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

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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 SCF^{Grr1} 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 SCF^{Grr1} 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)[2] 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 Agp1, 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 SCF^{Grr1} 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 SCF^{Grr1}-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 ($CuSO_4$, 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.

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[2] The abbreviations used are: Cat, catalytic; CKI, casein kinase I; Ub, ubiquitin.

TABLE 1

Yeast strains

Strain JA716 was constructed by introducing the six HA-encoding sequence (preceded by a *loxP* site) between codons 42 and 43 of the chromosomal *SSY5* gene according to the method of Gauss *et al.* (31). Strain CA080 was isolated by deleting the *GRR1* gene in strain JA716. Strains 29102a and 29105c were isolated by crossing. All strains are available upon request.

Strain	Genotype	Reference or source
23344c	*MATα ura3*	Laboratory collection
AA01	*MATα agp1::lacZ kanMX2 ura3*	Laboratory collection
FB90	*MATα ssy5Δ::kanMX2 ura3*	Ref. 7
30629c	*MATa gap1Δ::kanMX2 ura3*	Ref. 7
FB92	*MATa gap1Δ::kanMX2 ssy5Δ::kanMX2 ura3*	Ref. 14
34686b	*MATα gap1Δ ssy5Δ::kanMX2 Stp1-HA kanMX2 ura3*	Ref. 2
34692c	*MATα ssy5Δ::kanMX2 agp1::lacZ kanMX2 ura3*	Ref. 2
JA902	*MATα ssy5Δ::kanMX2 agp1::lacZ kanMX2 grr1Δ::HgB ura3*	This study
JA115	*MATα grr1Δ::kanMX2 ura3*	Ref. 7
32501c	*MATα ssy1Δ::kanMX2 ura3*	Ref. 7
JA716	*MATα Ssy5-42-HA$_6$ ura3*	This study
CA080	*MATα grr1Δ::kanMX2 Ssy5-42-HA$_6$ ura3*	This study
29102a	*MATα ptr3Δ::kanMX2 Ssy5-42-HA$_6$ ura3*	This study
29105c	*MATα ssy1Δ::kanMX2 Ssy5-42-HA$_6$ ura3*	This study
CA045	*MATα STP1-HA3 ura3*	Ref. 29
JA827	*MATα STP2-HA3 ura3*	Ref. 29
WGC4a-1/22a	*MATα 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-Δ1 yck2-2^{ts}*	Ref. 19
CMY763	*MATα ura3-52 leu2Δ1 his3Δ-200 cim3-1*	Ref. 20

TABLE 2

Plasmids

The pCJ353 plasmid was constructed by recombination in yeast between linearized pFA153 plasmid and a DNA fragment carrying the 6-HA-encoding sequence. This DNA fragment (flanked by appropriate 40-bp recombination sequences) was obtained by PCR using total DNA of strain JA716 as template. The same *in vivo* recombination approach was applied to isolate plasmid pCJ358 from pCJ353 and plasmid pCJ346 from pFA150 by insertion of two PCR fragments forming the *SSY5* mutated gene. The mutations resulting in the replacement of serines and threonines with alanines were introduced into the oligonucleotides used to amplify the *SSY5* fragment. The pCJ350 plasmid was constructed by *in vivo* recombination between the linearized pFA153 plasmid and a PCR fragment carrying the *SSY5* mutated gene. The accuracy of each newly isolated mutagenized plasmid was checked by sequencing. All plasmids and oligonucleotides are available upon request.

Plasmid	Description/Markers	Reference or source
pFA150	YCp-*HA$_3$-SSY5* (*URA3*)	Ref. 2
pFA153	YCp-*SSY5* (*URA3*)	Ref. 2
pCJ358	YCp-*SSY5-42-HA$_6^{7S/T:A}$ (URA3)*	This study
pCJ353	YCp-*SSY5-42-HA$_6$* (*URA3*)	This study
pCJ346	YCp-*HA$_3$-SSY5$^{7S/T:A}$ (URA3)*	This study
pCJ350	YCp-*SSY5$^{7S/T:A}$* (*URA3*)	This study
pFA200	YCp- *SSY5–42-HA$^{S\rightarrow A}$ (URA3)*	This study
pFL38	*CEN-ARS (URA3)*	Ref. 32
pOM10	HA$_6$- *KanMX6 (URA3)*	Ref. 31
pAGP1-lacZ	YCp-*AGP1-lacZ (URA3)*	Ref. 7
YEp96	YEp-CUP1-Ub *(TRP1)*	Ref. 22
YEp96 HIS	YEp-CUP1-HIS-Ub *(TRP1)*	Ref. 22

Yeast Cell Extracts, Immunoprecipitation, and Immunoblotting—Crude cell extracts were prepared and immunoblotted as described previously (2). After transfer to a nitrocellulose membrane (Shleicher & Schüell), 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 ($\sim 10^8$ 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 His$_6$-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 His$_6$-tagged ubiquitin (YEp96-HIS) or untagged ubiquitin (YEp96). Cells were grown in yeast nitrogen base medium with proline as the sole nitrogen source. $CuSO_4$ (0.1 mM) was added for 3 h to activate the *CUP1-Ub* 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. 1*A*). 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. 1*C*) and to induce expression of an *AGP1-lacZ* gene (Fig. 1*D*). 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. 1*B*). 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

