C (*gap1*Δ *ura3*), 37053a (*gap1*Δ *stp1*Δ *ura3*), KW034 (*gap1*Δ *stp2*Δ *ura3*), and KW037 (*gap1*Δ *stp1*Δ *stp2*Δ *ura3*) were spread on minimal medium with Phe, Leu, or Ile (100, 200, or 500 μM) as sole nitrogen source and incubated for 5 days at 29 °C. *B*, strains 30629C (*gap1*Δ *ura3*), 37053a (*gap1*Δ *stp1*Δ *ura3*), KW037 (*gap1*Δ *stp2*Δ *ura3*), and KW037 (*gap1*Δ *stp1*Δ *stp2*Δ *ura3*) were pre-grown to log phase on minimal urea medium. Equal quantities of cells were then added to a minimal medium with phenylalanine at 0, 50, 150, 200, 300, or 400 μM as the sole nitrogen source. The optical density (*O.D.*) at 660 nm reached by each culture after 2 days was then measured.

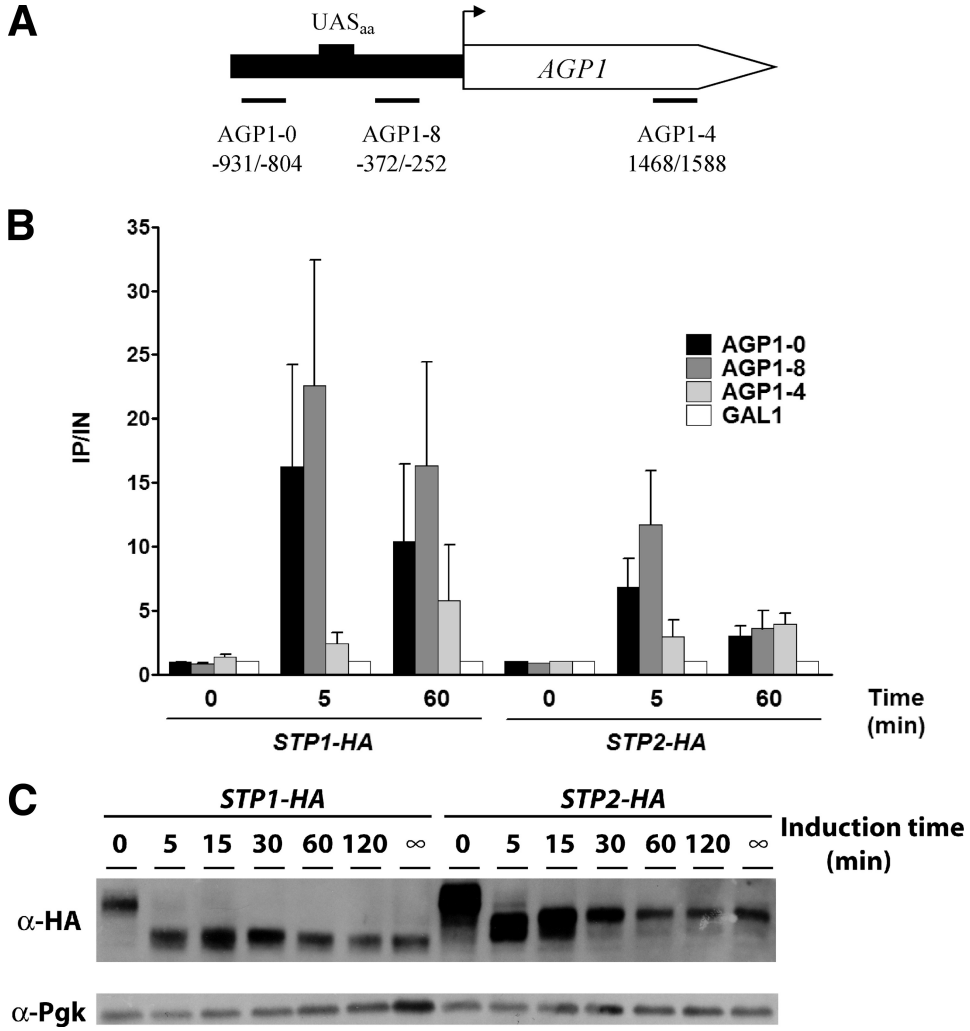


FIGURE 8. **Dissimilar occupancy of the *AGP1* upstream region by Stp1 and Stp2.** *A*, schematic representation of the *AGP1* gene is shown. The positions of the amplicons (AGP1-0, AGP1-8, and AGP1-4) used as probes in the chromatin immunoprecipitation experiments are indicated. The *white box* represents the coding region of *AGP1*. The position of UAS$_{AA}$ is also indicated. *B*, strains CA045 (*STP1-HA$_3$ ura3*) and JA827 (*STP2*-(GA)$_5$-*HA$_3$ ura3*) were grown on proline as the sole nitrogen source, after which Phe was included (+) or not (-) (at 5 mM concentration) for 5 or 60 min. Chromatin extracts were prepared, HA-tagged forms of the Stp factors were immunoprecipitated with anti-HA antibodies, and copurifying DNA was analyzed by qPCR using oligonucleotides hybridizing to the *AGP1* upstream region (AGP1-0 and AGP1-8), the *AGP1* coding sequence (AGP1-4), or the *GAL1* gene (the latter two being used as negative controls). The occupancy level for each specific fragment was defined as the ratio of immunoprecipitated to input DNA. The value 1 was arbitrarily given to the reference signal provided by amplifying the *GAL1* gene. *C*, strains CA045 (*STP1-HA$_3$ ura3*) and JA827 (*STP2*-(GA)$_5$-*HA$_3$ ura3*) were grown on proline as the sole nitrogen source, after which Phe was included (at 5 mM concentration) for 5–120 min. The same strains were also grown on Pro and Phe as sole nitrogen sources (α). Total cell extracts were then prepared, and immunoblotting was carried out with anti-HA and anti-phosphoglycerate kinase (*PgK*) antibodies.

