Amino Acid Signaling in Yeast: Activation of Ssy5 Protease Is Associated with Its Phosphorylation-induced Ubiquitylation*

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Fadi Abdel-Sater, Cathy Jean, Ahmad Merhi, Stéphan Vissers, and Bruno André

From the Laboratoire de Physiologie Moléculaire de la Cellule CP300, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, 11 Rue des Pr. Jeener et Brachet, 6041 Gosselies, Belgium

The yeast Ssy5 protein is a serine-type endoprotease autoprocessed into a catalytic domain and a large inhibitory prodomain. When external amino acids are detected by the plasma membrane Ssy1 sensor, Ssy5 is activated and catalyzes endoproteolytic processing of the Stp1 and Stp2 transcription factors. These Stp proteins then migrate into the nucleus and activate transcription of several amino acid permease genes. Previous studies showed that Ssy5 activation involves the SCFGrr1 ubiquitin ligase complex, but the molecular mechanisms of this activation remain unclear. We here report that the prodomain of Ssy5 is phosphorylated in a casein kinase I-dependent manner in response to amino acid detection. We describe a mutant form of Ssy5 whose prodomain is not phosphorylated and show that it is nonfunctional. Amino acid detection also induces ubiquitylation of the Ssy5 prodomain. This prodomain ubiquitylation requires its prior phosphorylation and the SCFGrr1 complex. When this ubiquitylation is defective, Ssy5 accumulates as a phosphorylated form but remains inactive. A constitutive Ssy5 form in which the prodomain fails to inhibit the catalytic domain does not need to be phosphorylated or ubiquitylated to be active. Finally, we provide evidence that ubiquitylation of the inhibitory prodomain rather than its subsequent degradation is the key step in the Ssy5 activation mechanism. We propose that the Ssy5 protease is activated by phosphorylation-induced ubiquitylation, the effect of which is relief from inhibition by its prodomain.

The yeast SSY5 gene (1) encodes a chymotrypsin-like endoprotease involved in a signaling pathway responding to external amino acids (2, 3). The enzyme is first synthesized as a precursor undergoing autoprocessing into an N-terminal prodomain and a C-terminal catalytic (Cat) domain (2–4). In the absence of any amino acid in the external medium, Ssy5 is processed but remains inactive. The lack of Ssy5 activity under these conditions is due to the prodomain, which somehow inhibits the Cat domain (3, 4). Ssy5 is activated when Ssy1, a plasma membrane permease-like sensor (5–7), detects the presence of amino acids in the external medium. Once activated, Ssy5 catalyzes the endoproteolytic removal of the N-terminal domains of two transcription factors, Stp1 and Stp2, present as precursors in the cytosol (3, 8). These Stp factors then migrate into the nucleus (8) and bind together with the Dal81/Uga35 transcription factor upstream from several amino acid permease genes to activate their transcription (9–11). One of these inducible permeases is Afg1, which catalyzes the uptake of all neutral amino acids into the cell (7).

The mechanism of Ssy5 activation in response to Ssy1-mediated detection of external amino acids remains unknown. Other factors known to be essential to processing of Stp factors might be involved in this activation. Among these factors is Ptr3 (1, 5), a protein containing several WD40 repeats and interacting with Ssy1 and Ssy5 (12, 13). Casein kinase I (CKI) is also crucial to Stp1 processing (2). One target of CKI is Ptr3, which undergoes CKI-dependent hyperphosphorylation in response to amino acids (13). Finally, the protein components of the SCFGrr1 ubiquitin (Ub) ligase complex are also required for Stp factor processing and AGP1 induction (2, 14–16). However, inhibition of the proteasome does not impair cleavage of the Stp factors (2, 8), suggesting that if a ubiquitylation reaction is needed for transduction of the amino acid signal to the Ssy5 endoprotease, the possible subsequent degradation of the ubiquitylated target protein is not (2). A recent study nevertheless reported that the prodomain of Ssy5 is destabilized by the proteasome in response to amino acids. Furthermore, forced destabilization of the Ssy5 prodomain by means of a conditional degron leads to Ssy5 activation (17).

