The transcriptional response to environmental changes has to be prompt but appropriate. Previously, it has been shown that the Gis1 transcription factor is responsible for regulating the expression of postdiauxic shift genes in response to nutrient starvation, and this transcription regulation is dependent upon the Rim15 kinase. Here we demonstrate that the activity of Gis1 is negatively modulated by proteasome-mediated limited proteolysis. Limited degradation of Gis1 by the proteasome leads to the production of smaller variants, which have weaker transcription activities than the full-length protein. The coiled-coil domain, absent from the smaller variants, is part of the second transcription activation domain in Gis1 and is essential for both the limited proteolysis of Gis1 and its full activity. Endogenous Gis1 and its variants, regardless of their transcription capabilities, activate transcription in a Rim15-dependent manner. However, when the full-length Gis1 accumulates in cells due to overexpression or inhibition of the proteasome function, transcription activation by Gis1 is no longer solely controlled by Rim15. Together, these data strongly indicate that the function of the limited degradation is to ensure that Gis1-dependent transcription is strictly regulated by the Rim15 kinase. Furthermore, we have revealed that the kinase activity of Rim15 is essential for this regulation.

Cell growth is modulated in response to the availability of nutrients and growth factors. Yeast cells, when starved for any of the core macronutrients (including carbon, nitrogen, phosphorus, and sulfur) cease growth, arrest cell cycle progression prior to “start,” and enter a poorly defined G0 state. In order to survive an extended period of starvation, during the transition to the stationary phase, cells also acquire a set of characteristics typical of the stationary phase. These include accumulation of storage carbohydrates (glycogen and trehalose), increased resistance to a variety of environmental stresses, and thickening of the cell wall (1, 2). However, when cells are subject to starvation for non-core nutrients, such as auxotrophic supplements, they fail to arrest uniformly as unbudded cells and lose their viability rapidly (3). Therefore, entry into the quiescent G0 state requires the cells to sense the availability of core nutrients and coordinate the cellular activities with their depletion rather than simply the cessation of growth (4).

The conserved signaling pathways, Ras/cAMP and TOR, which sense the presence of nutrients and regulate growth (5–8), have also been implicated in the regulation of entry into stationary phase in response to nutrient deprivation, in survival at the quiescent state, and in exit from the stationary phase (2, 9, 10). Both protein kinase A and TOR have been shown to regulate entry into the G0 phase of the cell cycle, converging on common targets, the Rim15 kinase and the downstream effectors. Consistent with this, the rim1Δ mutants exhibit many of the stationary phase defects, including diminished accumulation of trehalose and glycogen, decreased thermotolerance, reduced expression of stress response genes, and failure to arrest prior to S phase (11). As a member of the PAS family, the kinase activity of Rim15 is negatively regulated by protein kinase A (11), and its nuclear localization is controlled by TOR through Sch9 (12–14) and the Pho80-Pho85 (15) signaling pathways. The effect of Rim15 kinase on quiescence derives partly from changes in transcriptional profile of the cell, mediated by the stress response transcription factors Msn2/Msn4 and the postdiauxic shift transcription factor Gis1 (16). The transcription activity of Msn2/4 is controlled by protein kinase A and TOR through regulating its cellular localization (17, 18, 19, 20). Similarly, the expression of Gis1-dependent genes is also controlled by the protein kinase A, Sch9, and TOR signaling pathways (12, 21, 22). However, the means whereby the activity of Gis1 is controlled remains to be elucidated.

Previously, we discovered a group of genes whose transcription was elevated significantly upon starvation for any of the core macronutrients, including carbon, nitrogen, phosphorus, and sulfur (23). We termed these UES genes (for universally expressed at starvation). Bioinformatic and transcript analysis confirmed that the expression of many of these genes is dependent on the Gis1 transcription factor (24). Phenotypic and transcriptomic analyses demonstrated that Rim15 and Gis1 are indispensable at both diauxic and postdiauxic shift phases, suggesting that they are responsible for regulating the transition from exponential growth to quiescence (24). Gis1 has been shown to function as a transcription repressor (25, 26) as well as a transcription activator (21). Although the repression function of Gis1 may be independent of the control by Rim15 (24), the transcription activation by Gis1 is strictly dependent on Rim15 (16, 21, 22, 24). Here, we have demonstrated that Gis1 is subject to proteasome-mediated selective proteolysis to decrease the level of the full-length Gis1 and produce less active smaller
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fragments. The coiled-coil domain in Gis1 is identified as the essential element for both the limited proteolysis and its full activity. The function of the limited degradation is to ensure that Gis1-dependent transcription is strictly regulated by the Rim15 kinase. Failure of the degradation, as a result of GIS1 overexpression or inactivation of the proteasome function, leads to accumulation of the full-length Gis1 and transcription activation of Gis1 target genes in the rim15 mutants. These data indicate that there are Rim15-independent factors that promote the activity of the full-length Gis1. Furthermore, we have revealed that the kinase activity of Rim15 is essential for its function in regulating the transcription activity of Gis1. Together, these findings provide novel insights into the mechanisms by which the Gis1-specific transcription is modulated in response to nutrient depletion.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Yeast deletion strains were based on either BY4743 or BY4742 and generated by the Saccharomyces Genome Deletion Project (27, 28). Deletion of PDR5 in both BY4742 GIS1::kanMX4 and BY4742 rim15Δ::kanMX4 and deletion of RIM15 in BY4742 GIS1::kanMX4 was achieved using His3MX6 marker as described previously (29). Similarly, chromosomal tagging of Gis1 with Myc13 at its carboxyl terminus was carried out in the heterozygous strain BY4743 RIM15/ rim15. Sporulation of the resulting diploid gave haploid RIM15GIS1, RIM15GIS1Myc, and rim15ΔGIS1Myc strains following tetrad dissection. The chosen tetrads have the remainder of their genotype identical to that of BY4742. L40 (MATa his3D200 trp1-901 leu2-3112 ade2 lys2::ApxAop-HIS3 LRA3::ApxAop-lacZ GAL GAL) was used for the one-hybrid assays. Deletion of GIS1 or RIM15 in L40 was performed as described using the KanMX4 marker (30).

