Tor Signaling and Nutrient-based Signals Converge on Mks1p Phosphorylation to Regulate Expression of Rtg1p-Rtg3p-dependent Target Genes*

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The heterodimeric bZip/HLH transcription factors Rtg1p and Rtg3p regulate the expression of a concise set of metabolic genes (termed RTG target genes) required for de novo biosynthesis of glutamate and glutamine. Several components have now been identified that control both the intracellular localization as well as activity of the Rtg1p-Rtg3p complex, yet the precise upstream regulatory signals involved remain unclear. For example, it has been proposed that Rtg1p-Rtg3p activity is repressed by glutamate, acting through the mitochondrial retrograde response pathway or, alternatively, by glutamine, acting through the Tor kinase pathway. Here we demonstrate that RTG target gene regulation is remarkably complex, with glutamate and glutamine as well as ammonia collaborating as potentially distinct signals to regulate RTG target gene expression. We show that both Tor and these nutrient-based signals converge on Mks1p, the immediate upstream inhibitor of Rtg1p-Rtg3p, and that a direct correlation exists between the degree of Mks1p phosphorylation and the extent of RTG target gene repression. Finally, we find that Tor- and glutamine-mediated RTG-target gene repression can be experimentally uncoupled, indicating that glutamine and Tor act, at least in part, independently to inhibit this pathway.

Normal cell proliferation requires that cells adjust their protein biosynthetic and metabolic activity in response to nutrient availability and other environmental cues. Specific signaling pathways exist that enable cells to perceive and respond appropriately to these cues, and distinct pathogenic states are often contingent upon impaired regulation of these pathways (1-3). Accordingly, a detailed understanding of how these pathways function is likely to be important for improved treatment of many human diseases, including cancer. One signaling pathway utilized by cells to control their growth in response to nutritional signals is the Tor (Target of rapamycin) kinase pathway. This pathway was discovered through the action of the antibiotic rapamycin, which inhibits the activity of many rapidly proliferating cells and, because of this, is presently in clinical use as an anti-proliferative agent as well as in trials as a potential anti-cancer tool (1, 3-5). Rapamycin inhibits the growth of a wide variety of eukaryotic cell types and organisms, including the budding yeast, Saccharomyces cerevisiae (4, 6-8). Treating yeast cells with rapamycin induces several responses that bear similarity to nutrient starvation, including a reduction in protein synthesis and ribosome biogenesis, cell cycle arrest, and ultimately, entry into G0 (4, 9). Remarkably, despite the importance of Tor as a regulator of cell growth and an important target for therapy, we still know very little about the physiologically relevant signals that control this pathway.

Recent studies demonstrate that Tor regulates the expression of many cell growth-related genes in yeast, including genes involved in carbon and nitrogen metabolism (10-14). In particular, we and others show that Tor negatively regulates a concise cluster of genes, termed RTG target genes, which encode a variety of enzymes involved in the anaplerotic production of α-ketoglutarate for use in de novo biosynthesis of glutamate and glutamine (13, 14). These genes were characterized originally by Butow and co-workers (15-17) as being under the control of a mitochondria-to-nucleus signaling pathway or retrograde response pathway that adjusts their transcription in response to the respiratory state of the cell. RTG target genes include enzymes involved in the tricarboxylic acid and glyoxylate cycles, for example peroxisomal citrate synthase 2 (CIT2), and are positively regulated by a heterodimeric transcription factor complex composed of the bHLH/Zip proteins Rtg1p and Rtg3p (15, 16, 18). In response to impaired mitochondrial function, as occurs in rho- petite cells that lack mitochondrial DNA or after rapamycin treatment of cells grown under rich nutrient conditions, the Rtg1p and Rtg3p proteins translocate from the cytoplasm into the nucleus and activate their target genes (13, 19). Several proteins have been identified that are important for this regulated nucleocytoplasmic trafficking event, including two cytoplasmic proteins, Rtg2p (13, 16, 19) and Mks1p (20-22). Results of several studies indicate that Mks1p is an inhibitor of the Rtg1p-Rtg3p complex and that Rtg2p in turn acts to inhibit Mks1p (20-22). Indeed, dynamic interactions between Rtg2p and Mks1p as well as between Mks1p and Bmh1p and Bmh2p, the latter being the yeast homologues of mammalian 14-3-3 proteins, play critical roles in regulating the localization and activity of the Rtg1p-Rtg3p complex (23). An important focal point within this regulatory scheme is the phosphorylation state of Mks1p, which is responsive both to mitochondrial dysfunction as well as rapamycin treatment (20, 21, 23).