In this work, we show that detection of amino acids by the Ssy1 sensor leads to CKI-dependent phosphorylation of the Ssy5 prodomain. This phosphorylation is essential to Ssy5 activation, and its role is to trigger SCFGrr1-dependent ubiquitylation of the protease. Our data suggest that the short term effect of this ubiquitylation is activation of the protease by relief from autoinhibition.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Plasmids—**All Saccharomyces cerevisiae strains used in this study are presented in Table 1. The plasmids and the methods used to construct them are described in Table 2. Yeast strains were grown at 29 °C in the 165 minimal buffered medium (pH 6.1) with 3% glucose as the carbon source. The yeast nitrogen base was used instead of 165 medium when extra copper (CuSO₄·0.1 mm) was added to activate the CUP1 gene’s promoter. In all experiments, proline (10 mm) was added as a sole nitrogen source of the medium to which Phe or Leu (5 mm final concentration) was added to activate the Ssy5 endoprotease.
Phosphorylation-induced Ubiquitylation of the Ssy5 Protease

TABLE 1
Yeast strains

| Strain     | Genotype                          | Reference or source          |
|------------|-----------------------------------|-----------------------------|
| 23344c     | MATA ura3                         | Laboratory collection       |
| AA01       | MATA agg1-lacZ kanMX2 ura3        | Laboratory collection       |
| FB90       | MATA sss5Δ:kanMX2 ura3            | Ref. 7                     |
| 30629c     | MATA gap12:kanMX2 ura3            | Ref. 7                     |
| FB92       | MATA gap1Δ:kanMX2 sss5Δ:kanMX2 ura3|
| 34686b     | MATA gap1Δ sss5Δ:kanMX2 Stp1-HA kanMX2 ura3|
| 34692c     | MATA sss5Δ:kanMX2 aggl1-lacZ kanMX2 ura3|
| JA902      | MATA sss5Δ:kanMX2 aggl1-lacZ kanMX2 grr1Δ:Hgb ura3|
| JA115      | MATA grr1Δ:kanMX2 ura3            | Ref. 7                     |
| 32501c     | MATA ssy1Δ:kanMX2 ura3            | Ref. 7                     |
| JA716      | MATA Ssy5-42-HA, ura3             | This study                  |
| CA080      | MATA grr1Δ:kanMX2 Ssy5-42-HA, ura3| This study                  |
| 29102a     | MATA ptr3Δ:kanMX2 Ssy5-42-HA, ura3| This study                  |
| 29105c     | MATA ssy1Δ:kanMX2 Ssy5-42-HA, ura3| This study                  |
| CA045      | MATA STP1-HA ura3                 | Ref. 29                    |
| JA827      | MATA STP2-HA ura3                 | Ref. 29                    |
| WCG4a-1/22a| MATA his3-11 leu2-3112 pre1-1 pre2-1 ura3| Ref. 30                  |
| LRB341     | MATA his3 leu2 ura3-52             | Ref. 19                    |
| LRB346     | MATA his3 leu2 ura3-52 yck1-1 yck2-21a|
| CMY763     | MATA ura3-52 leu2Δ1 his3Δ-200 cinm3-1|

Yeast Cells, Immunoprecipitation, and Immunoblotting—Crude cell extracts were prepared and immunoblotted as described previously (2). After transfer to a nitrocellulose membrane (Schleicher & Schuell), mouse anti-HA (12CA5, Roche Applied Science), anti-FLAG (F1804, Sigma), or anti-UB (p4D1, Santa Cruz Biotechnology, Inc. [Santa Cruz, CA]) antibodies were used at 1:10,000, 1:2000, or 1:500 dilution, respectively. Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody (GE Healthcare). Treatment of cell extracts with alkaline phosphatase was performed as described previously (2). In Ssy5 immunoprecipitation experiments, exponentially growing cells (∼10⁸ cells) were first suspended in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40) supplemented with Complete EDTA-free protease inhibitor mixture tablets (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, and 10 mM N-ethylmaleimide. The cells were lysed by vortexing with glass beads. Cell extracts were incubated for 1 h on ice and centrifuged for 30 min at 12,000 × g. HA-tagged Ssy5 was immunoprecipitated from the lysates with anti-HA microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. Purification of His₆-Ubiquitin Protein Conjugates—A mutant strain with a reduced internal pool of ubiquitin (doa4Δ ura3 leu2) was transformed with a plasmid encoding a copper-inducible His₆-tagged ubiquitin (YPE96-HIS) or untagged ubiquitin (YPE96). Cells were grown in yeast nitrogen base medium with proline as the sole nitrogen source. CuSO₄ (0.1 mM) was added for 3 h to activate the CLIP1-Lib gene. Cells were harvested and disrupted with glass beads in a buffer containing 6 M guanidium. The extract was incubated with nickel-NTA-Sepharose beads (Qiagen, Hilden, Germany), which were then collected and washed several times with a buffer containing 8 M urea. Bound proteins were eluted in HIS elution buffer.

