JOURNAL OF BACTERIOLOGY, Apr. 2001, p. 2331–2334 Vol. 183, No. 7
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.7.2331–2334.2001

TOR Modulates *GCN4*-Dependent Expression of Genes Turned on by Nitrogen Limitation

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Received 26 October 2000/Accepted 5 January 2001

In *Saccharomyces cerevisiae*, the rapamycin-sensitive TOR signaling pathway plays an essential role in up-regulating translation initiation and cell cycle progression in response to nutrient availability. One of the mechanisms by which TOR regulates cell proliferation is by excluding the *GLN3* transcriptional activator from the nucleus and, in consequence, preventing its transcriptional activation therein. We examined the possibility that the TOR cascade could also control the transcriptional activity of Gcn4p, which is known to respond to amino acid availability. The results presented in this paper indicate that *GCN4* plays a role in the rapamycin-sensitive signaling pathway, regulating the expression of genes involved in the utilization of poor nitrogen sources, a previously unrecognized role for Gcn4p, and that the TOR pathway controls *GCN4* activity by regulating the translation of *GCN4* mRNA. This constitutes an additional TOR-dependent mechanism which modulates the action of transcriptional activators.

The yeast *Saccharomyces cerevisiae* is able to use a variety of compounds as nitrogen sources. When yeast cells are provided with poor nitrogen sources, such as proline, genes coding for the enzymes involved in the catabolism of these compounds are highly expressed. Conversely, in the presence of high-quality nitrogen sources, such as glutamine or asparagine, a decrease in the levels of catabolic enzymes and transport systems is observed. The reduced expression of the genes coding for enzymes involved in the utilization of poor nitrogen sources is brought about through the action of a regulatory system known as the nitrogen catabolite repression or nitrogen discrimination pathway (3, 6, 7, 9). It is now well established that nitrogen catabolite repression operates through the action of two transcriptional activators, Gln3p and Gat1p (also called Nil1p), each containing a GATA-binding zinc finger motif (7, 21).

Studies with the immunosuppressive drug rapamycin revealed the existence of a signal transduction cascade, conserved from the yeast *S. cerevisiae* to humans (10). Studies of the transcriptional activation profile of yeast cells treated with rapamycin showed that this drug inhibits Tor1p and Tor2p and that the Tor proteins directly modulate the nitrogen discrimination pathway (4, 11). Further experiments showed that the TOR signaling pathway prevented the transcription of genes expressed upon nitrogen limitation by promoting the association of the GATA transcription factor Gln3p with the cytoplasmic protein Ure2p, thus retaining Gln3p in the cytoplasm (2). A rapid dissociation of this complex occurs in the presence of rapamycin or when cells are transferred from a rich medium to one containing a poor nitrogen source, indicating that TOR-mediated regulation acts in response to nutrient limitation (2). The fact that in the presence of rapamycin Gln3p is readily localized in the nucleus indicates that translocation probably precedes transcription of *GLN3*-dependent genes. The above-mentioned studies indicate that one of the major rapamycin-sensitive functions of the TOR signaling pathway seems to be the sensing of the levels and/or quality of amino acids or other available nitrogen sources; nonetheless, the exact nature of the intracellular indicator(s) of nutrient availability has yet to be determined.

The general amino acid control (GCN) is elicited when yeast cells are deprived of any of 11 amino acids. At the onset of GCN, translation of the transcriptional activator *GCN4* increases, leading to increased transcription of more than 30 amino acid biosynthetic enzymes. It has been proposed that the signal eliciting this response could be uncharged tRNA (13). This regulatory mechanism is similar to the positive control of the stringent response that has been thoroughly studied for *Escherichia coli* (5). Yeast cells treated with rapamycin resemble ones deprived of nutrients, since this molecule represses rRNA transcription and induces G_1 cell cycle arrest, translation arrest, glycogen accumulation, sporulation, and autophagy (11). Thus, it might be expected that the *GCN4*-mediated GCN response could be elicited in the presence of rapamycin. However, when the transcriptional profile of yeasts grown on rich media was compared with that of cells treated with rapamycin, it was found that Tor proteins did not directly modulate the GCN control but regulated the expression of genes involved in the utilization of poor nitrogen sources (nitrogen-discriminating pathways) (11). Studies on the role of Gcn4p as a transcriptional activator have been conducted under conditions of extreme amino acid deprivation. However, it is possible that nitrogen-poor conditions under which yeast cells are grown could also lead to the accumulation of uncharged tRNA, resulting in increased *GCN4* mRNA translation; although this condition might not elicit the global GCN response, the possibility that another set of genes could respond to small increments of Gcn4p cannot be excluded. Since the presence of rapamycin mimics nutrient limitation, we decided to determine

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whether rapamycin increases *GCN4* translation and whether this increase in turn leads to increased expression of genes turned on by nitrogen limitation.