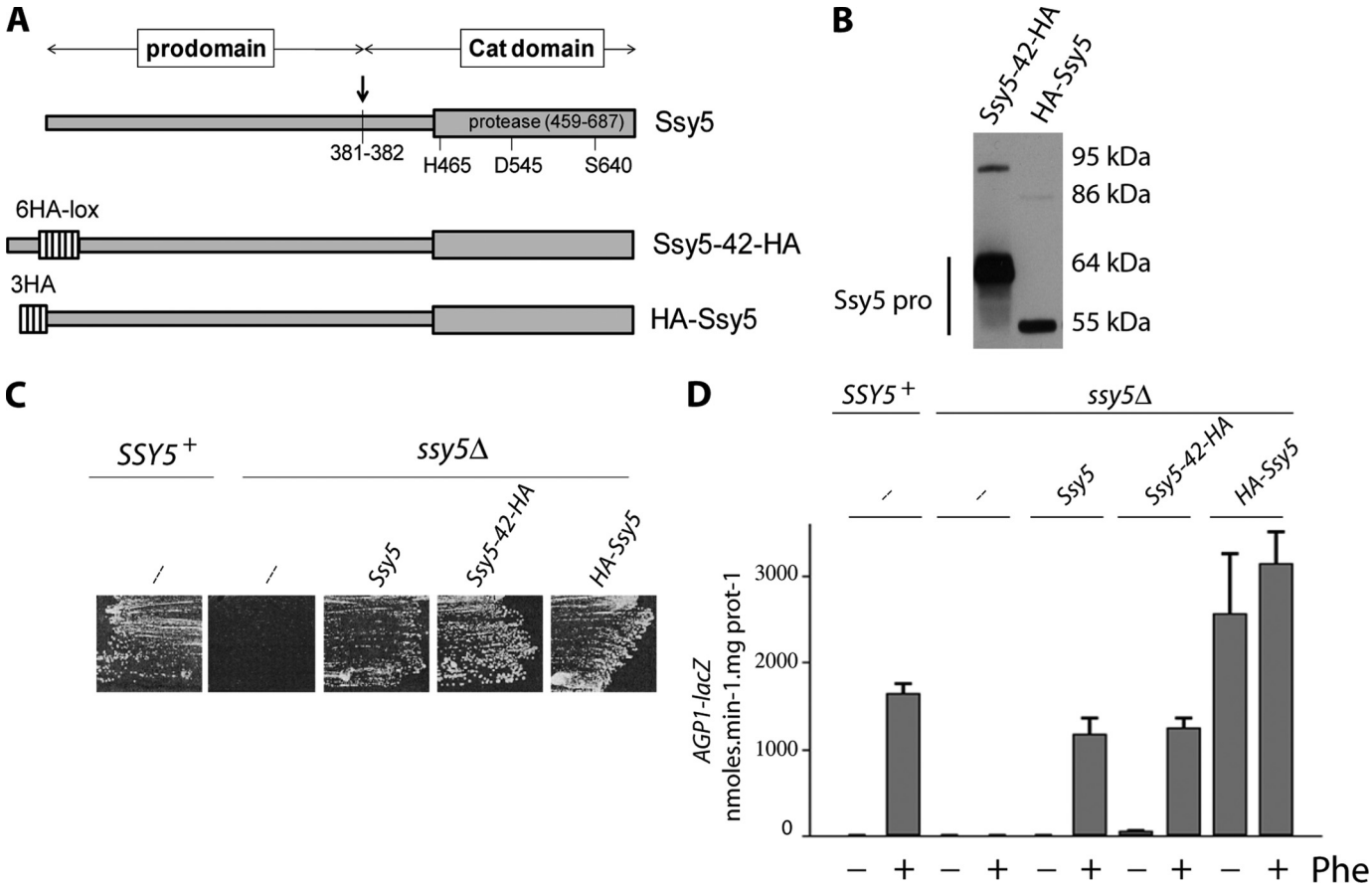

FIGURE 1. **The Ssy5-42-HA protease is functional and activatable by amino acids.** *A*, schematic representation of the Ssy5 endoprotease. Autocleavage between residues 381 and 382 (*vertical arrow*) yields the prodomain (42.2 kDa) and Cat domain (35.3 kDa). The Cat domain includes the His-Asp-Ser catalytic 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 (*ssy5*Δ) 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 (*ssy5*Δ) transformed with the CEN-based plasmid pFL38 (empty vector), pFA153 (Ssy5), pCJ353 (Ssy5-42-HA), or pFA150 (HA-Ssy5) and 30629c (*SSY5*$^+$) transformed with pFL38. All strains contained a *gap1*Δ mutation to prevent uptake of phenylalanine via the Gap1 permease. *D*, strains AA01 (*SSY5*$^+$) and 34692c (*ssy5*Δ) 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.

inducer amino acid is present. In other words, HA-Ssy5 is constitutively active (Fig. 1*D*). 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. 1*D*) and is thus better suited for investigating the mechanism of this activation.