RESULTS

Amino Acids Induce Accumulation of a Phosphorylated Form of the Ssy5 Prodomain in the grr1Δ Mutant—To detect Ssy5 in yeast cell extracts, we used an Ssy5 form (named Ssy5-42-HA) containing a 6-HA tag inserted between residues 42 and 43 of the prodomain (Fig. 1A). This region was chosen for tagging because of its poor conservation among Ssy5 orthologs, suggesting a nonessential function. Accordingly, Ssy5-42-HA is functional, as judged by its capacity to complement the inability of an ssy5Δ mutant to grow on specific amino acids as sole nitrogen source (Fig. 1C) and to induce expression of an AGP1-lacZ gene (Fig. 1D). Furthermore, Ssy5-42-HA seems to be normally processed because anti-HA immunoblots revealed a low intensity signal corresponding to the full-length (unprocessed) form of Ssy5 and a high intensity signal corresponding to the N-terminal prodomain (Fig. 1B). This profile is similar to that displayed by the HA-Ssy5 form containing a 3-HA tag at the extreme N terminus of the protease (2) (Fig. 1, A and B). However, HA-Ssy5 differs from Ssy5-42-HA in that the former activates transcription of the AGP1-lacZ gene even when no...
inducer amino acid is present. In other words, HA-Ssy5 is constitutively active (Fig. 1D). It has indeed been reported that in the absence of amino acids, the prodomain of Ssy5 prevents the C-terminal Cat domain from being active. The presence of the 3-HA tag at the N terminus apparently relieves this autoinhibition (3, 17). In contrast, the Ssy5-42-HA form remains activatable by amino acids (Fig. 1D) and is thus better suited for investigating the mechanism of this activation.

We expressed the Ssy5-42-HA form in a grr1Δ mutant strain and added Phe, one of the strongest amino acid inducers of AGP1 transcription (7), to the medium. We observed a gradual accumulation of higher molecular weight forms of the prodomain above the main signal (Fig. 2A). These upper bands correspond to phosphorylated forms of the prodomain because they disappear after treatment of cell extracts with alkaline phosphatase (Fig. 2B). Interestingly, these phosphorylated forms are observed neither in the wild type nor in ssy1Δ or ptr3Δ mutants (Fig. 2C). Furthermore, this phosphorylation is reversible because it tends to disappear when cells are transferred back to a medium without any inducer amino acid (Fig. 2D). This amino acid-induced phosphorylation was also observed in the case of Ssy5-42-HA with a serine-to-alanine substitution in the protease’s catalytic site (Fig. 2, E and F). This Ssy5-42-HAΔS mutant is inactive and fails to catalyze its own processing (2). Hence, induced phosphorylation of Ssy5 is observed even when Ssy5 is inactive and unprocessed.

Protein substrates of SCF complexes often need to be phosphorylated to be recognized and ubiquitylated by this class of Ub ligases. These proteins thus tend to accumulate as phosphorylated forms when the SCF function is defective (18). The above results thus raise the interesting possibility that Ssy5 might be a protein substrate of the SCFGrr1 complex.

Amino Acids Induce Transiently Detectable Phosphorylation of the Ssy5 Prodomain, a Modification Dependent on Ssy1, Ptr3, and Casein Kinase I—If the above model were true, phosphorylation of the Ssy5 prodomain should also be visible in wild-type cells. In the experiment of Fig. 2C, the Ssy5 prodomain was immunodetected 30 min after the addition of Phe. We considered that in wild-type cells, prodomain phosphorylation might be more readily detectable during the first minutes after amino