An outstanding question that remains is the nature of metabolic signals that regulate RTG target gene expression. Butow and Liu provide evidence that glutamate is important as a repressive signal for the pathway (17). This repression appears to involve, at least in part, the SPS amino acid sensor system (24), which includes the amino acid permease-like protein

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**Tor and Nutritional Control of Rtg1p and Rtg3p Target Genes**

**Table 1**

| Strain | Relevant genotype | Source |
|--------|------------------|--------|
| S288c  | Mata gal2 mal    | D. Botstein |
| Σ1278b | Mata             | E. Dubois |
| MY1384 | Σ2000 series, Mata | Microbia |
| MY1385 | Σ2000 series, Mata | Microbia |
| MY1389 | Σ2000 series, Mata, leu2Δ | Microbia |
| PLY192 | Same as MY1389, except mks1::LEU2 | Ref. 21 |
| MY1391 | Σ2000 series, Mata, his3Δ | Microbia |
| PLY377 | Same as MY1391, except dur2::HIS5 (Schizosaccharomyces pombe) | This study |
| PLY379 | Same as MY1391, except mks1::HIS5 | This study |
| PLY398 | Same as MY1391, except gen4::HIS5 | This study |
| PLY400 | Same as MY1391, except ure2::HIS5 | This study |
| JK9-3da| Mata leu2-3,112 ura3-52 rme1 trp1 his4 can1-100 GAL* HMLα | M. Hall |
| JC10-4d| Mata gln1::KANMX leu2-3,112 ura3-52 rme1 trp1 his4 GAL | M. Hall |
| EY0733 | Mata ade2-1 trp1-1 can 1-100 leu2-3,112 his3-11,15 ura3 Gal* | rtg1::TRP1 |

Ssy1p and its partner proteins Ptr3p and Shr3p (25). By contrast, in collaboration with Hall and co-workers (26), we have provided evidence that glutamine production is important for RTG target gene repression. Indeed, because of the correlation between the effects of glutamine starvation and rapamycin treatment, we suggested that glutamine is likely to act upstream of Tor within this pathway (26). Finally, Cooper and co-workers reported that providing proline as a sole source of nitrogen is also repressive for this pathway, but in a manner that is insensitive to rapamycin, suggesting the existence of a Tor-independent mode for RTG target gene repression. Here we have investigated further the role of these different putative repressive nutritional signals. We find that the role of proline can be attributed to its conversion to glutamate and that rapamycin-sensitive RTG target gene repression requires the availability of ammonia and/or glutamate. Importantly, we confirm a role for glutamine as a bona fide repressive signal for the pathway. However, our present results suggest that glutamine likely acts in concert with, rather than uniquely upstream of Tor. We find that all metabolic signals converge on Mks1p to influence its phosphorylation state and, ultimately, regulate the intracellular localization and activity of the Rtg1p-Rtg3p complex.

**Materials and Methods**

**Strains, Media, and General Methods—**S. cerevisiae strains used in this study are listed in Table 1. The following culture media were used: YPD (1% yeast extract, 2% peptone, and 2% dextrose); MD (minimal dextrose) with no heat and stored at 80 °C. Pellets were resuspended in 450 μl of 1 x 10,000 rpm at 4 °C. Supernatants were filtered, washed twice with distilled water, and resuspended in 1 ml of ice-cold methanol. The cell suspension was dried in a Speed-Vac with no heat and stored at −80 °C. Pellets were resuspended in 450 μl of distilled water by vortexing for 5 min followed by centrifugation at 10,000 rpm for 4 °C. Supernatants were filtered through a 0.2-μm syringe filter and submitted to the University of California Davis Molec-
characterization of rapamycin-insensitive RTG target gene repression—We were intrigued by the finding reported by Tate et al. (22) that the RTG target gene CIT2 remains repressed when Tor is inactivated by rapamycin in cells utilizing proline as a sole nitrogen source. This is in contrast to cells grown in glutamine, where RTG target genes are also repressed yet become induced by rapamycin treatment (13, 21, 26). Because proline is catabolized to glutamate within mitochondria (32), it was suggested that glutamate is likely to be the rapamycin-insensitive repressive signal (22). At odds with this conclusion is the fact that Sekito et al. (20) demonstrate that RTG target genes are repressed during growth in glutamate in a rapamycin-inducible manner. A key difference between these studies is that ammonia as well as glutamate was present in the growth medium used by Sekito et al. (20). Thus, we reasoned ammonia might act to convert RTG target gene expression from a rapamycin-insensitive to a rapamycin-sensitive mode during growth in glutamate.

