

The TOR signaling cascade regulates gene expression in response to nutrients

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Rapamycin inhibits the TOR kinases, which regulate cell proliferation and mRNA translation and are conserved from yeast to man. The TOR kinases also regulate responses to nutrients, including sporulation, autophagy, mating, and ribosome biogenesis. We have analyzed gene expression in yeast cells exposed to rapamycin using arrays representing the whole yeast genome. TOR inhibition by rapamycin induces expression of nitrogen source utilization genes controlled by the Ure2 repressor and the transcriptional regulator Gln3, and globally represses ribosomal protein expression. *gln3* mutations were found to confer rapamycin resistance, whereas *ure2* mutations confer rapamycin hypersensitivity, even in cells expressing dominant rapamycin-resistant TOR mutants. We find that Ure2 is a phosphoprotein in vivo that is rapidly dephosphorylated in response to rapamycin or nitrogen limitation. In summary, our results reveal that the TOR cascade plays a prominent role in regulating transcription in response to nutrients in addition to its known roles in regulating translation, ribosome biogenesis, and amino acid permease stability.

[Key Words: Rapamycin; TOR kinase; signal transduction]

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Rapamycin inhibits a conserved signaling cascade required for cell proliferation (Thomas and Hall 1997; Cardenas et al. 1998; Cutler et al. 1999). Rapamycin action is mediated by binding to the FKBP12 prolyl isomerase, and the FKBP12-rapamycin complex in turn inhibits conserved protein kinase homologs, Tor1 and Tor2 in yeast, and mTOR/FRAP/RAFT1 in mammalian cells (Heitman et al. 1991; Cafferkey et al. 1993; Kunz et al. 1993; Brown et al. 1994; Helliwell et al. 1994; Sabatini et al. 1994; Cardenas and Heitman 1995; Sabers et al. 1995; Zheng et al., 1995; Alarcon et al. 1996). The yeast Tor proteins and mammalian mTOR have intrinsic protein kinase activity (Brown et al. 1995; Brunn et al. 1997; Alarcon et al. 1999; Jiang and Broach 1999).

The TOR kinases are essential for viability and regulate translation initiation and cell cycle progression (Heitman et al. 1991; Kunz et al. 1993; Barbet et al. 1996). Depletion of TOR or exposure to rapamycin inhibits translation in yeast by destabilizing the initiation factor eIF4G (Berset et al. 1998). mTOR regulates translation in mammalian cells via p70 S6 kinase and the PHAS-I inhibitor of the CAP-binding protein eIF-4E (Graves et al. 1995; Lawrence and Abraham 1997). mTOR phosphorylates PHAS-I in vitro and regulates PHAS-I phosphorylation in vivo (Lin et al. 1995; von Manteuffel et al. 1996;

Brunn et al. 1997; Burnett et al. 1998). Phosphorylation causes PHAS-I to release eIF-4E, which then interacts with eIF4G to form a productive 5'CAP-binding complex and initiate translation.

The yeast Tap42 protein and a homologous mammalian protein $\alpha 4$ have been implicated as targets of the TOR pathway. Tap42 is an essential protein that associates with protein phosphatase 2A (PP2A) subunits and regulates translation initiation in yeast (Di Como and Arndt 1996). Rapamycin treatment or nutrient deprivation disassociates Tap42 from the PP2A subunits Sit4, Pph21, and Pph22. Tap42 is a phosphoprotein in vivo and the Tor2 kinase regulates Tap42 phosphorylation (Jiang and Broach 1999). The mammalian $\alpha 4$ protein is homologous to yeast Tap42 (Chen et al. 1998) and its association with PP2A is also perturbed by rapamycin (Murata et al. 1997; Inui et al. 1998).

The Tor kinases play a role in nutrient sensing in yeast and mammalian cells. In yeast, the TOR kinases regulate sporulation and autophagy (Zheng and Schreiber 1997; Noda and Ohsumi 1998). TOR also regulates the Npr1 protein kinase that is required for the stabilization or degradation of amino acid permeases in response to nutrients (Schmidt et al. 1998). Rapamycin represses transcription of rRNA and tRNA by inhibiting RNA polymerases I and III (Zaragoza et al. 1998), and inhibits ribosome biogenesis (Powers and Walter 1999). In the fission yeast *Schizosaccharomyces pombe*, rapamycin

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has no effect on vegetative growth, but blocks mating in response to nitrogen starvation (Weisman et al. 1997). In mammalian cells, the mTOR kinase is activated by amino acids (Hara et al. 1998; Xu et al. 1998; Kimball et al. 1999) and regulates autophagy in cultured hepatocytes (Shigemitsu et al. 1999).