All of the plasmids used in the study are listed in supplemental Table 1. Briefly, the expression vector, pGIS1tPGK2, was constructed by replacing the MET3 promoter with that of GIS1 (~800 bp of upstream sequences) in a centromeric vector, pMETtPGK2 (31). Full-length, N-terminally truncated, and internally deleted GIS1 open reading frames (ORFs) were cloned between the BamHI and NotI restriction sites in pGIS1tPGK2. To examine the effects of different deletions on the fate of the proteins so produced, C-terminal tagging with Myc13 was achieved in each of the constructs. Internal ATG codons were mutated to TTG by site-directed mutagenesis using the full-length version as the template and a kit from Stratagene. For overexpression studies, the full-length and truncated GIS1 constructs were also cloned between the BamHI and NotI sites into pCM190 under the control of the tetO7 promoter (32). The full-length Gis1 protein from overexpression studies was examined by cloning the TAP-tagged GIS1/ORF construct into pCM190 under the control of the ADH1 promoter in plexPd vector (33).

A stock solution (1 mg/ml) of rapamycin (Sigma) was made up in 90% (v/v) ethanol and 10% (v/v) Tween 20. 50 μM MG132 (Sigma) was prepared in DMSO. 50 μg/ml doxycycline (Sigma) was made up in 50% (v/v) ethanol. Unless otherwise stated, working concentrations were 200 ng/ml for rapamycin, 50 μM for MG132, and 20 μg/ml for doxycycline.

Microarray and Transcript Analysis—CML376 cells (36) bearing the tetO7-GIS1 construct in pCM190 were grown under repressive conditions (Dox+) to early/mid-exponential phase (A600 ~ 0.4). Cells were harvested, washed twice in sterile water, and resuspended into an equal volume of medium with (Dox+) or without (Dox−) doxycycline and grown for an additional 6 h. Samples were taken at 0, 3, and 6 h to determine the effect of overexpression of GIS1 on whole genome transcription profiles. Total RNA was isolated from cultures as described previously (23). Individual transcripts were assayed by Northern analysis (37). Transcription regulation of target genes was normalized against the level of ACT1. The level at time 0 (drug treatment or induction) was set at the arbitrary unit 1. Microarray experiments were carried out using the yeast genome S98 oligonucleotide arrays (Affymetrix, Inc.) according to the manufacturer’s instructions. Normalization and statistical analyses were carried out using Partek Genomics software (available on the World Wide Web). Microarray data were generated using two independent samples from cells grown under the conditions described above. The correlation coefficients between the duplicated microarray experiments were between 0.983 and 0.994.

Immunoblot Analysis—Cells from a 50–100 ml culture were collected by centrifugation, washed once in ice-cold extraction buffer (20 mM HEPES, pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, Roche Applied Science protease inhibitor mixture, and 20 μM MG132), and resuspended in 0.3 ml of extraction buffer. Cells were then broken with glass beads in a capped microcentrifuge tube in a FastPrep-24 device (MP Biomedicals). The concentration of protein was determined using the Bradford assay (Bio-Rad). Anti-Myc monoclonal antibody (α-Myc) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-tubulin TAT1 antibody (α-tub) was supplied by Cancer Research UK.

Growth Arrest Assay Using GIS1 Overexpression Constructs—Overexpression of GIS1 or its deletion constructs was achieved by transforming these constructs into yeast cells. Transformants were grown under repression conditions in SMM (36) medium containing doxycycline. Cells were resuspended in water and spotted in serial dilutions on SMM plates containing 20 μg/ml doxycycline or no doxycycline to determine the toxicity of overexpression of each of the constructs.

One-hybrid Assay—To determine the transcriptional activation capabilities of different Gis1 domains, truncated GIS1 constructs fused with LexA DNA binding domain in plexPd were transformed into cells of L40 yeast strain, which has two independent reporters, lacZ and HIS3. The level of HIS3 expression

2 The abbreviations used are: ORF, open reading frame; FL, full-length.
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was determined by growth assay in serial dilutions on SMM medium supplemented with or without histidine. β-Galactosidase activity was measured and normalized as described previously (39).

RESULTS

Gis1 Is Subject to Selective Proteolysis by the Proteasome—Activation of Gis1-dependent transcription in cells starved for glucose or treated with rapamycin is abolished in rim15 deletion mutants (21, 22, 24). However, overexpression of GIS1 activates the expression of a number of starvation-specific genes in nutrient-sufficient conditions (21), suggesting that the level of Gis1 protein in vivo is strictly controlled. We tagged the chromosomal GIS1 ORF (expressed from its native promoter) with a Myc tag at the C terminus and examined the level of Gis1 protein in exponentially growing cells and cells treated with rapamycin. To our surprise, at least six size variants of the Gis1 protein were seen in actively growing cells (Fig. 1A). Four variants were much smaller than the expected size (~120 kDa), based on the length of the GIS1 ORF (894 amino acids, ~100 kDa) plus the Myc13 tag (~20 kDa). The level of three of these small gene products (v3, v4, and v5) increased upon rapamycin treatment. In rim15Δ cells, the pattern of Gis1 variants remained the same, and the drug triggered a similar increase of v3, v4, and v5 (Fig. 1A). In contrast, the levels of the two largest forms, the full-length (FL) protein and v1 (~90 kDa), decreased following rapamycin treatment. Similar results were observed in exponential cells starved for glucose (Fig. 1B), and deletion of RIM15 had no effect on the levels of the different variants (data not shown).