To test this possibility directly, we used Northern blotting to examine expression of CIT2 when the prototrophic strain Σ1278b was grown in media containing different defined nitrogen sources followed by treatment with rapamycin (Fig. 1A). The results showed that rapamycin treatment had no effect on CIT2 expression when cells were grown in minimal dextrose (MD) media that contained either proline (MD-Pro) or glutamate (MD-Glu) as a sole nitrogen source (Fig. 1A). Significant induction was, however, observed when cells were grown in media that contained both glutamate and ammonia (MD-Glu/NH₄⁺) (Fig. 1A, lanes 9 and 10), in agreement with the above prediction. The level of CIT2 induction caused by rapamycin was similar in MD-Glu/NH₄⁺ as in MD-Glu (Fig. 1A, compare lanes 6 and 10). A higher level of CIT2 expression was observed in MD-NH₄⁺, and this level was induced further by rapamycin (Fig. 1A, lanes 11 and 12), in agreement with previous observations (22). The addition of ammonia to proline media also resulted in rapamycin-inducible CIT2 expression (data not shown). Together these results demonstrate that cells switch to a rapamycin-sensitive mode of CIT2 repression when both ammonia and glutamate (or proline) are present in the growth medium. While this work was in progress Tate et al. (33) also reported that using glutamate as a sole source of nitrogen results in rapamycin-insensitive CIT2 repression, in agreement with our present findings (33).

To determine the strain specificity of our results, we examined the effects of rapamycin treatment on CIT2 expression when a different prototrophic strain S288c was grown in glutamate media that either contained or lacked ammonia (Fig. 1B). Here similar results were obtained, namely, that ammonia converted repression of CIT2 to a rapamycin-sensitive mode during growth in glutamate (Fig. 1B, compare lanes 7 and 8 and lanes 9 and 10). By contrast, strain-dependent differences were observed in the expression of the Gln3p-dependent target gene GAP1, where ammonia alone repressed this gene in the Σ1278b but not in the S288c background (Fig. 1B, compare lanes 5 and 11). This latter result suggests that the effect of ammonia on CIT2 expression in glutamate-containing media is distinct from the well established role of ammonia in nitrogen

with 0.2 μg/ml rapamycin for 30 min where indicated. B, Σ1278b and S228c cells were grown to mid-log phase in the indicated medium and treated with 0.2 μg/ml rapamycin for 30 min as indicated. A and B, after incubation, cells were harvested and processed for Northern blot analysis, probing for the specified mRNAs.

FIG. 1. Rapamycin-sensitive repression of RTG target gene CIT2 involves glutamine and ammonia availability. A, Σ1278b cells were grown in MD media supplemented with the indicated nitrogen source(s), where each nitrogen source was present at a final concentration of 0.2% (w/vol). Cells were grown to 0.5 A₅₅₀/ml and treated with 0.2 μg/ml rapamycin for 30 min where indicated. B, Σ1278b and S228c cells were grown to mid-log phase in the indicated medium and treated with 0.2 μg/ml rapamycin for 30 min as indicated. A and B, after incubation, cells were harvested and processed for Northern blot analysis, probing for the specified mRNAs.
catabolite repression, where significant differences have been characterized between these two strain backgrounds (34, 35).