Signaling cascades enable yeast cells to shift from the utilization of abundant levels of good nitrogen sources to either poorer nitrogen sources or limiting concentrations of good nitrogen sources (Magasanik 1992). Glutamine synthetase plays a central role in nitrogen metabolism by converting ammonium to glutamine (Mitchell and Magasanik 1983). Intracellular glutamine is sensed by a pathway involving the Ure2 repressor, which binds and inhibits glutamine synthetase (Gln1) and the transcriptional activator Gln3 (Courchesne and Magasanik 1988; Coschigano and Magasanik 1991; Xu et al. 1995; Blinder et al. 1996). When cells are shifted to poor or limiting nitrogen sources, intracellular glutamine decreases, Ure2 is inactivated, and glutamine synthetase and Gln3 are activated, increasing glutamine and inducing nitrogen utilization genes. Gln3 activates genes encoding glutamine synthetase (Gln1), glutamate synthase (Glt1), glutamate dehydrogenase (Gdh1 and Gdh2), permeases for nitrogenous compounds (Gap1 and Mep2), enzymes involved in nitrogen source metabolism (Dal3 and Put1), and transcription factors that regulate gene expression (Dal80 and Dal82) (Mitchell and Magasanik 1984; Minehart and Magasanik 1991; Stanbrough et al. 1995). How cells sense nitrogen, how glutamine regulates Ure2, and how Ure2 regulates Gln3 are not known.

Gene expression analysis with yeast genome arrays reveals that rapamycin has profound effects on gene transcription, inducing nitrogen catabolite-repressed genes regulated by Gln3 and Ure2 and globally repressing ribosomal protein expression. Moreover, mutations in genes encoding nitrogen source regulatory proteins, including Ure2, Gln3, Npr1, and Npi1, were found to modulate rapamycin action. We show that Ure2 is a phosphoprotein in vivo whose phosphorylation state is regulated by nutrients and TOR kinase. In conclusion, the TOR kinases function in a signaling cascade that detects nitrogen sources and regulates gene expression.

Results

Rapamycin induces transcription of nitrogen catabolite-repressed genes and represses expression of ribosomal proteins

The TOR cascade is known to regulate translation in yeast and mammalian cells. Here, we tested whether the TOR cascade also regulates gene transcription. For this purpose, yeast cells were exposed to rapamycin and mRNA was isolated and hybridized to yeast whole-genome arrays. This analysis revealed that the expression of multiple genes was altered by rapamycin. Most importantly, a set of genes known to be transcriptionally induced by Gln3 was increased from 5- to 50-fold following rapamycin exposure (Table 1). These genes have been

characterized previously as the nitrogen catabolite-repressed genes, which are repressed in the presence of abundant rich nitrogen sources by Ure2-dependent inhibition of Gln3, and induced in a Gln3-dependent fashion when cells are grown with limiting quantities of good nitrogen sources (glutamine and ammonia) or poor nitrogen sources (proline and urea) (Magasanik 1992). These genes encode nitrogen metabolizing enzymes, including glutamate dehydrogenase (Gdh1 and Gdh2), plasma membrane permeases for nitrogenous compounds, including the general amino acid permease (Gap1), the high affinity ammonium permease (Mep2), and the high affinity proline permease (Put4), and enzymes involved in the catabolism of nitrogen sources, including allantoin utilization (Dal1 and Dal3), urea degradation (Dur1,2), and GABA catabolism (Uga1) (Table 1).

To confirm these findings obtained by hybridization to whole genome arrays, mRNA was isolated from cells exposed to rapamycin for 0, 15, 30, 60, or 120 min and subjected to Northern blot analysis (Fig. 1). This analysis provided independent confirmation that the *GAP1*, *MEP2*, and *GLN1* genes are induced by rapamycin, and also revealed that gene induction occurs rapidly following rapamycin addition. Other genes known to be induced by nutrient limitation, such as *SNZ1*, were induced by rapamycin only at later time points (Fig. 1). In contrast to rapamycin, the protein synthesis inhibitor cycloheximide did not affect *GAP1*, *MEP2*, or *GLN1* expression (Fig. 1). These findings support a model in which the TOR cascade regulates nitrogen source utilization gene expression in response to nutrients.