There are a number of ways in which the short variants of Gis1 might be generated. We first mutated all eight ATG codons in the GIS1 ORF, which could possibly lead to production of the small variants upon internal transcription or translation initiation. All eight alleles gave the same pattern as those in the wild type (data not shown), suggesting that the smaller Gis1 fragments are not the products of transcriptional or translational reinitiation. We then investigated the possibility that Gis1 is subjected to limited proteolysis by deleting the internal region(s) where hydrolysis must occur to give fragments of the observed length. Initially, three regions (corresponding to amino acids 592–623, 655–689, and 592–689; shown in Fig. 1C) were deleted from the GIS1 ORF, which could possibly lead to production of the small gene products (v3, v4, and v5). Deletion of amino acids 592–623, 655–689, and 592–689; shown in Fig. 1C) were deleted from the GIS1 ORF. These constructs, together with the full-length GIS1, were tagged with Myc13 and expressed under the control of the endogenous promoter to assess their role in the processing of the full-length protein. Deletion of the region 592–623 had a negative impact on the levels of FL and all variants, especially v1 (Fig. 1C). Deletion of amino acids 655–689 changed the pattern completely, with v3 disappearing and a new fragment appearing (indicated by the arrow in Fig. 1C), suggesting that this region contains a bona fide cleavage site. This new fragment was absent when the whole region (amino acids 592–689) was removed. These data indicate that the different shorter variants of Gis1 detected in actively growing cells or cells treated with rapamycin are the products of limited proteolysis.

Examples of proteasome-mediated limited proteolysis have been reported before (40). To find out if the processing of Gis1 is carried out by the proteasome, we transformed the above Myc13-tagged GIS1 plasmid into the pdr5Δ deletion
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Deletion of PDR5 renders cells sensitive to proteasome inhibitors, such as MG132 (41). Exponential phase cells were treated with rapamycin, MG132, or the two agents in combination, and the levels of Gis1 protein and its variants were analyzed by Western analysis. As shown in Fig. 1, when cells are treated with only MG132, the level of FL Gis1 increases significantly, and the truncated variants v1, v2, v3, and v4 are present at a very low level or are undetectable. This indicates that the smaller fragments are, indeed, the products of proteasome action. Moreover, inhibition of proteasome function with MG132 led to the disappearance of the smaller fragments within 30 min (compare time 0 samples treated with or without MG132), indicating that they are turned over quickly in vivo. The pattern remained the same even when rapamycin was added to the culture after MG132, suggesting that the processing of Gis1 in cells treated with rapamycin or starved for glucose (Fig. 1, A and B) is also carried out by the proteasome. v5 and a new fragment (indicated in Fig. 1D by an arrow), which is present at very low levels in exponentially growing cells, accumulated in cells treated with the proteasome inhibitor. This suggests that Gis1 may be subjected to proteasome-independent hydrolysis, enhanced when proteasome function is compromised.

Selective Degradation of Gis1 Leads to Down-regulation of Its Transcription Activation Ability—To find out the functional consequences of the selective degradation in vivo, an N-terminally truncated GIS1 clone was then constructed with the first 592 amino acids removed. The construct and its Myc13-tagged version were expressed under the control of the GIS1 promoter in gis1Δ/H9004 cells. As shown in Fig. 2, when cells are treated with only MG132, the level of FL Gis1 increases significantly, and the truncated variants v1, v2, v3, and v4 are present at a very low level or are undetectable. This indicates that the smaller fragments are, indeed, the products of proteasome action. Moreover, inhibition of proteasome function with MG132 led to the disappearance of the smaller fragments within 30 min (compare time 0 samples treated with or without MG132), indicating that they are turned over quickly in vivo (see legend to Fig. 1). The pattern remained the same even when rapamycin was added to the culture after MG132, suggesting that the processing of Gis1 in cells treated with rapamycin or starved for glucose (Fig. 1, A and B) is also carried out by the proteasome. v5 and a new fragment (indicated in Fig. 1D by an arrow), which is present at very low levels in exponentially growing cells, accumulated in cells treated with the proteasome inhibitor. This suggests that Gis1 may be subjected to proteasome-independent hydrolysis, enhanced when proteasome function is compromised.

FIGURE 2. Limited proteolysis of Gis1 down-regulates its transcriptional activation ability. A, Western analysis of Gis1 protein products encoded by the N592Δ construct. The GIS1 ORF and the N592Δ construct tagged with Myc13 were expressed from the endogenous promoter in pGIS1PGK2. gis1Δ cells bearing either construct were grown to midexponential phase and treated with rapamycin (Rap+) for 0, 1, and 3 h. B, Northern analysis of SSA3 and GRE1 transcripts in gis1Δ cells expressing the full-length GIS1 or N592Δ from its own promoter in pGIS1PGK2. Exponentially growing cells were treated with rapamycin (Rap+) for 0, 1, and 3 h. C, quantification and normalization of Northern analysis in B. Transcript levels of SSA3 and GRE1 were quantified and normalized against the level of ACT1. -Fold regulation at time 0 was set at 1. D, growth assay of cells overexpressing GIS1 or N592Δ under the control of the tetO7 promoter in PCM190. Dox+, 20 μg/ml doxycycline; Dox−, no doxycycline. E, Northern analysis of SSA3 and GRE1 transcripts in pdr5Δ deletion cells treated with rapamycin (Rap+) and/or MG132 for 0, 1, and 3 h. F, quantification and normalization of Northern analysis in E, following the procedures described in C.
toxic to cell growth than overexpression of the full-length protein, confirming that the N-terminal sequence is indeed crucial for the full activity of Gis1.