To determine whether these results extend to other known RTG target genes, we used DNA microarray analysis to compare global changes in gene expression when rapamycin was added to cells grown in MD-Glu versus MD-Gln, as described under “Materials and Methods.” For this analysis, we examined in parallel the expression profiles of the two prototrophic strains described above, S288c and Δ2178b, as well as a third prototroph, Δ2000 (Fig. 2). The results showed that rapamycin caused a significant increase in the expression of most RTG target genes in MD-Gln relative to MD-Glu in each of the three strains examined (Fig. 2). The sole exception was PYC1, where only minor differences were observed and only in two of the three strains examined, consistent with our previously published observation that the expression pattern of this gene is strain-specific (21). We, therefore, conclude that rapamycin-insensitive repression during growth in glutamate is a general feature of RTG target gene regulation. Genome-wide analysis further revealed that no other major class of genes displayed significant differences in rapamycin-induced expression in MD-Gln versus MD-Glu, indicating this phenomenon is restricted primarily to RTG target genes (data not shown).

We next characterized the interplay between glutamate, glutamine, and ammonia in mediating rapamycin-sensitive repression of CIT2 (Fig. 3). Similar levels of CIT2 induction were observed when rapamycin was added to cells grown in MD-Glu that had an equal concentration of either ammonia or glutamine (Fig. 3A, compare lanes 4 and 6). Decreasing the amount of ammonia or glutamine that was added to MD-Glu resulted in significantly less rapamycin-induced CIT2 expression (Fig. 3B and C). In addition, we found that urea could substitute for ammonia, demonstrating that at least a portion of this response specifically requires intracellular ammonia (Fig. 3D, compare lanes 2 and 4). An important control for this latter experiment was the demonstration that urea did not provide a similar response in a Δ12Δ2 mutant, which is unable to catabolize urea to ammonia (Fig. 3D, lane 10). Together these results suggest that the availability of glutamine either supplied exogenously or by de novo production using glutamate and ammonia, determines whether CIT2 is repressed by a rapamycin-inducible mechanism.

An alternative interpretation of our results would be that ammonia and glutamine provide independent signals that trigger the use of a rapamycin-sensitive mechanism for CIT2 repression. In this regard, while this work was in progress, Cooper and Tate (33) presented evidence suggesting RTG target gene expression is correlated with intracellular levels of ammonia. This conclusion was based primarily on an analysis of a strain deleted for URE2, an upstream negative regulator of the GATA-specific transcription factor Gln3p required for expression of nitrogen catabolite repression-regulated genes. In particular, it was shown that an approximate 5-fold increase in intracellular ammonia occurs in ure2Δ cells in comparison to wild type cells when grown in MD-NH₄⁺, a result that was attributed to increased expression of MEP2, a nitrogen catabolite repression-regulated gene encoding ammonia permease. Furthermore, this increase in intracellular ammonia coincided with a substantial increase in CIT2 expression in ure2Δ versus wild type cells, prompting the conclusion that ammonia metabolism is a major determinant in RTG target gene regulation (33).

We attempted to confirm these results by constructing a prototrophic ure2Δ strain in the Δ2178b background. In good agreement with the results of Tate et al. (33), we observed a significant (≥4-fold) increase in intracellular levels of ammonia in the mutant strain compared with wild type when both strains were grown in MD-NH₄⁺ (Fig. 4B). By contrast, no significant difference in basal CIT2 expression was observed between the two strains, and only a minor increase in rapamycin-induced CIT2 expression was observed in the ure2Δ strain (Fig. 4A). From these results we conclude that the intracellular level of ammonia is unlikely to be the primary factor that influences RTG target gene activity.

Convergence of Repressive Signals on Nucleocytoplasmic Trafficking of Rtg1p/Rtg3p and State of Mks1p Phosphorylation—Previous studies show that Tor controls the intracellular localization of Rtg1p and Rtg3p in response to available nitrogen sources, where these proteins are sequestered in the cytoplasm in cells grown in MD-Gln or MD-Glu/NH₄⁺ yet accumulate in the nucleus after treatment with rapamycin (13, 26). Our results presented above prompted us to ask whether this regulatory step would be influenced when glutamate was provided as a sole nitrogen source. Accordingly, we monitored the localization of an Rtg1p-GFP fusion protein in rapamycin-treated or untreated cells that were grown in MD-Glu or, for comparison, MD-Gln. As reported previously, rapamycin treatment resulted in the nuclear accumulation of Rtg1p-GFP when cells were grown in MD-Gln (Fig. 5A, upper panels). By contrast, no significant change in the distribution of Rtg1p-GFP was observed after rapamycin treatment when cells were grown in MD-Glu, where the majority of the protein remained cytoplasmic (Fig. 5A, lower panels). Similar results were also observed for an Rtg3p-GFP fusion protein (data not shown).
Based on these results, we conclude that the Rtg1p/Rtg3p complex remains sequestered within the cytoplasm when cells utilize glutamate as a sole nitrogen source despite inhibition of Tor by rapamycin.