Consistent with a recent report (Powers and Walter 1999), analysis of gene expression with whole-genome arrays revealed a marked and global repression of the vast majority of the genes encoding cytoplasmic, but not mitochondrial, ribosomal proteins (Table 2). Northern blot analysis confirmed that rapamycin represses expression of two specific cytoplasmic ribosomal proteins (Rpl9A and Rps26) (Fig. 1). In contrast, cycloheximide treatment modestly stimulated the transcription of these ribosomal protein genes (Fig. 1). These observations confirm that the TOR cascade functions to regulate transcription of ribosomal components in addition to the known effects of the TOR-signaling cascade on translation.

Proteins that regulate utilization of nitrogen sources play a role in TOR signaling

Next, we sought to establish whether the ability of the TOR cascade to regulate Ure2 and Gln3-regulated genes results in physiological alterations in rapamycin action. *gln3* mutant strains were partially resistant to rapamycin, whereas *ure2* mutant strains were rapamycin hypersensitive (Fig. 2A). The fact that the *ure2* and *gln3* mutations confer opposite phenotypes is in accord with their known effects on Gln3-mediated gene expression, namely that *ure2* mutations derepress Gln3-regulated genes, whereas *gln3* mutations impair gene induction by

Table 1. Rapamycin induces expression of nitrogen catabolite repressed genes

Gene	Fold induction	Gene product/function
Permeases		
<i>MEP2</i>	46.2	high-affinity ammonium permease
<i>DAL5</i>	19.2 ^a	allantoate and ureidosuccinate permease
<i>GAP1</i>	7.3	general amino acid permease
<i>DAL4</i>	5.1 ^a	allantoin permease
<i>PUT4</i>	4.5 ^a	high affinity proline permease
<i>CAN1</i>	4.3	arginine permease
General catabolism		
<i>GDH2</i>	5.0	glutamate dehydrogenase (NAD)
<i>GLT1</i>	3.0	glutamate synthase
<i>GDH1</i>	1.4	glutamate dehydrogenase (NADP)
<i>GLN1</i>	0.0	glutamine synthetase
Specific catabolism		
<i>DUR1,2</i>	16.0	urea degradation
<i>DAL3</i>	15.7 ^a	allantoin utilization (ureidoglycolate hydrolase)
<i>PUT1</i>	11.7 ^a	proline catabolism
<i>DAL1</i>	7.9 ^a	allantoin utilization (allantoinase)
<i>UGA1</i>	5.6	GABA catabolism
<i>PUT2</i>	2.8	proline catabolism
<i>DAL7</i>	1.6 ^a	allantoin degradation (malate synthetase)
<i>DAL2</i>	1.5 ^a	allantoin utilization (allantoicase)
Transcriptional regulation		
<i>DAL80/UGA43</i>	8.2	transcriptional repressor
<i>GAT1/NIL1</i>	3.1	transcriptional activator
<i>DAL82</i>	2.5	transcriptional repressor
Central regulators		
<i>GLN3</i>	2.0 ^a	transcriptional activator
<i>URE2</i>	2.2	inhibitor of Gln3 and glutamine synthetase
<i>NPR1</i>	4.2	serine/threonine kinase

Strain MLY41 was grown to early exponential phase and treated with or without 0.2 µg/ml rapamycin for 2 hr. Cells were harvested and poly(A) RNA was isolated and processed for yeast genome analysis. Note that the results shown are fold of gene induction (above) or repression (Table 2) observed in the rapamycin-treated sample using the untreated sample as the baseline. Note that maximal induction of the *GLN1* gene occurs at 1 hr after treatment (see Fig. 1), and at 2 hr expression has returned to the original uninduced level.

^aNo signal detected in untreated cells.

nitrogen limitation (Magasanik 1992). A *ure2 gln3* double mutant exhibited the rapamycin-resistant phenotype conferred by the *gln3* single mutation (not shown), indicating that the effects of the *gln3* mutation are epistatic to the *ure2* mutation, in accord with a linear pathway in which Ure2 inhibits Gln3.