MATERIALS AND METHODS

Strains. The wild-type strain CLA1 (*MAT*α *ura3 leu2*) (23) was transformed according to the method described by Ito et al. (14) with plasmids p180 (*GCN4-lacZ CEN4 ARS1 URA3*) (12), kindly provided by A. Hinnebusch, and pRS315 (*CEN6 ARS4 LEU2*) (20), yielding strain CLA-300 (*MAT*α *ura3 leu2*/p180 *GCN4-lacZ CEN4 ARS1 URA3*/pRS315 *CEN6 ARS4 LEU2*). An isogenic *gcn4*Δ derivative (*MAT*α *gcn4*Δ::*URA3 leu2*) was obtained from the CLA1 strain by gene replacement using the 3.7-kb *Bst*II-*Mlu*I restriction fragment of pM214 (12) and was transformed with pRS315, yielding strain CLA-301 (*MAT*α *gcn4*Δ::*URA3 leu2*/pRS315 *CEN6 ARS4 LEU2*). Correct insertion of the *Bst*II-*Mlu*I fragment was monitored by PCR amplification of genomic DNA obtained from CLA1 and CLA-301 with two deoxyoligonucleotides designed to amplify the *GCN4* coding sequence.

To prepare an isogenic *gln3*Δ kanMX derivative, two deoxyoligonucleotides were designed based on the nucleotide sequence of the *S. cerevisiae GLN3* gene obtained from the *Saccharomyces* genome database and on the sequence of the multiple-cloning site present in plasmid pFA6a (24). The deoxyoligonucleotide S1 (5′-TAG TCA TCT GGA CGT GCA TGG TCG AAG TAA TGA AGA GCC G **CGT ACG CTG CAG GTC GAC**-3′) comprised 40 bp from the 5′ end of the *GLN3* sequence and 18 bp (indicated in bold lettering) of the pFA6a multiple-cloning site. The deoxyoligonucleotide S2 (5′-TAT CCT CAC TGA TCT TTC CGC CTG CAC TCA CAT CTG CTT C **ATC GAT GAA TTC GAG CTC G**-3′) contained 40 bp from the 3′ end of the *GLN3* sequence and 19 bp (bold) from the pFA6a multiple-cloning site. Qiagen purified pFA6a DNA was used as a template for amplification by PCR, carried out in a Stratagene Robocycler 40 by following a previously described program (18). A 1,500-bp PCR product was obtained, gel purified, and used to generate a *gln3*Δ derivative of strain CLA1 (*MAT*α *ura3 leu2*) (23) by gene replacement. Correct insertion was monitored by PCR amplification on genomic DNA, using a pair of deoxyoligonucleotides designed to amplify the *GLN3* coding sequence. The isogenic *gln3*Δ derivative was transformed with plasmids p180 (*GCN4-lacZ CEN4 ARS1 URA3*) (12) and pRS315 (*CEN6 ARS4 LEU2*) (20), yielding strain CLA-302 (*MAT*α *ura3 leu2 gln3*Δ::*kan MX*/p180 *GCN4-lacZ CEN4 ARS1 URA3*/pRS315 *CEN6 ARS4 LEU2*). The *gcn4*Δ *gln3*Δ double mutant was prepared by transforming strain CLA-301 with the 1,500-bp PCR product used to prepare the *gln3*Δ derivative by following the above-described procedure. Strains CLA-304 (Σ1278b *MAT*α *ura3-52*/p180) and CLA-305 (Σ1278b *MAT*α *ura3-52 TOR1-4*/p180) were prepared by transforming strains MLY40 (Σ1278b *MAT*α *ura3-52*) and MLY90-1 (Σ1278b *MAT*α *ura3-52 TOR1-4*), respectively, kindly provided by M. E. Cárdenas, with plasmid p180 (*GCN4-lacZ CEN4 ARS1 URA3*) (12).