Recent studies have identified Mks1p as an important upstream negative regulator of the RTG pathway (20–22). Importantly, Cooper and co-workers (22) demonstrated that deletion of MKS1 results in constitutive activation of CIT2 expression in cells utilizing proline as a sole nitrogen source, suggesting that rapamycin-insensitive repression of RTG target gene expression also requires Mks1p (22). In agreement with this conclusion, we observed constitutive CIT2 expression in mks1Δ cells grown in MD-Glu, where the level of expression of this gene was very similar to that observed when the mutant was grown in MD-Gln or MD-Glu/NH4+ (Fig. 5B). Moreover, rapamycin treatment had no significant effect on the level of CIT2 expression in mks1Δ cells (Fig. 5B). Together, these results are consistent with previous conclusions that Tor as well as nutrient-related signals converge on Mks1p (21). While this work was in progress, Cooper and Tate (33) independently reported that deletion of MKS1 alleviates glutamate-mediated repression of CIT2, in agreement with our present results.

Previous studies show that Mks1p is a phosphoprotein whose phosphorylation state responds to Tor signaling (20, 21). In particular, we have demonstrated that rapamycin treatment of cells grown in rich media results in dephosphorylation of Mks1p in a manner that correlates with activation of the pathway (21). Therefore, to extend our results presented above, we determined the effect of rapamycin treatment on Mks1p phosphorylation in cells grown in different defined nitrogen sources using Western blotting to monitor an epitope-tagged version of this protein (Mks1p-HA3). We observed a consistent correlation between the level of phosphorylation of Mks1p-HA3 and the degree of inhibition of CIT2 expression (Fig. 5C). Thus, growth in MD-Glu, the most repressive of conditions for the pathway, resulted in essentially all of Mks1p-HA3 displaying reduced mobility on SDS-PAGE, consistent with this protein existing in a hyperphosphorylated form (Fig. 5C, lane 3). By contrast, rapamycin-induced CIT2 expression of cells grown in YPD, MD-Glu/NH4+, or MD-Gln correlated with essentially all of

Fig. 3. Interplay between Tor, glutamine, glutamate, and ammonia during RTG target gene repression. A–C, S288c cells were grown in MD media that contained the indicated nitrogen source(s). In A each nitrogen source was present at a final concentration of 0.2% (w/vol), and in B–C the concentrations of nitrogen sources were as listed. D, wild type (S2000) and dur1,2Δ cells (PLY377) were grown in MD media that contained the indicated nitrogen sources, where each nitrogen source was present at a final concentration of 0.2%. In A–D cells were grown to 0.5 A600/ml and treated with 0.2 μg/ml rapamycin for 30 min were indicated. Cells were then harvested and processed for Northern blot analysis, probing for ACT1 and CIT2 mRNAs. WT, wild type.
Mks1p-HA3 displaying increased mobility, indicating it had become significantly dephosphorylated under these conditions (Fig. 5C, lanes 2, 6, and 8).

Rapamycin treatment of cells grown in MD-Glu resulted in an intermediate form of Mks1p-HA3 phosphorylation, where the majority of the protein remained in a hyperphosphorylated state (Fig. 5C, lane 4). Interestingly, this pattern of Mks1p-HA3 phosphorylation looked remarkably similar to untreated cells grown in either MD-Glu/NH4+ or MD-Gln (Fig. 5C, compare lane 4 with lanes 5 and 7). Together these results suggest that a minimum threshold of Mks1p dephosphorylation must be achieved to result in significant CIT2 gene expression. These findings are also consistent with recent results from the Butow laboratory (23) which demonstrate that the phosphorylation state of Mks1p correlates with dynamic associations with Rtg2p as well as Bmh1p/Bmhp2 and, moreover, is likely to lie at the core of the regulation of the Rtg1p/H18528Rtg3p complex.