**FIGURE 1.** The Ssy5-42-HA protease is functional and activatable by amino acids. A, schematic representation of the Ssy5 endoprotease. Autocleavage between residues 381 and 382 (vertical arrow) yields the prodomain (42.2 kDa) and Cat domain (35.3 kDa). The Cat domain includes the His-Asp-Ser catalytic triad of the protease. The Ssy5-42-HA variant contains six HA tags preceded by a loxP-encoded peptide inserted between residues 42 and 43 of the prodomain. The HA-Ssy5 form contains three HA tags at the extreme N terminus. B, the Ssy5-42-HA and HA-Ssy5 forms are processed. Strain FB90 (ssy5Δ) transformed with the CEN-based plasmid pCJ353 (Ssy5-42-HA) or pFA150 (HA-Ssy5) was grown in minimal proline medium. Crude cell extracts were immunoblotted with anti-HA antibodies. The migration position of the prodomains is indicated. C and D, the Ssy5-42-HA form is functional and activatable by amino acids. C, cells were spread on minimal medium with phenylalanine (1 mM) as the sole nitrogen source. The strains were FB92 (ssy5Δ) transformed with the CEN-based plasmid pFL38 (empty vector), pFA153 (Ssy5), pCJ353 (Ssy5-42-HA), or pFA150 (HA-Ssy5) and 30629c (SSY5Δ) transformed with pFL38. All strains contained a gap1Δ mutation to prevent uptake of phenylalanine via the Gap1 permease. D, strains AA01 (SSY5Δ) and 34692c (ssy5Δ) containing the agp1::lacZ reporter gene were transformed with plasmid pFL38, pFA153 (Ssy5), pCJ353 (Ssy5-42-HA), or pFA150 (HA-Ssy5) and grown in minimal proline medium. Phenylalanine (5 mM) was added (+) or not (–), and the cultures were incubated for 2 h before measuring β-galactosidase activity in cell extracts.
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A Non-phosphorylatable Form of Ssy5 Is Not Activated by Amino Acids—We next sought to identify the region in the Ssy5 prom is that is phosphorylated after the addition of amino acids. We compared the prom domain sequences of various Ssy5 orthologs and identified a conserved region rich in serines and threonines (Fig. 4A). We next applied site-directed mutagenesis to replace seven of these Ser/Thr residues with alanines. The resulting Ssy5-42-HA7S/T:A mutant is normally processed (Fig. 4B) but fails to be phosphorylated in the wild-type and grr1Δ mutant after the amino acid addition (Fig. 4C). Remarkably, this Ssy5 mutant is inactive, as judged by the results of growth assays (Fig. 4D) and AGP1-lacZ induction assays (Fig. 4E) and the inability of the Stp1 transcription factor to be processed (Fig. 4F). Induced phosphorylation of Ssy5-42-HA is thus essential to its activation.

The same mutations were then introduced into the constitutive HA-Ssy5 form. The HA-Ssy57S/T:A mutant conserved its capacity to complement the growth defects of the ssy5Δ mutant (Fig. 4D) and to constitutively activate AGP1-lacZ transcription (Fig. 4E). Hence, the constitutive HA-Ssy5 form does not need to be phosphorylated in its prom domain to be active. This strongly suggests that prom domain phosphorylation is part of the Ssy5 activation mechanism. Constitutive HA-Ssy5 is nevertheless still phosphorylated in response to amino acid detection.

Acid addition. To test this hypothesis, we collected cells at various time intervals after the addition of Phe to the medium. Phosphorylation of the Ssy5 prom is indeed only transiently detectable (Fig. 3D). We compared the prom domain sequences of various Ssy5 orthologs and identified a conserved region rich in serines and threonines (Fig. 4A). We next applied site-directed mutagenesis to replace seven of these Ser/Thr residues with alanines. The resulting Ssy5-42-HA7S/T:A mutant is normally processed (Fig. 4B) but fails to be phosphorylated in the wild-type and grr1Δ mutant after the amino acid addition (Fig. 4C). Remarkably, this Ssy5 mutant is inactive, as judged by the results of growth assays (Fig. 4D) and AGP1-lacZ induction assays (Fig. 4E) and the inability of the Stp1 transcription factor to be processed (Fig. 4F). Induced phosphorylation of Ssy5-42-HA is thus essential to its activation.