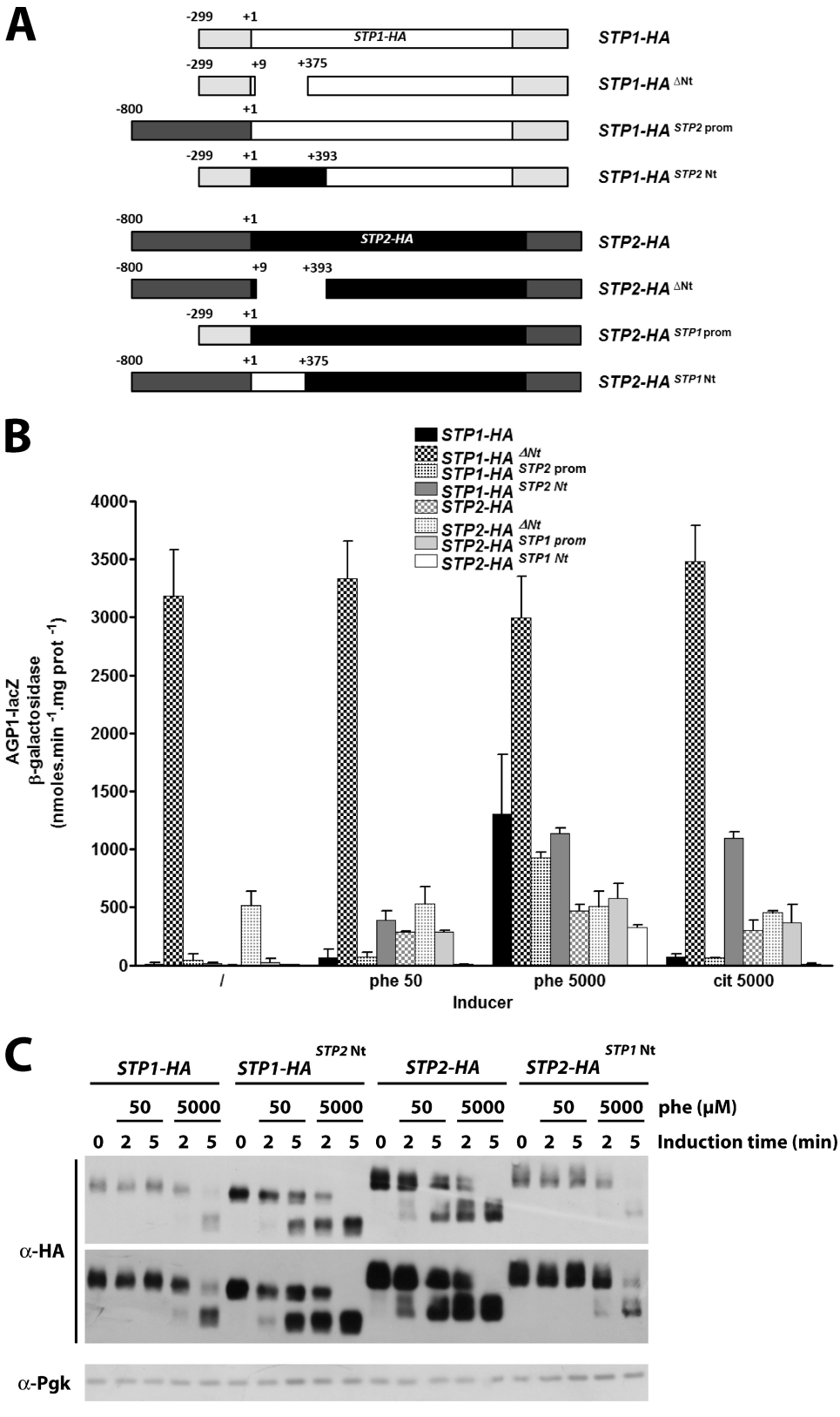
not Stp1, has the ability to activate Agp1 synthesis when amino acids are present at low concentration in the medium. These results were confirmed by comparing growth yields in liquid medium. Cultures containing various initial concentrations of Phe (from 0 to 400 μM) as the sole nitrogen source were inoculated with equal amounts of cells and incubated until stabilization of the optical density (Fig. 7*B*). At low Phe concentrations (100–250 μM), cell growth appeared to depend mostly on Stp2.

Dissimilar Occupancy of the AGP1 Upstream Region by Stp1 and Stp2—At high Phe concentrations, Stp1 and Stp2 are cleaved with similar efficiency (Fig. 5), but transcriptional induction of *AGP1* is largely Stp1-dependent under these conditions (Fig. 3). We, thus, used a chromatin immunoprecipitation assay to compare binding of Stp1 and Stp2 to the *AGP1* promoter. Five minutes after Phe addition, both Stp1 and Stp2 were found associated with the *AGP1* upstream region but not with the two DNA segments (the gene coding region and the *GAL1* gene) used as controls (Fig. 8*B*). One hour after Phe addition, Stp1-HA was the main factor associated with the *AGP1* regulatory region, Stp2 binding being less pronounced. The greater role of Stp1 in induction of *AGP1* in response to high concentrations of Phe, thus, correlates with a greater capacity of Stp1 to occupy the *AGP1* gene upstream region.