Relationship between Tor- and Glutamine-mediated RTG Target Gene Repression—We reported previously that specific depletion of glutamine results in increased RTG target gene expression (26). In this case, glutamine depletion was achieved either by removing glutamine from a gln1Δ mutant or, alternatively, by treating wild type cells with the compound MSX, a glutamate analogue and in vivo inhibitor of glutamine synthetase (Gln1p) (26). In these experiments the effect of MSX on CIT2 expression was observed in cells that were provided with both glutamate as well as NH4+ as nitrogen sources and correlated with rapamycin-induced expression of this gene (26). This correlation led to the suggestion that Tor senses glutamine as an upstream signal during RTG target gene repression (26).

Given our results presented here, a prediction of this simple linear model is that growth in MD-Glu should result in both rapamycin- as well as MSX-resistant CIT2 repression. However, we instead observed that MSX treatment caused significant CIT2 expression when cells were grown in MD-Glu to a level comparable with what was observed in MD-Glu/NH4+ media (Fig. 6A, compare lanes 3 and 9). In addition, MSX-induced CIT2 expression correlated with Mks1p dephosphorylation.

FIG. 4. Increased intracellular ammonia does not impact significantly upon RTG target gene expression. A, wild type (WT) (22000) and ure2Δ (PLY400) cells were grown to 0.5 A600/ml in MD-NH4+ and treated with 0.2 μg/ml rapamycin for 30 min where indicated. Cells were harvested and processed for Northern blot analysis, probing for the indicated mRNAs. B, intracellular ammonia in WT and ure2Δ cells, where both strains were grown to 0.5 A600/ml in MD-NH4+ and ammonia levels were determined as described under “Materials and Methods.”

FIG. 5. Repressive signals converge on Rtg1p-Rtg3p nuclear import and Mks1p phosphorylation state. A, fluorescence microscopy examining Rtg1p-GFP localization in cells grown in MD-Gln or MD-Glu and treated either with rapamycin (right panels) or drug vehicle alone (left panels). Results show significant nuclear accumulation of Rtg1p-GFP when rapamycin is added to cells grown in MD-Gln but not in MD-Glu. B, Northern blot analysis of specified mRNAs isolated from wild type (WT) (22000) and mks1Δ (PLY192) cells after growth in the indicated media. C, Northern blot analysis of ACT1 and CIT2 mRNAs (upper panels) and Western blot analysis of Mks1p-HA3 (lower panel). Strain PLY379 was grown in the designated media to 0.5 A600/ml and treated with 0.2 μg/ml rapamycin for 30 min where indicated, and cells were harvested and processed for Northern or Western blot analysis. In A–C, each nitrogen source was present at a final concentration of 0.2%.
FIG. 6. Testing the relationship between Tor and glutamine during RTG target gene repression. A, Northern blot analysis of ACT1 and CIT2 mRNAs (upper panels) and Western blot analysis of Mks1p-HA3 (lower panel). Strain PLY379 was grown in the designated media to 0.5 A₆₀₀/ml and treated with 0.2 μg/ml rapamycin or 2 mM MSX for 30 min where indicated, and cells were harvested and processed for Northern or Western blot analysis. B, Northern blot analysis of specified mRNAs in wild type (WT; JK9–3da) and gln1Δ (JC10–4d) cells grown in MDN-Gln or after a shift to MDN-Glu. Where indicated, cells were treated with 2 mM MSX for 30 min at different times after transfer to MD-Glu. In A and B, the indicated nitrogen sources were present at a final concentration of 0.2%. In B media also contained 0.003% uracil, 0.008% tryptophan, 0.006% histidine, and 0.008% leucine to supplement the auxotrophic requirements of these strains.

While this work was in progress, Cooper and Tate (33) suggested that glutamine was unlikely to act as a repressive signal during RTG target gene repression. Nevertheless, these results also confirm that both Tor as well as glutamine ultimately converge on a common regulatory step, namely phosphorylation of Mks1p (Fig. 6A) (21).