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FIGURE 3. Phosphorylation of the Ssy5 prodomain is dependent on Ssy1, Ptr3, and casein kinase I. A, strain CA080 (Ssy5-42-HA grr1Δ) was grown in minimal proline medium, and phenylalanine (5 mM) was added at time 0. Crude cell extracts were immunoblotted with anti-HA antibodies. B, strain CA080 (grr1Δ) expressing Ssy5-42-HA was grown in proline minimal medium. Phenylalanine (5 mM) (Phe) was added or not, and the cultures were incubated for 5 min. Total cell extracts were incubated (+) or not (−) in the presence of alkaline phosphatase (Pse) and immunoblotting was carried out with anti-HA antibodies. C, strains JA716 (wild type), CA080 (grr1Δ), 29102a (ptr3Δ), and 29105c (sSy1Δ) expressing Ssy5-42-HA were grown in proline minimal medium. Phenylalanine (5 mM) was added (+) or not (−), and the cultures were incubated for 5 min. Crude cell extracts were immunoblotted with anti-HA antibodies. D, the wild-type (LRB341) and yck1Δ yck2-2ts (LRB346, yck1Δ) strains were transformed with plasmids pCJ353 (Ssy5-42-HA) and YCpJYS-20 (bearing the HIS3 and LEU2 genes for complementation of auxotrophies). The strains were grown at 24°C on minimal medium containing proline as the sole nitrogen source. Cultures were then transferred to 37°C for 20 min. Phenylalanine was then added (+) or not (−), and the cultures were incubated for 5 min. Crude cell extracts were immunoblotted with anti-HA antibodies.

(Amino Acids Induce Grr1-dependent Ubiquitylation of Ssy5—) That the Ssy5 prodomain accumulates as a phosphorylated form in the grr1Δ mutant suggests that it is a substrate of the SCFGrr1 complex. Furthermore, the HA-Ssy5 form remains constitutively active in grr1Δ mutant cells (Fig. 5A). This means that the ubiquitylation reaction catalyzed by SCFGrr1 is dispensable when the prodomain of Ssy5 fails to inhibit its Cat domain. To test whether activation of Ssy5 involves its ubiquitylation, the prodomain of Ssy5-42-HA was immunoprecipitated and probed with anti-Ub antibodies (Fig. 5B). An Ub signal was clearly detected after the addition of Phe and was not observed in the ssy1Δ and grr1Δ mutants. Furthermore, no Ub signal was detected in the case of the non-phosphorylatable Ssy5-42-HA^73T/A mutant. Hence, the prodomain of Ssy5 or some associated factor undergoes Grr1-dependent ubiquitylation in response to Ssy1-mediated detection of amino acids. Furthermore, this ubiquitylation requires prior phosphorylation of the prodomain.

Amino acid-induced ubiquitylation of the Ssy5-42-HA prodomain was also observed using cells expressing Ub fused to a polyhistidine tag (Fig. 5C). The gene encoding this Ub variant was placed under the control of the copper-inducible CLIP1 promoter. An untagged Ub variant was used as a control. Copper was provided or not to the cells for 3 h, Phe was then added, and the ubiquitylated proteins present in denatured cell extracts were purified on nickel columns before immunoblotting with anti-HA antibodies. The results confirm that the Ssy5 prodomain is ubiquitylated in response to Phe (Fig. 5C). This ubiquitylation was more readily detectable in the early minutes after the amino acid addition. As shown above, it was not detected when using the non-phosphorylatable Ssy5-42-HA^73T/A mutant. We also performed this experiment using the constitutive HA-Ssy5 form (Fig. 5D). The results show that the prodomain of HA-Ssy5 is not constitutively ubiquitylated; this modification is nevertheless observed upon amino acid addition (Fig. 5D). Finally, we observed that the inactive Ssy5-42-HA^73T/A mutant also undergoes amino acid-induced ubiquitylation (Fig. 5E), indicating that Ssy5 does not need to be processed and active to be ubiquitylated. This suggests that a minor fraction of the ubiquitylated Ssy5 proteins detected in Fig. 5C may correspond to the unprocessed form.