Why Stp2 binding to the *AGP1* upstream region is less pronounced 60 min after the addition of Phe is not known. Stp1 might bind with higher affinity to the *AGP1* upstream sequences and progressively displace Stp2 from common binding sites. The Stp2 protein might also be less stable than Stp1. The latter explanation is in fact supported by the results presented in Fig. 8*C*. The Stp1 and Stp2 proteins tagged with the same HA epitope were detected in the same immunoblot before and several time intervals after the addition of Phe (5 mM) to the cells. In the absence of any amino acid, the signal corresponding to Stp2 was of higher intensity. After the addition of Phe, Stp1 and Stp2 were efficiently processed. Interestingly, whereas the Stp1 signal remained largely stable, the intensity of the Stp2 signal declined and tended to reach that of Stp1 (Fig. 8*C*). Further experiments showed that Phe-induced degradation of Stp2 is mediated by the proteasome and does not occur in the *ssy5*Δ mutant in which Stp2 is not processed (data not shown). Hence, Stp2 is more abundant than Stp1 in the absence of any amino acid, but after processing Stp2 is destabilized, and its cellular level turns similar to that of Stp1. It is likely that this destabilization accounts for the reduced occupancy by Stp2 of the *AGP1* promoter observed 60 min after Phe addition. Noteworthy, although Stp2 is destabilized upon processing, it promotes efficient *AGP1* induction. This destabilization was indeed also observed after the addition of a high Phe concentration to the *stp1*Δ mutant (data not shown) in which the role of Stp2 in *AGP1* transcription is clearly visible (Fig. 3).