FIG. 7. Testing the role of Gcn4p in RTG target gene expression during glutamine starvation. Wild type (WT; MY1391) and gcn4Δ (PLY398) cells were grown in YPD to 0.45 A₆₀₀/ml, washed with distilled water, and transferred to either MDN-Glu or MDN-Glu/Gln media for 30 min. Where indicated samples were then treated with MSX for 30 min, and cells were harvested and processed for Northern blot analysis, probing for the specified mRNAs.

cells to mid-log phase in glutamine-containing media, where strong repression of CIT2 was observed, as expected (Fig. 6B, lanes 1 and 8). Next we transferred cells to media that lacked glutamine but instead contained glutamate and monitored CIT2 expression at different times after transfer. The results showed that CIT2 expression remained fully repressed in wild type cells but became increasingly induced over time in gln1Δ cells (Fig. 6B, compare lanes 2, 4, and 6 with lanes 9, 11, and 13). Finally, at each time point during growth in glutamine-free media, portions of each culture were treated with MSX for 30 min. The results showed that MSX caused significant (20–40-fold) induction of CIT2 in wild type cells but had only a marginal effect (~1.2-fold induction) in gla Δ cells (Fig. 6B). From these results we conclude that glutamine starvation has a dramatic effect on CIT2 expression despite the continuing presence of glutamate, in agreement with our previous conclusions (26). Moreover, we conclude that Gln1p is in fact the primary in vivo target of MSX in these experiments, at least with respect to the expression of RTG target genes.

MSX-induced CIT2 Expression Occurs Independently from Gcn4p—Previous studies have shown that expression of Gcn4p, the master regulatory transcription factor responsible for general amino acid control, is induced after starvation for a variety of amino acids, including glutamine (36). In addition, Hinnebusch and co-workers (37) show that a subset of RTG target genes, including CIT2, respond to amino acid starvation in a Gcn4p-dependent manner and, moreover, that their promoters contain sequences corresponding to the upstream activating sequence specific for Gcn4p. Finally, a connection between Tor signaling and Gcn4p activation has been established recently (38, 39). Together these results prompted us to ask to what extent CIT2 expression after glutamine starvation is dependent upon Gcn4p. Toward this end we performed the following experiment using a prototrophic gcn4Δ strain constructed in the Σ2000 background (Fig. 7). First, we grew wild type and gcn4Δ cells in rich media (YPD) to mid-log phase to compensate for observed growth defects of the mutant strain in minimal
media (data not shown). Next, cells were transferred either to MDN-Glu or MDN-Glu/Gln for 30 min, and then a portion of each culture was treated with MSX for an additional 30 min. As expected, MSX treatment resulted in no CIT2 induction when either strain was grown in the presence of glutamine (Fig. 7, lanes 4 and 8). By contrast, significant MSX-induced CIT2 expression was observed when gcn4Δ cells were grown in MDN-Glu to a level that was comparable with what was observed in wild type cells (Fig. 7, lanes 2 and 6). From these results we conclude that RTG target gene expression resulting from glutamine starvation is largely independent from the action of Gcn4p. This conclusion is consistent with our previous demonstration that MSX-induced RTG target gene expression requires RTG1-RTG3 (26).

**DISCUSSION**

We have presented evidence for complex interplay between distinct metabolic conditions as well as Tor signaling during regulated expression of RTG target genes. Our observations can be explained in terms of a model wherein these different factors influence the phosphorylation state of Mks1p, a central negative regulator of the RTG system (Fig. 8). Much of this model derives from recent results from Butow and co-workers (23), who have shown that Mks1p forms mutually exclusive interactions with Rtg2p or Bmh1p/Bmh2p in a manner that correlates with its state of phosphorylation. Thus, when Mks1p is phosphorylated, it interacts preferentially with Bmh1p/Bmh2p to form a complex that inhibits nuclear localization of Rtg1p/Rtg3p. By contrast, dephosphorylation of Mks1p enables it to associate with Rtg2p, where it is prevented from forming an inhibitory complex with the 14-3-3 proteins. An important elaboration of this model that we suggest, based on our present analyses of Mks1p, is that distinct states of Mks1p phosphorylation contribute to the responsiveness of the RTG system under different cellular conditions (Fig. 8). This model is consistent with observations that Mks1p is a multiply phosphorylated protein (20, 21, 23). Moreover, this model helps to clarify the observed effects of rapamycin treatment on RTG target gene expression under different nutrient conditions.