To further determine the functional consequences of the limited proteolysis of Gis1, the pdr5Δ cells were treated with MG132 and rapamycin, and expression of SSA3 and GRE1 was analyzed by Northern analysis. Exponential cells were treated with rapamycin (Rap+) for 0, 1, and 3 h. C, quantification and normalization of Northern analysis in B. D, Western analysis of Gis1 variants encoded by the Myc13-tagged N330Δ and N400Δ constructs in gis1Δ cells treated with rapamycin (Rap+) for 0, 1, and 3 h. E, Western analysis of Gis1 variants encoded by the Myc13-tagged N330Δ and N400Δ constructs in gis1ΔΔpdr5Δ cells treated with MG132 for 0, 1, and 3 h. F, Northern analysis of SSA3 and GRE1 transcripts activated by N330Δ and N400Δ constructs in the gis1ΔΔpdr5Δ cells treated with both rapamycin (Rap) and MG132 for 0, 1, and 3 h. Both drugs were added at time 0. G, quantification and normalization of Northern analysis in F. The transcript level of SSA3 and GRE1 was quantified and normalized against the level of ACT1 and the level of full-length protein displayed in E. H, growth assay of cells overexpressing the N- and C-terminal deletion constructs. These constructs were expressed in pCM190 under the control of the tetO7 promoter. Dox+, 20 μg/ml doxycycline; Dox−, no doxycycline.

The Coiled-coil Domain Is Required for Both the Limited Proteolysis of Gis1 and Its Full Activation Ability—The above results suggest that the N-terminal part of Gis1 is critical for its activity. Analysis of the Gis1 protein (SMART; available on the World Wide Web) revealed three known functional domains, including jumonji and coiled-coil domains at its N terminus and a DNA-binding domain (two zinc fingers) at its C terminus.

FIGURE 3. The coiled-coil domain is essential for both limited proteolysis of Gis1 by the proteasome and its full activity. A, strategy for N-terminal and C-terminal deletions; B, Northern analysis of SSA3 and GRE1 transcripts in gis1Δ cells transformed by GISH, N330Δ, N400Δ, and C816Δ constructs expressed from the GISH promoter in the single copy plasmid pGIS1PGK2. Exponential cells were treated with rapamycin (Rap+) for 0, 1, and 3 h. C, quantification and normalization of Northern analysis in B. D, Western analysis of Gis1 variants encoded by the Myc13-tagged GISH, N330Δ, and N400Δ constructs in gis1Δ cells treated with rapamycin (Rap+) for 0, 1, and 3 h. E, Western analysis of Gis1 variants encoded by the Myc13-tagged N330Δ and N400Δ constructs in gis1ΔΔpdr5Δ cells treated with MG132 for 0, 1, and 3 h. F, Northern analysis of SSA3 and GRE1 transcripts activated by N330Δ and N400Δ constructs in the gis1ΔΔpdr5Δ cells treated with both rapamycin (Rap) and MG132 for 0, 1, and 3 h. Both drugs were added at time 0. G, quantification and normalization of Northern analysis in F. The transcript level of SSA3 and GRE1 was quantified and normalized against the level of ACT1 and the level of full-length protein displayed in E. H, growth assay of cells overexpressing the N- and C-terminal deletion constructs. These constructs were expressed in pCM190 under the control of the tetO7 promoter. Dox+, 20 μg/ml doxycycline; Dox−, no doxycycline.
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The coiled-coil domain is part of the second transcription activation domain in Gis1. A, positions of primers used for cloning different GIS1 constructs into the plexPd plasmid bearing the LexA DNA-binding domain. The number in parentheses denotes the amino acid position at which the 5'-end of each primer located. jnmN, jumoni N domain; jnmC, jumoni C domain; C/C, coiled-coil domain; ZinF, zinc fingers; TAD1, transcription activation domain 1; TAD2, transcription activation domain 2. B, growth assay of L40 cells expressing the above constructs. Exponential cells bearing the constructs were spotted in serial dilutions on plates that were incubated at 30 °C for 3–5 days. HIS+, SMM medium supplemented with histidine; HIS−, SMM medium without histidine. C, the normalized β-galactosidase activities activated by the above GIS1 constructs in L40 cells. Data were obtained from quadruplicates (two biological and two technical replicates). Mean and S.D. values from these measurements were plotted.

expressed (C816Δ; Fig. 3H). Deletion of the jumoni domain from GIS1 (N330Δ; Fig. 3A) did not change the pattern of Gis1 variants (Fig. 3D), as compared with those produced from the full-length protein. This suggests that deletion of the jumoni domain does not affect the proteolytic processing of Gis1 by the proteasome. In contrast, the transcript levels of the target genes (SSA3 and GRE1) activated by the N330Δ construct were increased by 2–4-fold in cells treated with rapamycin (Fig. 3, B and C). This indicates that the presence of the jumoni domain has a negative effect on the ability of Gis1 as a transcriptional activator. However, further deletion of the coiled-coil domain in addition to the jumoni domain (N400Δ; Fig. 3A) led to the disappearance of all forms of Gis1 (Fig. 3D) and hence abolishes its ability to activate the expression of Gis1-dependent genes (Fig. 3, B and C). This suggests that the coiled-coil domain is essential for the stability of Gis1.