The N-terminal Domains of Stp1 and Stp2 Are Responsible for These Proteins Being Cleaved at Different Amino Acid Concentrations—Stp1 and Stp2 are synthesized as latent cytoplasmic proteins with 10-kDa N-terminal domains crucial for their regulation. A study of Stp1 revealed that this N-terminal domain contains a sequence (amino acids 65–97) required for binding to the Ssy5 protease and for Ssy5-dependent processing. The N-terminal domain contains another sequence (amino acids 16–35) required for cytoplasmic retention (or promoter exclusion) of Stp1 when amino acids are not present in the medium (38).

To further study the specific functional properties of Stp1 and Stp2, we constructed genes encoding Stp proteins whose N-terminal domains have been deleted or swapped. We also isolated two gene constructs in which the upstream non-coding sequences of *STP1* and *STP2* have been swapped (Fig. 9*A*). These *STP* genes were first introduced into the *stp1*Δ *stp2*Δ mutant, and induction of *AGP1* was monitored under the conditions that Stp1 (high Phe concentration) or Stp2 (low Phe concentration or citrulline) normally confers most of the *AGP1* induction (Fig. 9*B*). The results show that the Stp factors lacking their N-terminal domains confer constitutive expression to *AGP1*, the level of which is much higher in the case of Stp1 *versus* Stp2. These data are consistent with the proposed role of the N-terminal domains in cytoplasmic retention of Stp factors and confirm that the transcriptional activation power of Stp1 is higher than that of Stp2. The *STP* genes in which the promoter regions have been swapped behaved like the native *STP* genes, suggesting that the functional divergence between Stp1 and Stp2 is not due to some differences at gene promoter levels. Finally, the data show that Stp1 containing the N-terminal domain of Stp2 gained the ability to activate *AGP1* expression in response to low Phe or citrulline, a typical property of Stp2. Conversely, this property was lost for Stp2 containing the N-terminal domain of Stp1. The latter chimeric protein conserved the ability to respond to a high Phe concentration, but the induction level was limited and close to that conferred by native Stp2. These results show that it is the N-terminal domains of Stp1 and Stp2 that confer to these proteins different abilities to respond to the amino acid concentration. They also confirm that the different