For example, we have shown that when glutamate is provided as a sole nitrogen source, Mks1p exists in a hyperphosphorylated state that we propose correlates with strong repression of the pathway (Fig. 8A). Because rapamycin treatment causes partial dephosphorylation of Mks1p, we take this as an indication that this protein remains responsive to Tor under these conditions. However, because dephosphorylation is only partial, Mks1p remains sufficiently phosphorylated to maintain strong associations with Bmh1p/Bmh2p and keep the pathway in a repressed state. By contrast, when either ammonia or glutamine is additionally provided or, alternatively, when glutamine is used as a sole nitrogen source, Mks1p shifts to an intermediate state of phosphorylation, where we predict interactions with Bmh1p/Bmh2p may become weakened (Fig. 8B). Under such conditions, rapamycin treatment contributes to further dephosphorylation of the protein that ultimately crosses a threshold required for de-repression of the pathway (Fig. 8C). Thus, different metabolic conditions give rise to distinct states of Mks1p phosphorylation that ultimately determine whether the pathway will respond at the transcriptional level to loss of Tor activity. Partial phosphorylation of Mks1p, as occurs after rapamycin treatment of cells grown in glutamate, suggests either that there are Tor-independent phosphorylation sites in this protein or, alternatively, that a stochastic process is involved that utilizes a common set of phosphorylation sites. Identifying and characterizing these sites of phosphorylation as well as the exact kinase(s) and phosphatase(s) that act upon Mks1p are necessary steps toward addressing this issue.

Our present results confirm our previous conclusion that glutamine starvation acts as an inducer of RTG target gene expression (26). In particular, we have shown here that this response is independent from Gcn4p-mediated general amino acid control and instead correlates with dephosphorylation of Mks1p. Together with our previous finding that glutamine starvation results in nuclear entry of the Rtg1p/Rtg3p complex (26), we conclude that this response is likely to involve the same regulatory scheme outlined above (Fig. 8). Unlike rapamycin treatment, however, glutamine starvation appears to be completely dominant with respect to the phosphorylation state of Mks1p that is established during growth in glutamate. Thus, we suggest that glutamine starvation represents an override signal that must act, at least in part, independently from Tor (Fig. 8, dashed line). Although the precise nature of this signal remains to be determined, we have confirmed here that it is directly related to the loss of activity of glutamine synthetase (Gln1p). In this respect it is interesting to note that a proteome-wide screen has identified Mks1p and Gln1p as interacting proteins, suggesting the intriguing possibility that glutamine synthetase itself participates in the regulated expression of RTG target genes (40). However, we have so far been unable to confirm an interaction between these two proteins using co-immunoprecipitation approaches. Additional experiments will be required to address the possible role of Gln1p in the regulation of this pathway.

An important question raised by our results presented here is regarding the precise role of ammonia in the RTG system. In this regard, Butow and Avadhani (41) recently pointed out that there must be a proper balance between the anaplerotic production of α-ketoglutarate (a function of the RTG pathway) and intracellular levels of ammonia, both, to maintain adequate *de novo* glutamate and glutamine biosynthesis as well as prevent toxicities associated with the build up of metabolites, in particular ammonia. Here it is relevant that Cooper and Tate (33) suggested recently that ammonia acts as an independent, positive signal that regulates the activity of the RTG system. Alternatively, Butow and Guaragnella (42) argue that ammonia could play a more indirect role, for example by influencing the relative intracellular levels of α-ketoglutarate, glutamate, and glutamine, by modulating their rates of inter-conversion. Our present results are more in agreement with this latter

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2 I. Dilova, S. Aronova, J. C.-Y. Chen, and T. Powers, unpublished results.
possibility. According to this scheme, we envision that these metabolites may play distinct regulatory roles, specifically by modulating the phosphorylation state of Mks1p and thereby regulating its association with specific partners. An outstanding question raised by our present findings is whether Tor activity is itself regulated by these different metabolites discussed here. We believe that the development of better tools to characterize intracellular pools of different metabolites as well as understanding their potential effects on the regulatory proteins described here represent crucial steps toward further deciphering the complexity that is observed within this system.

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