Growth conditions. For the treatment with rapamycin, cells were grown at 30°C with agitation to an optical density (OD) of 0.8 on a rich medium containing 1% yeast extract, 2% peptone, and 2% dextrose (YPD). Pertinent aliquots of these cultures were used to inoculate flasks containing 300 ml of YPD to an OD of 0.05. These cultures were allowed to grow to an OD of 0.50, and 50-ml aliquots were independently collected by centrifugation. The rest of the culture was treated with 200 ng of rapamycin per ml for 30 and 120 min, after which 50-ml aliquots were independently collected and centrifuged.

Growth of strains in the presence of rapamycin was tested on plates prepared with YPD plus 2% agar with or without 200 ng of rapamycin per ml.

Northern analysis. Northern analysis was carried out by preparing total RNA from 50-ml samples of the pertinent cultures as described by Struhl and Davis (22). Prehybridization was carried out at 65°C for 1 h (1). Filters were sequentially hybridized with different probes for 18 h and after each hybridization were washed with a 10-fold dilution of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min and then with a 130-fold dilution of 20× SSC containing 0.1% SDS at 65°C for 30 min. The signal was quantified using STORM 840 and ImageQuant software (Molecular Dynamics). Before the addition of each probe, filters were boiled for 15 min in 0.1% SDS and prehybridized.

Determination of β-Gal activity. Soluble extracts were prepared by suspending whole cells in the pertinent buffer (19) and grinding them with glass beads in a Vortex mixer. β-Galactosidase (β-Gal) activities were determined as previously described (19). Specific activity was expressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein. Protein was measured by the method of Lowry et al. (15), with bovine serum albumin as a standard.

TABLE 1. β-Gal specific activity fostered by a *GCN4*::*lacZ* reporter carried on plasmid p180 in *S. cerevisiae* strains treated or not treated with rapamycin[a]

Duration (min) of treatment	β-Gal sp act (nmol/min/mg)[b] in strain:			
	CLA-300 (*TOR1*/p180)		CLA-304 (*TOR1*/p180) with rapamycin	CLA-305 (*TOR1-4*/p180) with rapamycin
	Without rapamycin	With rapamycin		
0	10	13	4	2
30	13	52	12	2
120	10	135	64	4

[a] Strains were grown as stated in the text and treated with 200 ng of rapamycin per ml. Only relevant genotypes are shown.
[b] Means of three independent experiments. Variations were ≤15%.

RESULTS AND DISCUSSION

In order to analyze whether Gcn4p had a role in the TOR signaling pathway, we determined the levels of *GCN4* translation in two wild-type strains and in a *TOR1-4* derivative (Table 1) and the expression of a representative group of genes in the wild-type strain CLA-300 and in its isogenic *gcn4*Δ and *gln3*Δ mutant derivatives CLA-301 and CLA-302, in the presence and absence of rapamycin (Fig. 1). As Table 1 shows, β-Gal activity fostered by the translational *GCN4-lacZ* gene fusion increased 10-fold after rapamycin treatment of wild-type strain CLA-300 indicating that in the presence of this immunosuppresor, translation of *GCN4* mRNA was increased. In order to confirm that this was a TOR-dependent response, we determined β-Gal activity in wild-type strain CLA-304 (*TOR1*) and its isogenic derivative CLA-305, which carries the *TOR1-4* mutation that renders the cells rapamycin resistant (2, 4). β-Gal activity in the CLA-304 strain increased 16-fold following rapamycin treatment (Table 1), confirming the results obtained with wild-type strain CLA-300. Conversely, β-Gal activity was not increased in the CLA-305 strain in the presence of rapamycin. These results indicate that the TOR cascade regulates *GCN4* transcriptional activity by preventing *GCN4* mRNA translation, suggesting the existence of alternative TOR-dependent mechanisms that in addition contribute to modulating the transcription factor functions besides controlling their translocation to the nucleus.

It is worth mentioning that when the CLA-300 strain was treated with 3-aminotriazole (3-AT) to elicit histidine deprivation, the values for β-Gal activity rose from 50 $nmol^{-1}$ mg^{-1} on ammonium to 463 $nmol^{-1}$ mg^{-1} on ammonium with 3-AT, as has been previously reported (12). The observed value on 3-AT is higher than those shown in Table 1 (12), indicating that the presence of rapamycin induces only a subtle limitation which is not equivalent to the deprivation obtained with 3-AT. Northern analysis was carried out in total RNA samples obtained from wild-type, *gcn4*Δ, and *gln3*Δ strains grown in the presence and absence of rapamycin. As Fig. 1 shows, *HIS3* expression showed a twofold increase in expression after rapamycin treatment, which was abolished in a *gcn4*Δ mutant; as expected, the presence of the *gln3*Δ mutation did not affect *HIS3* expression. Figure 1 also shows that neither *GLN3* nor *GCN4* expression was significantly increased after rapamycin treatment. When the expression of genes whose products are involved in the transport or degradation of secondary nitrogen