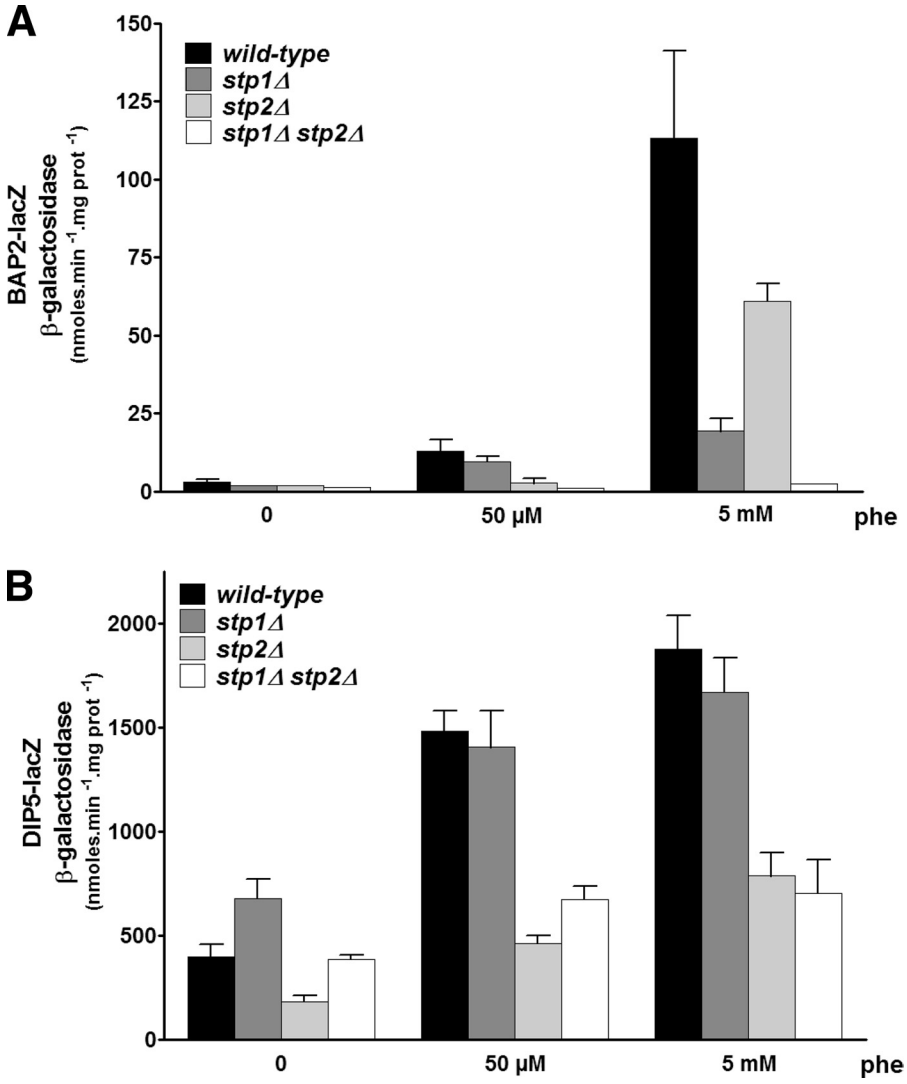


FIGURE 10. **Induction of *DIP5* expression by amino acids is mediated mainly by Stp2.** Strains 23344c (*ura3*), KW018 (*stp1*Δ *ura3*), KW021 (*stp2*Δ *ura3*), and KW023 (*stp1*Δ *stp2*Δ *ura3*) transformed with the CEN-based plasmid YCpBAP2-lacZ (*A*) or YCpDIP5-lacZ (*B*) were grown on minimal proline medium, and phenylalanine was then added at 50 μM or 5 mM concentration for 2 h before cell harvesting.

transcriptional activation capacities of Stp1 and Stp2 are associated with their C-terminal domains.

We next compared the processing of the native and chimeric Stp factors in response to low or high Phe concentrations (Fig. 9*C*). As previously shown, Stp1 is only processed at high Phe concentrations, whereas Stp2 is processed at low or high Phe concentrations. Remarkably, the Stp1 factor with the N-terminal domain of Stp2 was efficiently processed at low Phe concentrations, and the Stp2 factor with the N-terminal domain of Stp1 was only processed at high Phe concentrations. These results correlate with those of the *AGP1* induction assay presented above and show that the N-terminal domains of Stp1 and Stp2 are responsible for these proteins being differently cleaved by the Ssy5 endoprotease according to the amino acid concentration.

Stp2, but Not Stp1, Mediates Induction of the DIP5 Gene by Amino Acids—The functional divergence of Stp1 and Stp2 during evolution might have led to situations where a single Stp factor mediates amino acid-induced expression of specific amino acid permease genes. To test this we examined the role of each Stp factor in induction by Phe of two additional amino acid permease genes, namely *BAP2* and *DIP5* (Fig. 10). *BAP2* showed an expression profile similar to that of *AGP1*; that is, moderate, largely Stp2-dependent induction by low Phe and higher, largely Stp1-dependent induction by high Phe. In contrast, the expression of *DIP5* was similarly and moderately increased whatever the Phe concentration and in a solely Stp2-dependent manner. Thus, induction of *DIP5* by Phe appears mediated specifically by Stp2. It is also entirely dependent on Uga35/Dal81 (data not shown).