We expressed the Ssy5-42-HA form in a *grr1*Δ 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. 2*A*). These upper bands correspond to phosphorylated forms of the prodomain because they disappear after treatment of cell extracts with alkaline phosphatase (Fig. 2*B*). Interestingly, these phosphorylated forms are observed neither in the wild type nor in *ssy1*Δ or *ptr3*Δ mutants (Fig. 2*C*). Furthermore, this phosphorylation is reversible because it tends to disappear when cells are transferred back to a medium without any inducer amino acid (Fig. 2*D*). 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-HA$^{S\rightarrow A}$ 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 SCF^{Grr1} 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. 2*C*, 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

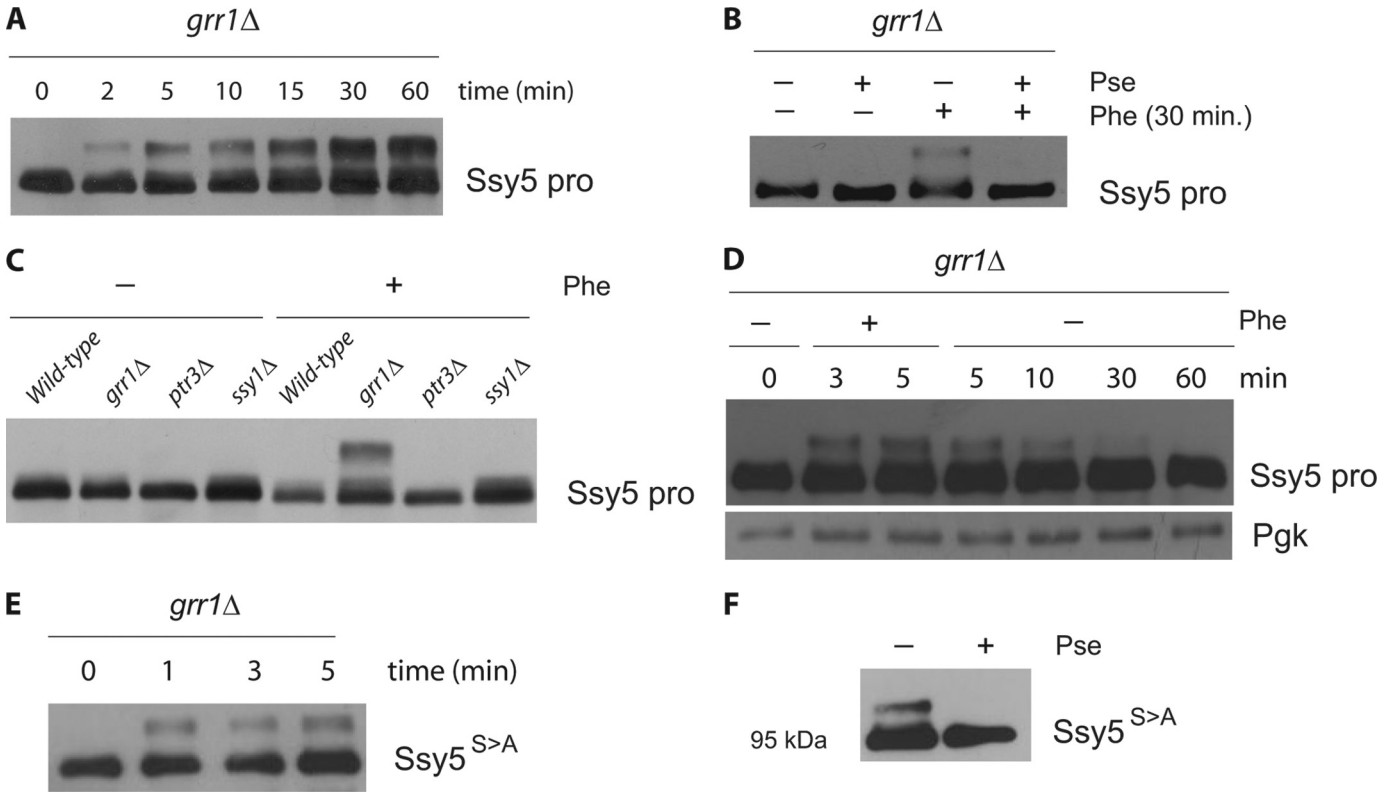

FIGURE 2. **The prodomain of Ssy5 accumulates as a phosphorylated form in the *grr1*Δ mutant after amino acid addition.** *A*, strain CA080 (*Ssy5-42-HA grr1*Δ) was grown in minimal proline medium, and phenylalanine (5 mM) was added at time 0. Crude cell extracts were immunoblotted with anti-HA antibodies. *B*, strain CA080 (*grr1*Δ) expressing Ssy5-42-HA was grown in proline minimal medium. Phenylalanine (5 mM) (*phe*) was added or not, and the cultures were incubated for 30 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 (*grr1*Δ), 29102a (*ptr3*Δ), and 29105c (*ssy1*Δ) expressing Ssy5-42-HA were grown in proline minimal medium. Phenylalanine (5 mM) was added (+) or not (−), and the cultures were incubated for 30 min. Crude cell extracts were immunoblotted with anti-HA antibodies. *D*, experiment as in *A* except that 5 min after phenylalanine addition, the cultures were filtered, washed, and transferred back to a fresh minimal proline medium. Cell extracts were also immunoblotted with anti-Pgk (phosphoglycerate kinase) antibodies. *E*, experiment as in *A* except that strain CA080 (*grr1*Δ) expressed the inactive and unprocessed Ssy5-42-HA$^{S\rightarrow A}$ mutant. *F*, experiment as in *B* except that strain CA080 (*grr1*Δ) expressed the inactive and unprocessed Ssy5-42-HA$^{S\rightarrow A}$ mutant.