In conclusion, the prodomain of Ssy5 is ubiquitylated in a Grr1-dependent manner when cells detect external amino acids, and this modification is essential to Ssy5 activation. This ubiquitylation is conditioned by prior CKI-dependent phosphorylation of the prodomain and is dispensable for Ssy5 activity if the prodomain fails to inhibit the Cat domain. Activation of Ssy5 Is a Short Term, Proteasome-independent Process Not Correlating with Degradation of Its Prodomain—In previous studies, it was reported that Leu, a strong amino acid inducer of AGP1 transcription, promotes proteasomal degradation of the Ssy5 prodomain. Furthermore, the reduced prodomain levels correlated with Stpl processing (17). These observations suggest that proteasomal degradation of the inhibitory prodomain is part of the mechanism of Ssy5 activation (e.g. this degradation would relieve the negative effect exerted by the prodomain on the protease’s Cat domain). This model is also supported by the observation that destabilization of the prodomain by means of a conditional degron or mutations results in activation of Ssy5 (17). However, other studies showed that processing of Stpl transcription factor and AGP1 induction occur normally when the proteasome is inhibited by means of conditional mutations (8) or inhibitors (2). Furthermore, it was also reported that the level of the prodomain does not always correlate with the degree of activation of the amino acid-sensing pathway (13).

These apparent discrepancies might be resolved if one considers that Ssy5 activation results from ubiquitylation of its prodomain (e.g. this modification alone would relieve the...
FIGURE 4. A non-phosphorylatable mutant form of Ssy5 is not activated by amino acids. A, a prodomain region rich in Ser and Thr residues is conserved among Ssy5 orthologs. The sequences of Ssy5/YJL156C (residues 65–88) and Ssy5 orthologs were retrieved from the SGD database (available on the World Wide Web) and aligned using Clustal B. The Ssy5-42-HA7S/T:A form is processed. Extracts were prepared from cells transformed with plasmids pCJ353 (Ssy5-42-HA) or pCJ358 (Ssy5-42-HA7S/T:A) and immunoblotted with anti-HA antibodies. C, strains 23344C (wt) and JA115 (grr1Δ/H9004) transformed with plasmids pCJ353 (Ssy5-42-HA) or pCJ358 (Ssy5-42-HA7S/T:A) were grown in proline minimal medium. Phenylalanine (5 mM) was added or not for 5 min. Cell extracts were immunoblotted with anti-HA antibodies.

D–F, the Ssy5-42-HA7S/T:A form is not functional, whereas HA-Ssy57S/T:A is functional and constitutively active. D, growth tests as in Fig. 1C except that cells were also transformed with plasmids pCJ358 (Ssy5-42-HA7S/T:A) and pCJ346 (HA-Ssy57S/TA). E, experiment as in Fig. 3B except that extracts were prepared from strain JA115 (grr1Δ/H9004) transformed with plasmid pFA44 (HA-Ssy5).