DISCUSSION

Traces of WGD events have been found in the genomes of all four eukaryotic kingdoms, including fungi (39). In fungi, the event occurred in the common ancestor of a group of yeast species that includes *S. cerevisiae*. Only 10% of the resulting duplicated genes survived as duplicates, and a significant fraction of these appear to be transcription factors (39). Here we report that the *STP1* and *STP2* genes of *S. cerevisiae* most likely derive from this WGD. This is supported by the presence of only one *STP* orthologue in pre-WGD yeast species and by the syntenic context conservation of *STP* genes in post-

FIGURE 9. **The N-terminal domains of Stp1 and Stp2 are responsible for these proteins being cleaved at different amino acid concentrations.** *A*, schematic representation of the plasmid born *STP* genes used in this experiment is shown. *STP1-HA* and *STP2-HA*, *STP1* and *STP2* genes coding for triple-HA-tagged Stp proteins; *STP1-HA*$^{\Delta Nt}$ and *STP2-HA*$^{\Delta Nt}$, coding for Stp proteins deleted of the N-terminal sequences removed by Ssy5-mediated cleave; *STP1-HA*^{STP2prom} and *STP2-HA*^{STP1prom}, in which the upstream non-coding sequences were swapped; *STP1-HA*^{STP2Nt} and *STP2-HA*^{STP2Nt}, coding for Stp factors in which the N-terminal sequences were swapped. *B*, strain KW109 (*AGP1-lacZ stp1*Δ *stp2*Δ *ura3*) transformed with the CEN-based plasmids pKW010 (*STP1-HA*$_3$), pKW012 (*STP1-HA*$_3^{\Delta Nt}$), pKW014 (*STP1-HA*$_3^{STP2\ prom}$), pKW016 (*STP1-HA*$_3^{STP1Nt}$), pKW018 (*STP2*-GA$_5$-*HA*$_3$), pKW021 (*STP2*-GA$_5$-*HA*$_3^{Nt}$), pKW022 (*STP2*-GA$_5$-*HA*$_3^{STP1prom}$), or pKW024 (*STP2*-GA$_5$-*HA*$_3^{STP1Nt}$) was grown on minimal medium with proline as sole nitrogen source. Phe (50 or 5000 μM) or citrulline (5000 μM) was then added or not (/) to the medium for 2 h. *C*, strain KW022 (*stp1*Δ *stp2*Δ *ura3*) transformed with the CEN-based plasmids pKW010 (*STP1-HA*$_3$), pKW016 (*STP1-HA*$^{switch\ Nt}$), pKW018 (*STP2*-GA$_5$-*HA*$_3$), or pKW024 (*STP2*-GA$_5$-*HA*$_3^{switch\ Nt}$) was grown on minimal medium with proline as sole nitrogen source. Phenylalanine (50 or 5000 μM) was then added or not to the medium for 2 or 5 min. Total cell extracts were then prepared, and immunoblotting was carried out with anti-HA or anti-phosphoglycerate kinase (*Pgk*) antibodies. Two exposures times are presented for the anti-HA antibody.

versus pre-WGD species (Fig. 1). It is worth noting that some pre-WGD species (like *S. kluyveri*, *K. thermotolerans*) contain one more distantly related *STP* gene in addition to the single *STP* orthologue, suggesting that *STP* genes have sometimes undergone isolated duplication events. In other pre-WGD species, however, such duplications do not seem to have occurred (*e.g.* in *K. lactis*, *Z. rouxii*). Isolated gene duplication is likely at the origin of the *STP1* and *STP2* genes existing in *Candida albicans*. Interestingly, these Stp factors exhibit a clear dichotomy in the genes they transactivate (40).