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 prodomain was indeed only transiently detectable (Fig. 3, *A* and *B*). Interestingly, this phosphorylation is deficient in the *ssy1*Δ and *ptr3*Δ mutants; however, it occurs in the *grr1*Δ mutant (Fig. 3*C*). These results show that amino acid detection by the Ssy1 sensor triggers a transiently detectable phosphorylation of the Ssy5 prodomain. We next tested whether this phosphorylation is dependent on CKI. This kinase is essential to processing of Stp1 and to *AGP1* induction (2). The Ssy5-42-HA form was expressed in a thermosensitive *yck*^{ts} mutant. In this strain, the *YCK1* gene encoding one CKI isoform is deleted, and the *YCK2* gene encoding the other contains a mutation inactivating CKI at the restrictive temperature of 37 °C (19). The results show that the prodomain of Ssy5 fails to be phosphorylated in response to Phe when the *yck*^{ts} strain is transferred for 20 min to 37 °C before adding the amino acid (Fig. 3*D*). In conclusion, detection of external amino acids by Ssy1 leads to CKI-dependent phosphorylation of the Ssy5 prodomain. Although we favor a direct role of CKI in this phosphorylation, an indirect effect cannot be ruled out. This phosphorylated form of the prodomain is only transiently detectable in wild-type cells but tends to accumulate in cells defective for Grr1, the F-box protein of the SCF^{Grr1} complex.

A Non-phosphorylatable Form of Ssy5 Is Not Activated by Amino Acids—We next sought to identify the region in the Ssy5 prodomain that is phosphorylated after the addition of amino acids. We compared the prodomain sequences of various Ssy5 orthologs and identified a conserved region rich in serines and threonines (Fig. 4*A*). We next applied site-directed mutagenesis to replace seven of these Ser/Thr residues with alanines. The resulting Ssy5-42-HA$^{7S/T:A}$ mutant is normally processed (Fig. 4*B*) but fails to be phosphorylated in the wild-type and *grr1*Δ mutant after the amino acid addition (Fig. 4*C*). Remarkably, this Ssy5 mutant is inactive, as judged by the results of growth tests (Fig. 4*D*) and *AGP1-lacZ* induction assays (Fig. 4*E*) and the inability of the Stp1 transcription factor to be processed (Fig. 4*F*). 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-Ssy5$^{7S/T:A}$ mutant conserved its capacity to complement the growth defects of the *ssy5*Δ mutant (Fig. 4*D*) and to constitutively activate *AGP1-lacZ* transcription (Fig. 4*E*). Hence, the constitutive HA-Ssy5 form does not need to be phosphorylated in its prodomain to be active. This strongly suggests that prodomain phosphorylation is part of the Ssy5 activation mechanism. Constitutive HA-Ssy5 is nevertheless still phosphorylated in response to amino acid detection

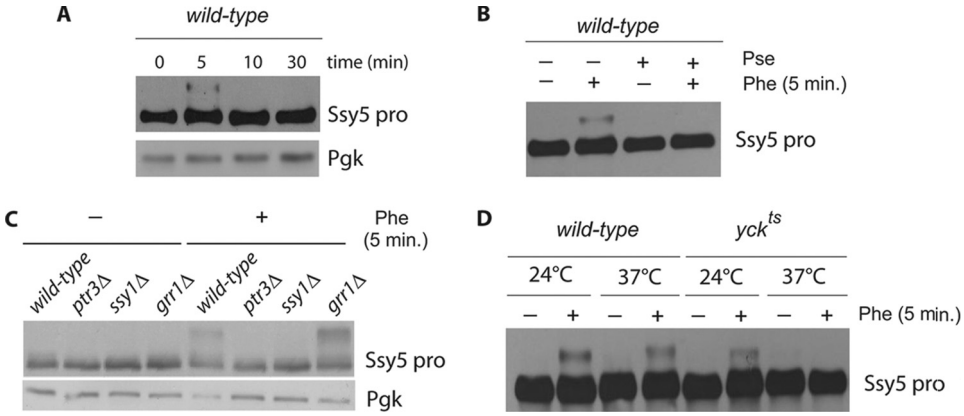

FIGURE 3. **Phosphorylation of the Ssy5 prodomain is dependent on Ssy1, Ptr3, and casein kinase I.** *A*, strain CA080 (*Ssy5-42-HA grr1*Δ) was grown in minimal proline medium, and phenylalanine (5 mM) was added at time 0. Crude cell extracts were immunoblotted with anti-HA antibodies. *B*, strain CA080 (*grr1*Δ) 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 (*grr1*Δ), 29102a (*ptr3*Δ), and 29105c (*ssy1*Δ) 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 *yck1*Δ *yck2-2ts* (LRB346, *yck*^{ts}) 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.

(Fig. 4*G*). Hence, although prodomain phosphorylation is not needed for HA-Ssy5 to be active, it still occurs when cells detect external amino acids.

Amino Acids Induce Grr1-dependent Ubiquitylation of Ssy5—That the Ssy5 prodomain accumulates as a phosphorylated form in the *grr1*Δ mutant suggests that it is a substrate of the SCF^{Grr1} complex. Furthermore, the HA-Ssy5 form remains constitutively active in *grr1*Δ mutant cells (Fig. 5*A*). This means that the ubiquitylation reaction catalyzed by SCF^{Grr1} 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. 5*B*). A Ub signal was clearly detected after the addition of Phe and was not observed in the *ssy1*Δ and *grr1*Δ mutants. Furthermore, no Ub signal was detected in the case of the non-phosphorylatable Ssy5-42-HA$^{7S/T:A}$ 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. 5*C*). The gene encoding this Ub variant was placed under the control of the copper-inducible *CUP1* 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. 5*C*). 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$^{7S/T:A}$ mutant. We also performed this experiment using the constitutive HA-Ssy5 form (Fig. 5*D*). The results show that the prodomain of HA-Ssy5 is not constitutively ubiquitylated; this modification is nevertheless observed upon amino acid addition (Fig. 5*D*). Finally, we observed that the inactive Ssy5-42-HA$^{S\rightarrow A}$ mutant also undergoes amino acid-induced ubiquitylation (Fig. 5*E*), 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. 5*C* 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 Stp1 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 Stp1 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