To decide whether the domain is required for the integrity of Gis1 protein before its proteasome-mediated selective proteolysis or for the selective proteolysis itself, the two Myc13-tagged constructs of N330Δ and N400Δ were expressed in the pdr5 deletion cells. As shown in Fig. 3E, the level of Gis1 lacking the jumoni domain (N330Δ) increased after the proteasome inhibitor was added, similar to that seen with the FL Gis1 (Fig. 1D). However, without the coiled-coil domain (N400Δ), the abundance of Gis1 protein increased initially but decreased with prolonged treatment with MG132, indicating that the coiled-coil domain is critical for the proteasome-mediated limited proteolysis. Without it, Gis1 would either be completely degraded by the proteasome or subject to proteasome-independent degradation when proteasome function is compromised. The addition of both rapamycin and MG132 to cells did not change the pattern of Gis1 protein encoded by either construct (data not shown). The protein encoded by the N400Δ construct (shown by an arrow in Fig. 3, D and E) is slightly larger in size than v1, indicating that v1 does not contain the coiled-coil domain.

To determine whether the coiled-coil domain is also required for the full activity of Gis1, the transcript levels of target genes, SSA3 and GRE1, were determined and normalized against the abundance of Gis1 protein in pdr5Δgis1Δ cells treated with both MG132 and rapamycin. As shown in Fig. 3, F and G, the normalized transcript levels for both SSA3 and GRE1 decreased significantly when the coiled-coil domain was absent, especially at the early stages of the treatment (1 h). Overexpression of the Gis1 construct without the jumoni domain confers slightly more severe toxicity to cells (N330Δ; Fig. 3H), whereas further removal of the coiled-coil domain (N400Δ) significantly attenuates its ability to arrest cell growth, further confirming that the coiled-coil domain is not only essential for selective degradation of Gis1 by the proteasome but also critical for its full transcription activity.

The Coiled-coil Domain Is Essential for the Activity of the Second Transcription Activation Domain in Gis1—To discover why the coiled-coil domain is required for full activity of Gis1, we conducted one-hybrid assays by fusing different truncated GIS1 constructs (lacking the two zinc fingers at the C terminus) with a LexA DNA-binding domain. These constructs were expressed under the control of the ADH1 promoter in plexPd (see “Experimental Procedures”). The positions of the different primers used to amplify the constructs are shown in Fig. 4A. These constructs were transformed into L40 cells, and the expression of the two independent reporters, HIS3 and lacZ, was monitored. As shown in Fig. 4, B and C, cells bearing the LexA-Gis1 construct (FOR1) grow well on medium lacking histidine (HIS−) and express β-galactosidase activity. Removing the C-terminal sequences adjacent to the zinc fingers (clone FOR2) severely decreases cell growth on HIS− medium and completely abolishes the β-galactosidase activity, indicating that the region upstream of the two zinc fingers is indispensable for the transcription activity of FL Gis1. We name this region TAD1, for transcription activation domain 1. Similarly, expression of either the jumoni and coiled-coil domains together (clone FOR4) or only the jumoni domain (clone FOR5) failed to support cell growth on HIS− medium or to induce the expres-
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A) 

B)

C)

D)

FIGURE 5. Endogenous Gis1 variants rely on Rim15 to activate transcription. A, β-galactosidase activity in wild-type (L40) and rim15 deletion cells activated by expression of TAD2 (F1R3), TAD1 (F3R1), and both TAD1 and TAD2 (F1R1) in plexPd. Midexponential phase cells were treated with either rapamycin (Rap+) or its solvent (Rap−) for 2 h. B, growth assay of the wild-type (L40) and rim15Δ cells expressing TAD1 (F3R1); HIS+, SMM medium supplemented with histidine; HIS−, SMM medium without histidine. C, Northern analysis and quantification of the SSA3 and GRE1 transcripts activated by N330Δ in gis1Δ rim15Δ mutant cells bearing the empty vector or the Rim15 plasmid. Exponential cells were treated with rapamycin (Rap+) for 0, 1, and 3 h. Quantification and normalization was performed following the procedures described in the legend to Fig. 2C. Normalized -fold regulation for SSA3 and GRE1 is displayed. D, Northern analysis and quantification of the SSA3 transcript activated by N592Δ in gis1Δ rim15Δ mutant cells bearing the empty vector or the Rim15 plasmid. The transcript level of GRE1 was not analyzed due to its low expression activated by the construct (see Fig. 2, B and C).

sion of lacZ. However, expressing the TAD1 domain alone (clone F3R1) restores cell growth on HIS− medium and expression of the β-galactosidase activity, confirming that TAD1 is indeed a transcription activation domain.

To confirm that the coiled-coil domain is critical for full transcription activity of Gis1, we then assayed the transcription activation enabled by the N- and C-terminal deletion constructs. With the jumoni domain removed (clone F1R1), cells grow well on HIS− medium (Fig. 4B) and express much higher β-galactosidase activity (~10-fold) than the FL Gis1 (Fig. 4C). Although further deletion of the coiled-coil domain (clone F2R1) did not affect cell growth on medium lacking histidine (Fig. 4B), the expression of β-galactosidase decreased significantly (~4-fold; Fig. 4C), as compared with clone F1R1. This confirmed the previous observations that the jumoni domain negatively impacts transcriptional activation by Gis1 but the coiled-coil domain is required for its full activity. Similarly, deletion of the TAD1 domain from clone F1R1 had no effect on growth of cells on medium without histidine (F1R2; Fig. 4B) but the expression of β-galactosidase was reduced by more than 8-fold (F1R2; Fig. 4C). Further deletion of the coiled-coil domain from F1R2 completely abolished its ability to support cell growth on HIS− medium (F2R2; Fig. 4B) and the expression of lacZ (F2R2; Fig. 4C), confirming that the coiled-coil domain is critical for full activity of the Gis1 transcription factor.