Conservation of both the *STP1* and the *STP2* gene in some post-WGD species like *S. cerevisiae* and *C. glabrata* suggests that the proteins they encode are not fully redundant. Our results indeed show that Stp1 and Stp2 diverged functionally during evolution; unlike Stp1, Stp2 is efficiently processed by the Ssy5 endoprotease when the Ssy1 sensor system is weakly stimulated, *e.g.* when good amino acid inducers like Phe are present only at low concentration or when the sole external amino acid is a poor inducer like citrulline. Stp2 is also the main factor responsible for limited *AGP1* expression in mutant strains such as *ptr3-35* or *rts1*Δ, where the signaling pathway is only weakly stimulated. The ability of Stp2 to be efficiently activated when amino acids are not abundant is also reflected in its specific ability to sustain Agp1-dependent growth on amino acids at low concentration as the sole nitrogen source. Stp2 also differs from Stp1 in that it exhibits a lesser capacity to activate transcription of the *AGP1* gene and probably of other amino acid permease genes as well. Furthermore, this activation is fully dependent on Uga35/Dal81, a protein acting as a transcriptional coactivator of multiple nitrogen source-inducible genes. The lesser transcription-activating capacity of Stp2 as compared with Stp1 is probably related to its more important role when external amino acids are not abundant; under such conditions, high level synthesis of an amino acid permease like Agp1 seems unnecessary.

Unlike Stp2, Stp1 is efficiently processed by the Ssy5 protease only when the amino acid sensing system is highly stimulated, *i.e.* when a good amino acid inducer is present at a sufficiently high concentration. Furthermore, Stp1 promotes higher level transcription of the *AGP1* gene. Another difference between Stp1 and Stp2 is that Stp2 is more abundant that Stp1 in the absence of any amino acid in the medium but is destabilized upon processing so that its cellular level tends to reach that of Stp1.

We could further show by domain swapping experiments that the N-terminal domains of Stp1 and Stp2 are responsible for these proteins being cleaved at different amino acid concentrations. In contrast, their different transcriptional activation capacities, as expected, are associated with the C-terminal parts released after processing. Further experiments will be needed to determine the precise mechanism allowing the N-terminal domains of Stp1 and Stp2 to promote differential processing according to the amino acid concentration. For instance, these domains might interact with the Ssy5 endoprotease with different affinities.

The functional divergence of Stp1 and Stp2 raises interesting questions about the evolutionary scenario that led to this situation. We could envisage that the divergence between *STP1* and *STP2* has been accompanied by neofunctionalization (39). For instance, the ancestral Stp factor might have been more similar to Stp1, activating, in the presence of amino acids at relatively high concentration, transcription of several genes encoding moderate-affinity amino acid permeases. After WGD, one of the Stp factors (Stp2) would have diverged, acquiring the specific ability to be activated by the Ssy5 protease in response to lower amino acid concentrations along with the ability to activate moderately the transcription of some amino acid permease genes. In parallel to *STP* gene divergence, other genes might have evolved. For instance, some genes encoding amino acid permeases may have become specifically or more efficiently activable by a single Stp factor. This would notably apply to *DIP5*, which is regulated only by Stp2. This scenario seems supported by the observation that the unique Stp factor present in pre-WGD species is more closely related to Stp1, as is the unique Stp factor having subsisted in *K. polysporus*, a post-WGD species.

Our results show that the system enabling yeasts to activate amino acid permease genes in response to external amino acids provides an interesting model for addressing the question of how a signaling pathway can evolve for improved fitness or for adaptation to different environments. This is also true for the glucose signaling pathway activated by the transporter-like Snf3 and Rgt2 glucose sensors. The latter proteins also derive from the WGD and display high and low affinity for glucose, respectively. Furthermore, a recent study reported some functional divergence between Mth1 and Std1, two other factors deriving from WGD and playing a key role in transducing the glucose signal from the cell surface to the nucleus (41). Investigating these nutrient signaling pathways in other pre- and post-WGD yeast species certainly appears as a promising direction of future investigation.

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