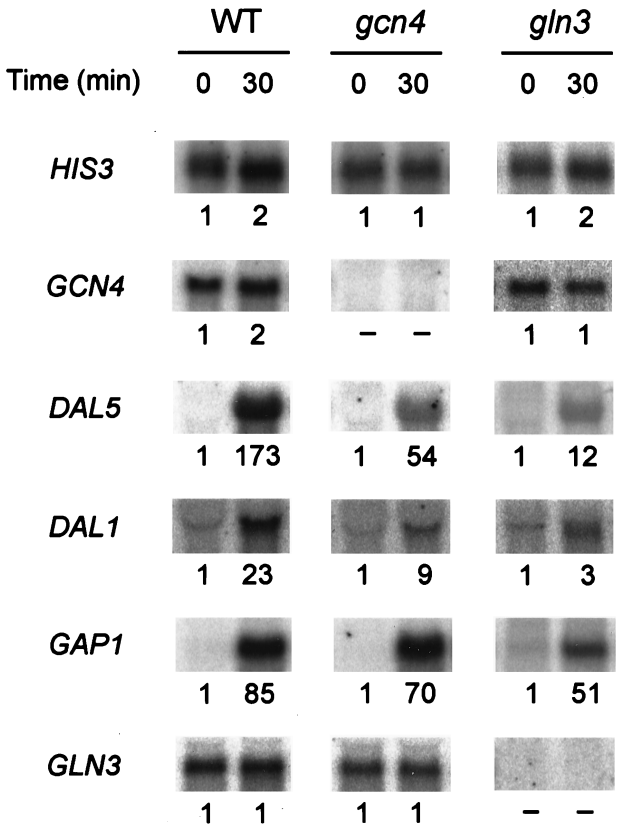

FIG. 1. Northern blot of total RNA from strains CLA-300 (wild type [WT]), CLA-301 (*gcn4*Δ), and CLA-302 (*gln3*Δ) after 30 min of treatment with rapamycin. RNA samples were separated by electrophoresis on a denaturing 1% agarose gel and transferred to a Hybond-N filter. Several filters were prepared with total RNA obtained from the wild-type strain and mutant strains CLA-301 and CLA-302. All of them were probed with a 1.5-kb PCR fragment of *ACT1* and alternatively with PCR fragments of 2.2, 1.0, 1.3, 1.78, 0.84, and 1.0 kb from *GLN3, GAP1 DAL1, HIS3, GCN4*, and *DAL5*, respectively; labeled with [α-^{32}P]CTP. Representative results from three experiments are shown. Numbers indicate mRNA quantitative values after normalizing with actin.

sources like *DAL1* (allantoinase), *DAL5* (allantoate permease), and *GAP1* (general amino acid permease) was analyzed, we found that as previously reported (4), a considerable increase in expression for these three genes was observed after 30 min of rapamycin treatment; the increases in expression of *DAL5* and *DAL1* were dependent on both *GCN4* and *GLN3* (Fig. 1), while that of *GAP1* was only *GLN3* dependent. These results suggest that the increased transcriptional activation of a group of genes involved in nitrogen utilization in the presence of rapamycin can be attributed to the combined actions of Gln3p and Gcn4p. The role of Gln3p in the expression of genes involved in nitrogen utilization has long been recognized and has been thoroughly studied (3, 8, 9, 16, 17), and for some time it was thought that Gln3p was the only transcriptional activator determining the expression of genes involved in nitrogen catabolism. Further studies showed that the GATA factor encoded by *GAT1* also played a role, modulating the expression of some of the *GLN3*-regulated genes, like *GAP1* (6, 7, 21). So, it was concluded that the transcriptional activation of the genes involved in nitrogen utilization was determined by the actions of both *GLN3* and *GAT1*. Conversely, Gcn4p has been shown to play a crucial role in the expression of the amino acid biosynthetic pathways, but no role for this transcriptional activator has been assigned in the expression of genes turned on when yeast cells are grown on poor or secondary nitrogen sources (12, 13). Thus, it has been considered that *GLN3* and *GAT1* modulate nitrogen catabolism whereas *GCN4* regulates amino acid biosynthesis, and no interaction between these two networks has been recognized. The above results indicate that Gcn4p can also contribute to the expression of some catabolic genes, suggesting physiological interactions between the *GCN4* and the *GLN3-GAT1* networks.