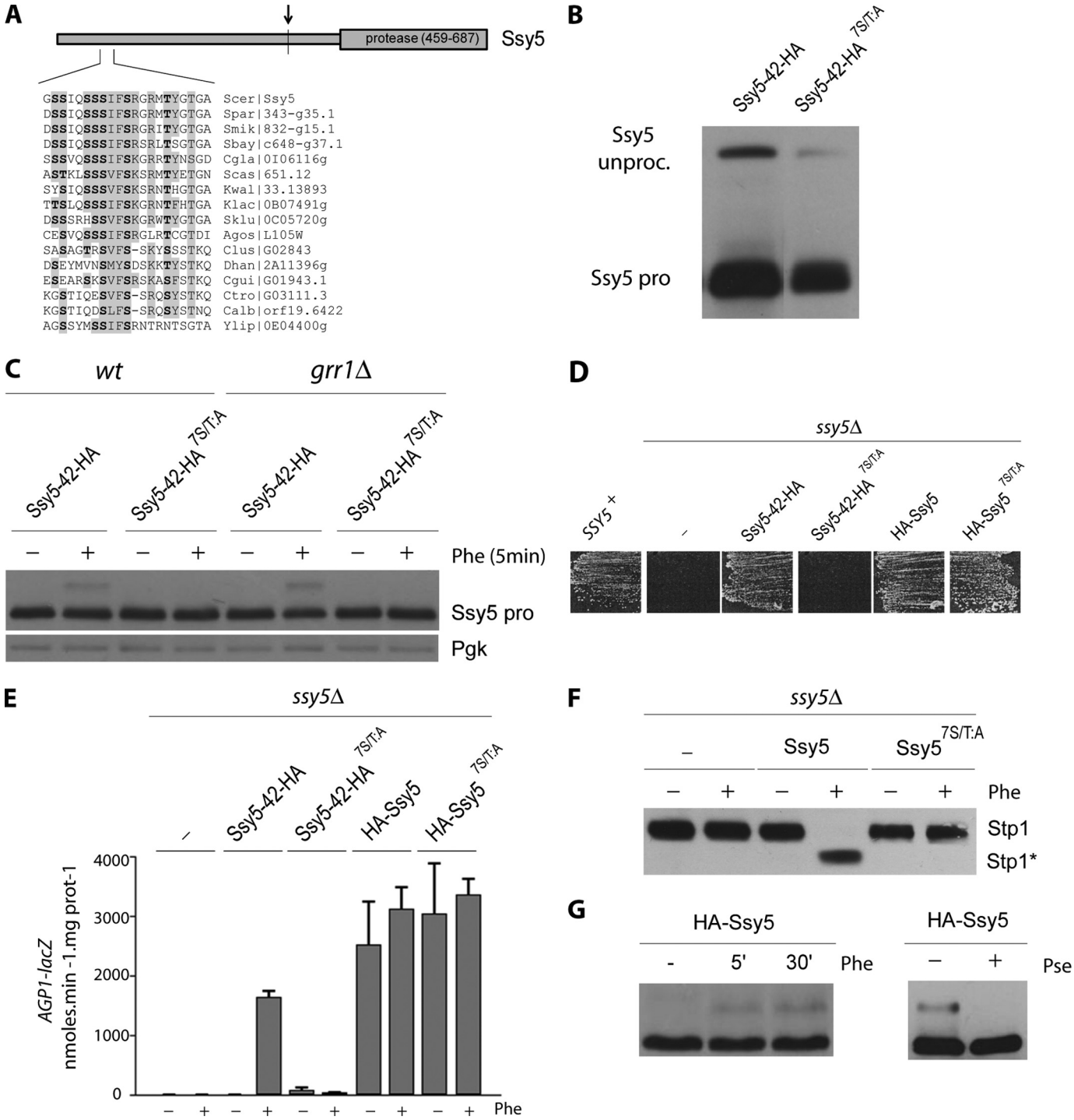

FIGURE 4. **A non-phosphorylatable mutant form of Ssy5 is not activated by amino acids.** *A*, a prodomain region rich in Ser and Thr residues is conserved among Ssy5 orthologs. The sequences of Ssy5/YJL156C (residues 65–88) and Ssy5 orthologs were retrieved from the SGD data base (available on the World Wide Web) and aligned using Clustal. *B*, the Ssy5-42-HA$^{7S/T:A}$ form is processed. Extracts were prepared from cells transformed with plasmids pCJ353 (Ssy5-42-HA) or pCJ358 (Ssy5-42-HA$^{7S/T:A}$) and immunoblotted with anti-HA antibodies. *C*, strains 23344C (*wt*) and JA115 (*grr1*Δ) transformed with plasmids pCJ353 (Ssy5-42-HA) or pCJ358 (Ssy5-42-HA$^{7S/T:A}$) 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-HA$^{7S/T:A}$ form is not functional, whereas HA-Ssy5$^{7S/T:A}$ is functional and constitutively active. *D*, growth tests as in Fig. 1*C* except that cells were also transformed with plasmids pCJ358 (Ssy5-42-HA$^{7S/T:A}$) and pCJ346 (HA-Ssy5$^{7S/TA}$). *E*, experiment as in Fig. 1*D* except that cells were also transformed with plasmids pCJ358 (Ssy5-42-HA$^{7S/T:A}$) and pCJ346 (HA-Ssy5$^{7S/TA}$). *F*, strain 34686b (*ssy5*Δ *STP1-HA*) transformed with the CEN-based plasmid pFA153 (Ssy5) or pCJ350 (Ssy5$^{7S/TA}$) or the empty vector (−) was grown on minimal proline medium. Phenylalanine (5 mM) was added (+) or not (−) for 30 min. Crude cell extracts were immunoblotted with anti-HA antibodies. *G*, the constitutive HA-Ssy5 form is phosphorylated in response to amino acids. *Left*, strain JA115 (*grr1*Δ) 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. 3*B* except that extracts were prepared from strain JA115 (*grr1*Δ) transformed with plasmid pFA44 (HA-Ssy5).