To find out why the coiled-coil domain is necessary for Gis1 to function as a transcription activator, we analyzed its ability to activate gene transcription. With only the coiled-coil domain and the downstream 65 amino acids fused with LexA, the TAD2 to activate the expression of either HIS3 or lacZ (data not shown), supporting the view that the TAD2 domain may function as a transcription activator on its own.

Transcription Activation by Gis1 Constructs Expressed from the Native Promoter Is Strictly Regulated by Rim15—The above one-hybrid assays revealed that there are two transcription activation domains (TAD2 and TAD1) in FL Gis1 and that its selective degradation generates smaller variants bearing only one of them (TAD1), leading to their weaker transcription capabilities. To determine if the transcription activities enabled by these domains are still regulated by the Rim15 kinase, we deleted RIM15 from the host strain (L40) and conducted the one-hybrid assays in the rim15 deletion cells. As shown in Fig. 5A, expression of the β-galactosidase activity from TAD2 (F1R3) is only marginally decreased in the rim15Δ mutants as compared with that in the wild-type cells growing either exponentially or treated with rapamycin, suggesting that Rim15 is not necessary for transcriptional activation by TAD2. However, expression of lacZ activated by the TAD1 domain (F3R1) is severely compromised in the rim15Δ mutants (Fig. 5A), and the rim15Δ cells grow more slowly than their wild-type counterparts in medium without histidine (Fig. 5B), indicating that the Rim15 kinase is essential for the transcriptional activity by TAD1 domain. The addition of rapamycin triggered a 4-fold increase of the galactosidase activity in wild-type cells, and a similar increase was seen in the rim15 deletion cells, indicating that RIM15 is required for the activity of TAD1 but not for its regulation. The construct F1R1 containing both the TAD1 and TAD2 domains was also transformed into the rim15 mutant cells, and its ability to activate the expression of lacZ was deter-
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FIGURE 6. Overexpression of GIS1 induces the expression of starvation-specific genes in both the wild-type and rim15 deletion cells. A, Gis1 protein produced from GIS1 overexpression. Samples were taken at 0 and 6 h after the tetO7 promoter was switched on (Dox−). Anti-tubulin antibody (α-tub) detects both the TAP-tagged Gis1 and tubulin due to presence of protein A in the TAP tag. B, hierarchical clustering of genes regulated by GIS1 overexpression. Cells bearing the plasmid, pCM190-GIS1, were grown in repression conditions (Dox+) to early exponential phase. They were harvested and then washed to remove doxycycline. Half of the cells were resuspended in medium with doxycycline (Dox+), and the other half were resuspended in the same medium without doxycycline (Dox−). Samples were taken 0, 3, and 6 h after resuspension. C, comparison between the set of genes up-regulated in cells treated with rapamycin (S3) and those up-regulated by GIS1 overexpression. D, growth arrest induced by GIS1 overexpression in the wild-type and rim15 deletion cells. Dox+, 20 μg/ml doxycycline; Dox−, no doxycycline. E, Northern analysis and normalization of SSA3 and GRE1 transcripts induced in the wild-type and rim15 deletion cells overexpressing GIS1 for 0, 3, and 6 h.

mined. As shown in Fig. 5A, deletion of RIM15 leads to a small reduction (by 20%) of the galactosidase activity in rim15 mutants as compared with that in wild-type cells. In contrast, rapamycin treatment has little effect on its activity in either the wild-type or rim15Δ mutant cells. These data indicate that only the transcription activity of TAD1 is tightly regulated by the Rim15 kinase.

To find whether the stringency of the regulation by Rim15 is linked to the transcription capabilities of different GIS1 constructs or to their expression levels, transcription activation by endogenous GIS1 constructs (expressed from the native promoter) was then determined in gis1Δrim15Δ double mutant cells, bearing either the RIM15 plasmid or the empty vector. N330Δ (its activation domain corresponding to that in F1R1) has a much higher transcription activity than the full-length GIS1 (Fig. 3, B and C). In contrast, N592Δ (its activation domain corresponding to that in F3R1) has a much lower transcription activity than full-length GIS1 (Fig. 2, B and C). The transcript levels of GRE1 and/or SSA3 in exponential cells treated with rapamycin were determined by Northern analysis, quantified, and normalized. As shown in Fig. 5C, transcription of either SSA3 or GRE1 activated by N330Δ was seen in cells carrying the RIM15 plasmid but completely abolished in cells bearing the empty vector. Similarly, transcription activation of SSA3 by N592Δ was observed only in cells harboring the RIM15 plasmid (Fig. 5D). The full-length GIS1, expressed from its own promoter, was previously shown to activate the transcription of postdiauxic shift genes, strictly dependent on the presence of Rim15 (21, 22, 24). Together, these data indicated that transcription activation by endogenous GIS1 constructs, regardless of their transcription capabilities, is strictly regulated by Rim15. Therefore, the less stringent control exerted by Rim15 on the transcription activity of F1R1 (Fig. 5A) may be due to the fact that the construct was expressed from the strong ADH1 promoter in the one-hybrid system.