In order to clearly establish that Gcn4p and Gln3p are the targets of the TOR signaling cascade, a *gln3*Δ *gcn4*Δ double mutant strain was constructed. As Fig. 2 shows, the double mutant was highly resistant to rapamycin, while single *gcn4*Δ and *gln3*Δ mutants were rapamycin sensitive, indicating that

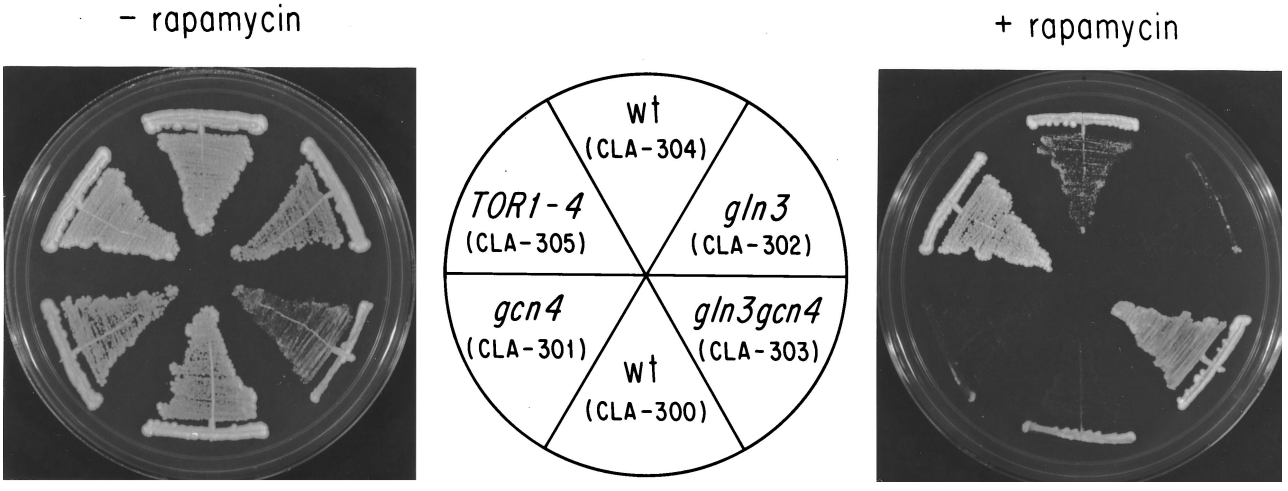

FIG. 2. Strains CLA-300 (wt), CLA-301 (*gcn4*Δ), CLA-302 (*gln3*Δ), CLA303 (*gcn4*Δ *gln3*Δ), CLA304 (*TOR1*), and CLA-305 (*TOR1-4*) were streaked on YPD and YPD with 200 ng of rapamycin per ml and incubated at 30°C for 2 and 5 days, respectively.

both Gln3p and Gcn4p are necessary for the inherent sensitivity of yeast cells to rapamycin and that these two transcriptional activators act independently on the target promoters.

The novel finding was that in the presence of rapamycin, Gcn4p regulates the expression of genes involved in the catabolism or transport of nitrogenous compounds but not of those involved in amino acid biosynthesis. This could be explained by proposing that Gcn4p plays a TOR-dependent role, elicited by a subtle amino acid limitation generated when yeast cells are grown on poor nitrogen sources, in addition to that of regulating the GCN TOR-independent pathway in response to extreme amino acid deprivation (11, 13).

The above results indicate that in yeast, as well as in mammalian cells (10), the TOR pathway responds to nutrient limitation and is thus able to promote the function of a proliferation pathway by preventing *GCN4* and *GLN3* transcriptional activities when yeast cells are grown on a rich nitrogen source and by promoting it when cells are shifted to a poor nitrogen source.

ACKNOWLEDGMENTS

We acknowledge Fernando Bastarrachea for critical review of the manuscript, José Esparza for helpful technical assistance, Allan Hinnebusch for kindly providing plasmids p180 and pM214, and M. E. Cárdenas and M. Hall for providing yeast strains MLY40 (Σ1278b *MAT*α *ura3-52*) and MLY90-1 (Σ1278b *MAT*α *ura3-52 TOR1-4*).

This work was supported in part by the DGAPA, Universidad Nacional Autónoma de México (IN212898), and by the Consejo Nacional de Ciencia y Tecnología (31774-N).

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