G, the constitutive HA-Ssy5 form is phosphorylated in response to amino acids. Left, strain JA115 (grr1Δ) transformed with plasmid pFA44 (HA-Ssy5) was grown in proline minimal medium. Phenylalanine (5 mM) was added or not for 5 or 30 min. Cell extracts were immunoblotted with anti-HA antibodies. Right, experiment as in Fig. 3B except that extracts were prepared from strain JA115 (grr1Δ) transformed with plasmid pFA44 (HA-Ssy5).
Amino acids induce Grr1-dependent ubiquitylation of the Ssy5 prodomain. A, the HA-Ssy5 form remains constitutively active in the grr1Δ mutant; experiment as in Fig. 4 except that the agp1::lacZ ssy5mutant strains were 34.692c (wild type) and JA902 (grr1Δ). B–E, Ssy5 is ubiquitylated in response to amino acids. B, the wild-type (23344c), grr1Δ (JA115), and ssy1Δ (32501c) strains transformed with the CEN-based plasmid pFA153 (Ssy5), pCJ353 (Ssy5-42-HA), or pCJ358 (Ssy5-42-HA7S:T/A) were grown in minimal proline medium. Phenylalanine (5 mM) was added (+/H11001) or not (−/H11002) for 30 min. Crude cell extracts were immunoprecipitated with anti-HA antibody, and immunoblotting was carried out with anti-HA or anti-Ub antibodies. C, strain 27071b (doa4Δ/ura3 leu2) was co-transformed with plasmids pCJ353 (Ssy5-42-HA) and Yep96 or Yep96 HIS bearing the CUP1-Ub gene or CUP1-Ub-His gene, respectively, and grown in yeast nitrogen base medium with proline as the sole nitrogen source. CuSO4 (0.1 mM) was added (+) or not (−) for 3 h to induce the CUP1-Ub gene. Phenylalanine (5 mM) was then added (+) or not (−) at time 0 of the experiment. Crude extracts were prepared under denaturing conditions and incubated with nickel-NTA-Sepharose beads. His6-tagged (ubiquitylated) proteins retained on the beads were then eluted, and immunoblotting was carried out with anti-HA antibodies. Crude extracts were also immunoblotted with anti-HA and anti-Pgk antibodies. D, experiment as in C except that cells were transformed with plasmid pFA150 (HA-Ssy5), and phenylalanine was added for 5 min. E, experiment as in C except that cells were transformed with plasmid pFA200 (Ssy5-42-HA3S:A), and phenylalanine was added for 5 min.
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A, strain JA716 (Ssy5-42-HA) was grown in minimal proline medium, and phenylalanine or leucine (each at 5 mM) was added at time 0. Crude cell extracts were prepared and immunoblotted with anti-HA and Pgk antibodies. B, wild-type cells (23344c) transformed with a plasmid bearing the SSSY5-42-HA gene under the G418 promoter were grown on minimal proline medium containing raffinose as the carbon source. The SSSY5-42-HA gene was induced by galactose for 1 h, and glucose was then added to the cultures (time 0) to repress its transcription. Leucine or phenylalanine was also eventually added (time 0), and cells were collected at various time intervals. Cell extracts were prepared, and immunoblotting was carried out using anti-HA and anti-Pgk antibodies. C, strains CA045 (Stp1-HA) and JA827 (Stp2-HA) were grown in minimal proline medium, and phenylalanine or leucine (each at 5 mM) was added at time 0. Total cell extracts were prepared, and immunoblotting was carried out with anti-HA antibodies. D, strains CMY763 (cim-3) and WGC4a-11/22a (pre1-1 pre2-1) were transformed with plasmid pKW10 (Stp1-HA) or pKW18 (Stp2-HA) and grown in minimal proline medium at 24 °C. Cultures were split into two of equal volumes, and one was transferred to 37 °C for 30 min prior to the addition of cycloheximide (100 μg/ml). Leucine was then added, and the cultures were incubated for 5 min. Crude cell extracts were immunoblotted with anti-HA antibodies. Strain pdr5Δ (JA547) transformed with plasmid pKW10 (Stp1-HA) or pKW18 (Stp2-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. Leucine was then added, and the cultures were incubated for 5 min. Crude cell extracts were immunoblotted with anti-HA antibodies.
Phosphorylation-induced Ubiquitylation of the Ssy5 Protease

The Ssy5 Cat domain remains associated with the prodomain after amino acid detection. A, processing of the double-tagged Ssy5-HA-42-FLAG form. Strains JA716 (Ssy5-42-HA) and FA150 (Ssy5-42-HA-FLAG) were grown in minimal proline medium. Crude cell extracts were immunoblotted with anti-HA and anti-FLAG antibodies. B, the Ssy5-FLAG and Ssy5-42-HA-FLAG forms are functional. Strains FB90 (ssy5Δ), JA716 (Ssy5-42-HA), FA140 (Ssy5-FLAG), and FA150 (Ssy5-42-HA-FLAG) were transformed with the plasmid YCpAGP1-lacZ and grown in proline minimal medium. Phenylalanine (5 mM) was added (+) or not (−), and the cultures were incubated for 2 h before measuring β-galactosidase activities in cell extracts. C, the prodomain and Cat domain remain associated after detection of amino acids. Strains FA140 (Ssy5-FLAG), FA150 (Ssy5-42-HA-FLAG), and JA716 (Ssy5-42-HA) were grown in minimal proline medium. Phenylalanine (5 mM) was added to strain FA150 for the time indicated. Crude cell extracts were immunoprecipitated with anti-HA antibody before immunoblotting with anti-HA and anti-FLAG antibodies.