Overexpression of GIS1 Induces Accumulation of the Full-length Protein and Expression of Starvation-specific Genes in both Wild-type and rim15Δ Cells—To confirm that the transcription activity of Gis1, when overproduced, is no longer solely regulated by Rim15, we overexpressed GIS1 and compared transcription activation of Gis1 target genes in both wild-type and rim15 mutant cells. Cells bearing the tetO7-GIS1 plasmid or its TAP-tagged version were grown to early exponential phase in the presence of doxycycline, washed, and then resuspended in medium lacking doxycycline to allow Gis1 overproduction. As shown in Fig. 6A, the Gis1 protein was induced to a level similar to that of tubulin in wild-type cells after the tetO7 promoter was switched on for 6 h. Compared with the FL Gis1, very little shorter variants were observed (Fig. 6A), suggesting that overproduction of Gis1 would severely compromise its degradation by the proteasome. Similar results were observed in rim15 mutant cells overexpressing TAP-tagged GIS1 (data not shown). The transcriptome profiles in wild-type cells with or without GIS1 overexpression were compared over a 6-h period (see “Experimental Procedures”). Although there are very few genes whose transcription is down-regulated by GIS1 overexpression, the expression of 88 ORFs is up-regulated by more than 2-fold (p < 0.01; Fig. 6B). These include genes involved in response to nutrient starvation stress (ADH2, PDC6, GND2, TTK2, ICL1, PCK1, SSA3, SSA4, HSP26, and GRE1), in oxidoreductase activity (NDE2, TSA2, CT11, GND2, TRR2, RNR3, YJL045W, ALD3, ALD2, ADH2, FRE4, and YNR073C), and those encoding cell wall proteins (PHO5, DAN3, TIR1, SPS100, YPS6, DAN1, ZPS1, and FIT3). Analysis of the promoter sequences of these genes identified the most overrepresented motifs, dharq and waggga, which resemble the PDS element (twaggga) recognized by Gis1. Among the 88 genes, the transcription of nearly 40% of them was also shown to be up-regulated by more than 2-fold in cells treated with
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To decide if the transcription activity of the overproduced Gis1 is strictly regulated by the Rim15 kinase, we compared the toxicity and transcription activation enabled by Gis1 overexpression in wild-type and rim15 deletion cells. As shown in Fig. 6D, the toxicity to cell growth induced by Gis1 overexpression remained the same in rim15Δ mutants as that seen in their wild-type counterparts. Similarly, overexpression of Gis1 induced the transcription of SSA3 and GRE1 in rim15 mutant cells, albeit to a lesser degree than that observed in wild-type cells (Fig. 6E). These data confirmed that the transcription activated by full-length Gis1, when overproduced, is no longer solely regulated by Rim15, suggesting that Rim15-independent factors are involved in regulating the activity of Gis1.

Limited Degradation of Gis1 by the Proteasome Ensures that the Gis1-dependent Transcription Is Strictly Controlled by Rim15—To further confirm the above observation, we determined if the transcription activation by endogenous Gis1 is still strictly regulated by Rim15 when the proteasome function is inhibited. A pdr5Δrim15Δ double mutant strain was constructed. The mutant cells bearing the Myc13-tagged GIS1 plasmid (Fig. 2A) were treated with MG132, and the same pattern of Gis1 fragments was observed as that seen in the wild-type cells treated with the proteasome inhibitor (Fig. 1D). Exponential phase cells of the mutant were treated with rapamycin, MG132, or both agents. The level of the SSA3 transcript was monitored by Northern analysis. As shown in Fig. 7, A and B, transcription activation of SSA3 seen in wild-type cells (Figs. 2, E and F) was abolished in the rim15 deletion cells treated only with rapamycin. Treating the rim15 mutant cells with MG132 does not significantly induce the expression of SSA3, similar to that observed in wild-type cells treated with the proteasome inhibitor (Fig. 2, E and F). However, with both drugs added, the transcription of SSA3 was dramatically induced (Fig. 7, A and B). The degree of the induction was about half of that detected in wild-type cells treated with both drugs for 1 h, and a similar level of induction was observed after 3 h of treatment (compare Figs. 7B and 2F). These results clearly demonstrate that the transcription of Gis1 target genes in cells treated with rapamycin is no longer solely regulated by the Rim15 kinase when the proteasome function is inactivated. These data further indicate that Rim15-independent factors are involved in promoting the activity of the full-length Gis1 in response to inactivation of TOR. Put together, these data strongly support the view that the function of the proteasome-mediated degradation is to ensure that Gis1-dependent transcription is strictly regulated by Rim15.

The Gis1-dependent Transcription Relies on the Kinase Activity of Rim15—To discover whether the kinase activity of Rim15 is necessary for its function to regulate the activity of Gis1, we used the above one-hybrid system and determined the activities of two constructs (F1R1 and F3R1; Figs. 4A and 5A) in rim15 mutant cells bearing either the wild-type Rim15 or the kinase-inactive alleles (Rim15K823Y or Rim15C1176Y (15)). Both Rim15 and the kinase-inactive alleles were expressed under the control of the Rim15 promoter in a centromeric plasmid. As shown in Table 1, the introduction of the wild-type Rim15 restored the activities of both F1R1 and F3R1 to induce the expression of the lacZ gene. In contrast, neither of the kinase-inactive alleles could complement the deletion of Rim15, indicating that the kinase activity of Rim15 is essential for its function to regulate the transcription activity of Gis1.