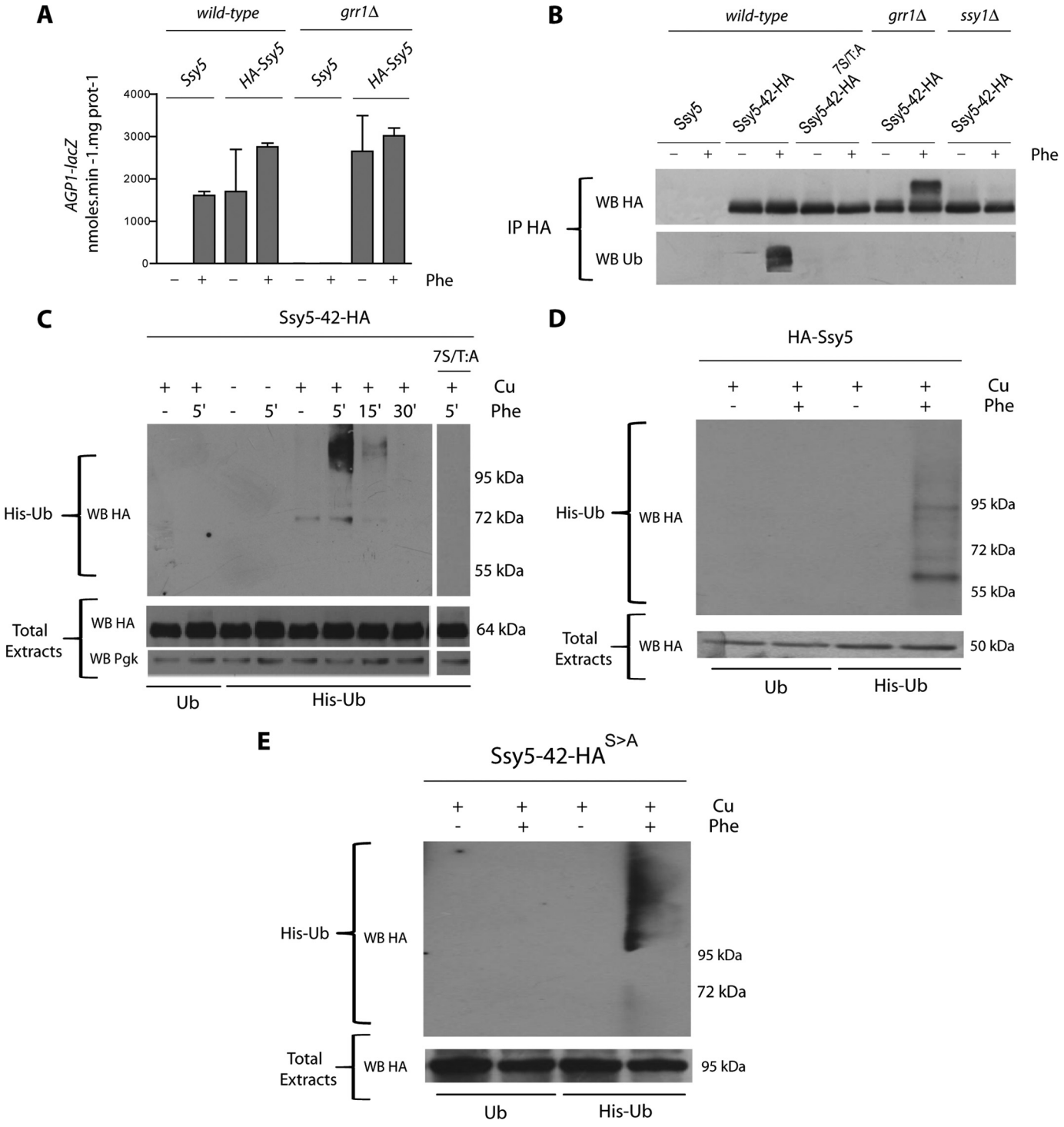

FIGURE 5. **Amino acids induce Grr1-dependent ubiquitylation of the Ssy5 prodomain.** *A*, the HA-Ssy5 form remains constitutively active in the *grr1*Δ mutant; experiment as in Fig. 4*C* except that the *agp1*::*lacZ ssy5* mutant strains were 34.692c (wild type) and JA902 (*grr1*Δ). *B–E*, Ssy5 is ubiquitylated in response to amino acids. *B*, the wild-type (23344c), *grr1*Δ (JA115), and *ssy1*Δ (32501c) strains transformed with the CEN-based plasmid pFA153 (Ssy5), pCJ353 (Ssy5-42-HA), or pCJ358 (Ssy5-42-HA$^{7S:T/A}$) were grown in minimal proline medium. Phenylalanine (5 mM) was added (+) or not (−) 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 (*doa4*Δ *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. $CuSO_4$ (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 in the presence of nickel-NTA-Sepharose beads. His_6-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-HA$^{S\rightarrow A}$), and phenylalanine was added for 5 min.