DISCUSSION

In this study, we report that the inhibitory prodomain of yeast Ssy5 endoprotease is phosphorylated in a CKI-dependent manner in response to Ssy1-mediated detection of external amino acids. This phosphorylation is also dependent on Ptr3, the protein interacting with Ssy1 and Ssy5 and also undergoing CKI-dependent phosphorylation in response to amino acid detection (13). We show that induced phosphorylation of the Ssy5 prodomain is essential to activation of the protease because an Ssy5 mutant with a non-phosphorylatable prodomain fails to process Stp1 in response to amino acids. This phosphorylation, however, is not sufficient to activate the protease. Our results rather show that its role is to promote ubiquitylation of the prodomain. This ubiquitylation is dependent on Grr1, the F-box protein of the SCF<sup>Grr1</sup> complex. Several ubiquitin ligases of the SCF category recognize protein substrates after these have been phosphorylated, and this is probably true for Ssy5 as well. The phosphorylation-induced ubiquitylation of the prodomain catalyzed by the SCF<sup>Grr1</sup> complex is obviously the key step in the Ssy5 activation mechanism because the protease remains totally inactive in the <i>grr1Δ</i> mutant in which the Ssy5 prodomain is phosphorylated but not ubiquitylated.

It has recently been reported that amino acids promote proteasomal degradation of the Ssy5 prodomain (17). Therefore, a simple model of Ssy5 activation is that ubiquitylation of the inhibitory prodomain leads to its degradation and thus to inhibition relief of the Cat domain. However, our study rather suggests that ubiquitylation of the Ssy5 prodomain itself causes relief of the inhibition exerted on the Cat domain, an effect similar to the activation of Ssy5 observed when peptides such as HA tags are inserted at the extreme N terminus of its prodomain. Another consequence of this ubiquitylation would be the proteasomal degradation of the prodomain, but this degradation would not be needed for initial activation of the protease.
Phosphorylation-induced Ubiquitylation of the Ssy5 Protease

An Ssy5 activation mechanism based on ubiquitylation suggests an obvious mean for rapid deactivation of the enzyme, by deubiquitylation. It may indeed be advantageous for the cell to first activate Ssy5 in a manner that is reversible. In contrast, once the prodomain has been degraded, the Ssy5 protease should in principle be irreversibly activated, unless other control mechanisms operate on the Cat domain. That the prodomain of Ssy5 undergoes degradation is consistent with preliminary data indicating that it seems mainly associated with Lys\(^{48}\)-linked poly-Ub chains.\(^3\) Our results also indicate that Ssy5 activation does not coincide with a marked dissociation of its prodomain and Cat domain. That both protease subunits remain bound to each other after Ssy5 activation is also consistent with a model in which the protease can eventually shift back into its autoinhibited state upon deubiquitylation. The identification of the deubiquitylating enzyme potentially involved in Ssy5 deactivation represents an obvious future direction of work. We also observed that the prodomain of Ssy5 undergoes dephosphorylation when cells are shifted back to a medium devoid of amino acids. It will also be interesting to identify the phosphatase catalyzing this dephosphorylation. The intervention of a phosphatase could also account for the observation that the Ssy5 prodomain is not entirely converted into its phosphorylated form after the addition of amino acids to the gpr1A mutant.

Our results also raise the question of how the prodomain of Ssy5 inhibits the Cat domain and how ubiquitylation of the prodomain eventually relieves this inhibition. Many ubiquitylated proteins are recognized by factors exposing Ub-binding domains, but no such protein has been identified in the various screens for yeast factors involved in amino acid signaling. It is more likely that Ub directly interferes with the prodomain action through some steric hindrance effect (23). For instance, the attached Ub molecules might displace the prodomain N terminus, which otherwise binds to and inhibits the Cat domain of the protease (e.g. by obstructing substrate entry). Allosteric control of serine proteases is well documented, and most allosteric effectors act by interfering with the process in which the newly formed N terminus of the protease (after processing of the zymogen) inserts into a preformed binding pocket so that the protease adopts an active conformation (24). The prodomain of Ssy5 might interfere with such an activation mechanism unless it is modified with Ub moieties.

Interestingly, activation of a protease by relief from autoinhibition has recently been reported (25). Furthermore, non-degradative ubiquitylation has recently emerged as a mechanism regulating caspases, the cysteine proteases involved in apoptosis (26). For instance, ubiquitylation of caspase-8 promotes its aggregation, processing, and thus activation (27), whereas in Drosophila, attachment of Lys\(^{48}\)-linked poly-Ub chains to the drICE effector caspase (a homolog of caspase-3 and -7) seems to directly reduce its proteolytic potency (28). Further investigation of the molecular details of Ub-dependent Ssy5 activation will certainly shed new light on the role of Ub as a potential regulator of protease function.

\(^3\) F. Abdel-Sater and B. André, unpublished results.

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