To confirm this, we then examined the transcription activities of the two corresponding endogenous GIS1 constructs (N330Δ and N592Δ in Fig. 5, C and D) in the rim15Δgis1Δ mutant cells harboring either wild-type Rim15 or the kinase-
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N
P
C

TOR
PKA
Pho80/85

Rim15

Gis1(FL)

Proteasome

Gis1(v)

FIGURE 8. The model proposing that the activity of Gis1 is modulated by the proteasome, the Rim15 kinase (negatively regulated by various signaling pathways), and an unidentified regulator (negatively controlled by TOR). The arrows and bars denote positive and negative controls, respectively. N, ammonium; C, glucose; P, phosphate; Gis1(FL), full-length Gis1 protein; Gis1(v), smaller Gis1 variants.

inactive alleles. Exponential phase cells were treated with rapamycin to determine the transcription activation of Gis1 target genes. As seen from Fig. 7C, the transcription of both SSA3 and GRE1 is activated by N330Δ in cells bearing the wild-type Rim15 but not in cells containing either of the kinase-inactive alleles. Similarly, although the wild-type Rim15 restored the activity of N592Δ, neither of the two kinase-inactive alleles could enable the SSA3 transcription activated by N592Δ (Fig. 7D). These data give further support to the conclusion that the kinase activity of Rim15 is essential for its function to regulate the transcription activity of Gis1.

DISCUSSION

The transcriptional response to nutrient starvation and stress involves a large proportion of the genome (42). An appropriate response to environmental changes ensures proper utilization of cellular resources and, indeed, cell growth and survival. In this study, we have demonstrated that starvation-specific transcription activated by the Gis1 transcription factor is negatively controlled by the proteasome-mediated proteolysis. Limited degradation of Gis1 by the proteasome is constitutive during all the growth phases to reduce the level of the full-length protein (Fig. 1B), which has a much higher transcriptional proficiency than the smaller fragments (Figs. 2 and 3). The biological function of the degradation is to ensure that the transcription of Gis1 target genes is strictly regulated by the Rim15 kinase. Based on our work and that of others, we propose a model of how the activity of Gis1 is modulated (Fig. 8).

Transcription activation by endogenous Gis1 or its variants is strictly regulated by the Rim15 kinase, regardless of their transcription capabilities (Fig. 5C and D) (21, 22, 24). Overexpression of GIs1 induces the accumulation of the full-length protein (Fig. 6A) and the expression of starvation-specific genes (Fig. 6B) in exponentially growing cells. Activation of these genes under the same conditions is only moderately attenuated in rim15 deletion cells (Fig. 6E). Similarly, the β-galactosidase activity activated by F1R1 (expressed from the strong ADH1 promoter) was only reduced by 20% in rim15 mutant cells as compared with that in the wild-type cells (Fig. 5A). Furthermore, when the proteasome function is inactivated, the full-length Gis1 (endogenous) accumulates (Fig. 5A), and the transcription of SSA3 is highly activated in rim15 mutant cells treated with rapamycin (compare Figs. 2, E and F, and 7, A and B). Together, these data strongly indicate that the function of the selective degradation is to ensure that the transcription activated by endogenous Gis1 and its variants is strictly modulated by the Rim15 kinase. The Gis1 protein, constitutively located in the nucleus (22), is required for activation of a set of genes that are universally expressed in cells starved for different core nutrients, including glucose, ammonium, and phosphate (23, 24). Given that the kinase activity of Rim15 is negatively regulated by protein kinase A (11) and that its nuclear localization is antagonized by the TOR (12) and Pho80/Pho85 (15) signaling pathways, the control exerted by the Rim15 kinase may enable cells to respond to starvation of different macronutrients and thus different nutrient-sensing pathways (Fig. 8), including glucose (protein kinase A), ammonium (TOR), and phosphate (Pho80/Pho85).

Transcription activation of Gis1 targets was seen in rim15 mutant cells overexpressing Gis1 (Fig. 5E) and in rim15 deletion cells treated with both rapamycin and the proteasome inhibitor (Fig. 7, A and B), indicating that Rim15-independent factors (denoted by a question mark in Fig. 8) are involved in promoting the activity of the full-length Gis1. Because the SSA3 transcript level was not significantly induced in rim15Δ cells treated with rapamycin or the proteasome inhibitor (Fig. 7, A and B), the effect of this factor(s) on Gis1-dependent transcription is probably determined by both the nutrient status and the amount of full-length Gis1 protein. Therefore, the transcription activity of Gis1 in vivo is modulated by at least three factors (Fig. 8): the proteasome, the Rim15 kinase, and the yet to be identified regulator(s).

The transcription activity of Gis1 and its variants is regulated by Rim15 in cells grown exponentially (Figs. 5A and 6E), in cells treated with rapamycin (Fig. 5A, C, D, and E), and in cells starved for glucose (21). The kinase activity of Rim15 is essential for its function (Table 1 and Fig. 7, C and D), indicating that the regulation of its kinase activity and nuclear localization by nutrients and the signaling pathways (12, 14, 15, 21) may be more quantitative than qualitative. Intriguingly, Pedruzzi et al. (12) reported that the localization of Rim15 in the nucleus and the transcription of SSA3 was observed when half of the initial glucose in the medium was consumed, independent of the absolute glucose concentration. It will be interesting to decide how the signaling pathways (protein kinase A, TOR, and Pho80/Pho85) sense the kinetics of nutrient signals and how they impinge on the Rim15 kinase cooperatively in response to depletion of different core nutrients. Because Rim15 regulates the transcriptional activity of Gis1 at different nutrient conditions (Figs. 5A, C, D, and E) and within different genomic contexts.
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