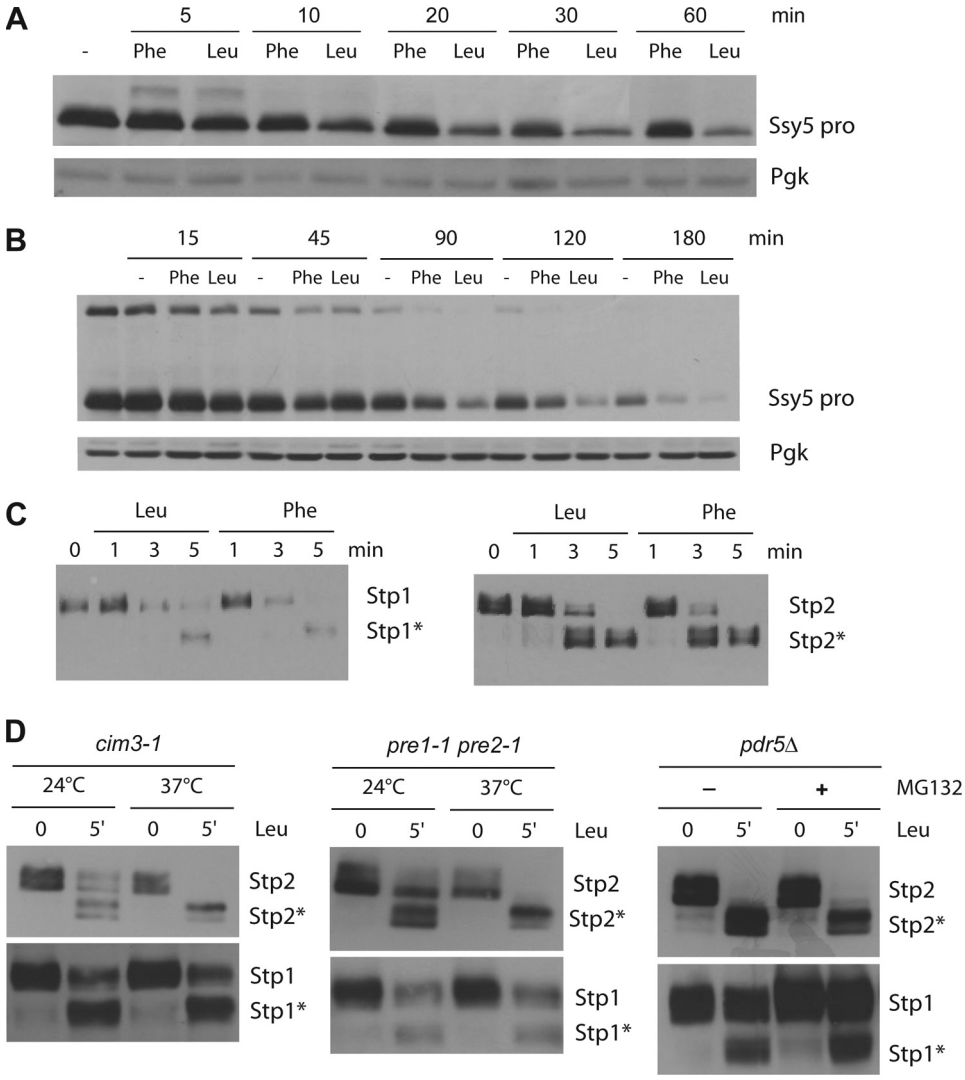

FIGURE 6. **Activation of Ssy5 is a short term, proteasome-independent process not correlating with degradation of its prodomain.** *A*, strain JA716 (*Ssy5-42-HA*) was grown in minimal proline medium, and phenylalanine or leucine (each at 5 mM) was added at time 0. Crude cell extracts were prepared and immunoblotted with anti-HA and Pgk antibodies. *B*, wild-type cells (23344c) transformed with a plasmid bearing the *SSY5-42-HA* gene under the *GAL* promoter were grown on minimal proline medium containing raffinose as the carbon source. The *SSY5-42-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 phenylanine 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 (*cim3-1*) 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 prepared and immunoblotted with anti-HA antibodies. Strain *pdr5*Δ (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.

prodomain's inhibitory effect on the Cat domain). Another consequence of this ubiquitylation would be the eventual proteasomal degradation of the prodomain, but this degradation would not be needed for the initial activation of Ssy5. We collected several results supporting this model. First, although we also observed that Leu destabilizes the Ssy5 prodomain, this degradation was less pronounced when using Phe as an amino acid inducer (Fig. 6*A*). This was also confirmed in an experiment in which neosynthesis of Ssy5 was arrested after the addition of the amino acid to the cells (Fig. 6*B*). Second, Phe was nevertheless as efficient as Leu in promoting processing of Stp1 and Stp2 transcription factors (Fig. 6*C*). Furthermore, this processing is complete 5 min after the amino acid addition (*i.e.* Ssy5 activation is a fast process). Within this time range, the level of the Ssy5 prodomain observed after the Phe addition is not significantly reduced (Fig. 6, *A* and *B*), and this was confirmed by quantification of the immunoblot signals. Third, we reexamined the role of the proteasome in short term (5 min) processing of Stp factors. We first analyzed Stp1 and Stp2 processing in a strain carrying a thermosensitive mutation in *RPT6* (*cim-3*), which encodes one of the ATPases of the proteasome 19 S regulatory particle (20). We used this mutant because the prodomain of Ssy5 was found to be stabilized in this strain transferred at the nonpermissive temperature of 37 °C (17). We found that Stp1 and Stp2 are similarly processed by Leu in *cim-3* cells growing at 24 °C or transferred for 30 min to 37 °C

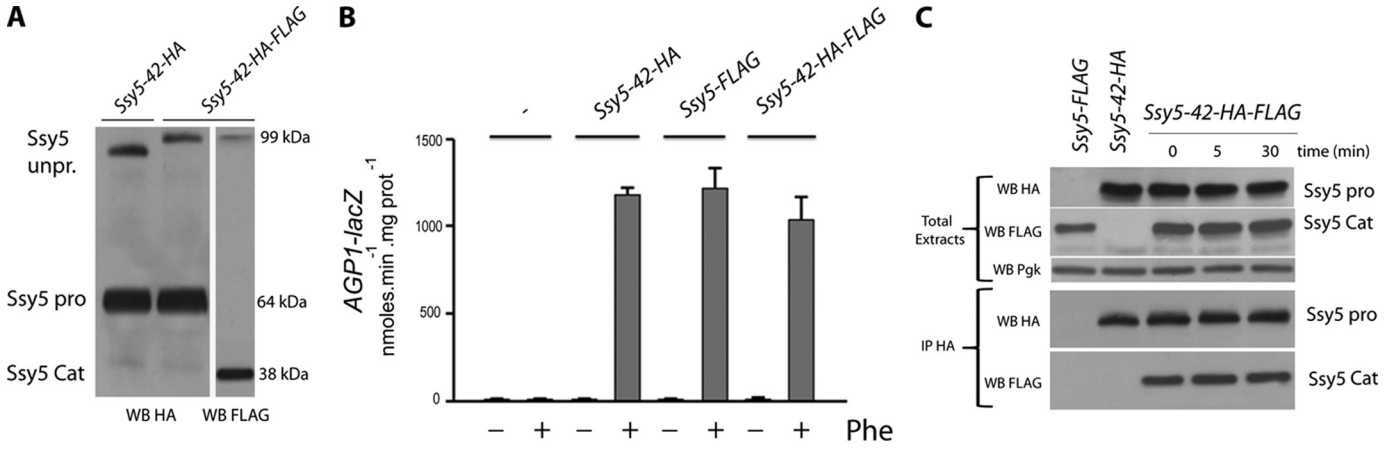

FIGURE 7. **The Ssy5 Cat domain remains associated with the prodomain after amino acid detection.** *A*, processing of the double-tagged Ssy5-HA-42-FLAG form. Strains JA716 (*Ssy5-42-HA*) and FA150 (*Ssy5-42-HA-FLAG*) were grown in minimal proline medium. Crude cell extracts were immunoblotted with anti-HA and anti-FLAG antibodies. *B*, the Ssy5-FLAG and Ssy5-42-HA-FLAG forms are functional. Strains FB90 (*ssy5*Δ), 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.

(Fig. 6*D*). The same experiment was applied to a strain carrying thermosensitive mutations in the *PRE1* and *PRE2* genes encoding catalytic subunits of the proteasome, and the result was similar (Fig. 6*D*). We finally tested the influence of MG132, a proteasome inhibitor. Because this toxic compound can be extruded from cells via the Pdr5 multidrug resistance pump (21), the experiment was carried out in a *pdr5*Δ mutant. Cells were incubated for 2 h in the presence of MG132 before the addition of Leu (Fig. 6*D*). The results showed that both Stp factors are similarly processed whether or not cells were incubated with MG132. These observations confirm that Ssy5 is normally activated in response to amino acid detection when the proteasome is inhibited. This suggests that the amino acid-induced proteasomal degradation of the prodomain is not the main mechanism of Ssy5 activation. This activation is more likely due to phosphorylation-induced ubiquitylation of the inhibitory prodomain.

The Ssy5 Cat Domain Remains Associated with the Prodomain after Amino Acid Detection—We considered that phosphorylation-induced ubiquitylation of the Ssy5 prodomain results in its dissociation from the Cat domain. The Cat domain would thus be active, and the released ubiquitylated prodomain would then be degraded by the proteasome. To test this model, we first isolated an Ssy5-42-HA form additionally tagged at its extreme C terminus with the FLAG epitope. We found that this double-tagged Ssy5 form is normally processed (Fig. 7*A*) and functional (Fig. 7*B*). We next immunoprecipitated the prodomain and tested whether the Cat domain is also recovered. In keeping with previous reports (3, 4), the Cat domain was immunoprecipitated with the prodomain in cells growing in the absence of any inducer amino acid (Fig. 7*C*, *time 0*). Interestingly, similar levels of the Cat domain were recovered 5 or 30 min after the addition of Phe (Fig. 7*C*). Hence, amino acid-induced Ssy5 activation does not seem to correlate with marked dissociation of its prodomain and Cat domain.

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^{Grr1} 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^{Grr1} complex is obviously the key step in the Ssy5 activation mechanism because the protease remains totally inactive in the *grr1*Δ 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.

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 *grr1*Δ 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, nondegradative 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^{63}-linked poly-Ub chains to the drICE effector caspase (a homolog of capsase-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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