To further test whether Ure2 is a component of the TOR cascade, we addressed whether the *ure2* mutation alters the level of rapamycin resistance conferred by *TOR2*, *TOR1*, or *fpr1* mutations. As shown in Figure 2B, the *ure2* mutation significantly reduced the level of rapamycin resistance conferred by the dominant *TOR2-1* or *TOR1-4* mutations. Because rapamycin can only inhibit the wild-type Tor1 kinase in the *ure2 TOR2-1* mutant cells, or the wild-type Tor2 kinase in the *ure2 TOR1-4* mutant cells, the simplest explanation is that both Tor1 and Tor2 are required for rapamycin resistance in cells lacking Ure2. In comparison, the *ure2* mutation had no effect on rapamycin resistance conferred by FKBP12 mutations, because in these mutant cells, rapamycin is unable to interact with TOR (Fig. 2B).

The level of rapamycin resistance or hypersensitivity conferred by *gln3* and *ure2* mutations was found to be similar to that afforded by mutations affecting the Npr1 kinase and Npi1 ubiquitin ligase (Fig. 2A), which function in a nutrient-regulated TOR pathway controlling nutrient permeases (Schmidt et al. 1998). A kinase-inactive Npr1 mutant did not restore rapamycin sensitivity of *npr1* mutants, indicating kinase activity is important for function (not shown). In contrast, the *npi1* mutation conferred rapamycin hypersensitivity (Fig. 2A), and suppressed rapamycin resistance conferred by *npr1* or *TOR* mutations (not shown). Mutations in permeases regulated by Npr1 or Npi1, including Gap1, Mep1, Mep2, and Mep3, had little or no effect on rapamycin action (data not shown); hence, other Npr1 and Npi1 targets in the Tor pathway remain to be identified. Drug import assays revealed no effect of the *gln3* or *ure2* mutations on rapamycin import (data not shown), and taken together, these findings indicate altered rapamycin sensitivity is not attributable to alterations in drug uptake.

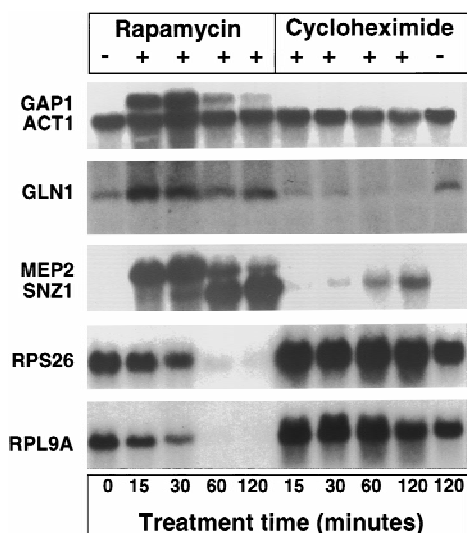


Figure 1. Rapamycin induces and represses gene expression. The wild-type yeast strain MLY41 was grown to early exponential phase, and treated with or without 0.2 μ g/ml rapamycin or 10 μ g/ml cycloheximide for 15, 30, 60, and 120 min, as indicated. RNA was prepared and analyzed by Northern blot with radioactive probes that hybridize to the genes indicated at left. Hybridization to the actin gene (*ACT1*) served as a loading control.

Tor regulates phosphorylation of nitrogen source regulator Ure2

The *Ure2*, *Gln3*, *Npr1*, and *Npi1* nitrogen source regulators could alter sensitivity to rapamycin by regulating *Tor1* or *Tor2* expression, or as regulators or effectors of the TOR cascade. By Western blot analysis, the *ure2*, *gln3*, *npr1*, and *npi1* mutations did not alter *Tor1* or *Tor2* expression (not shown).

Tor inhibition had only modest effects on *GLN3* and *URE2* gene expression, suggesting that rapamycin exerts a posttranscriptional effect. Next, we tested the hypothesis that *Ure2* is a phosphoprotein regulated by the TOR pathway. By Western blot analysis with specific polyclonal antisera, the *Ure2* protein migrated as either a single species, or, in some cases, as two species, in SDS-PAGE analysis of total protein extracts from vegetative cells grown in rich medium (Fig. 3). Importantly, treatment with 0.2 μ g/ml rapamycin led to a rapid increase in the electrophoretic mobility of the faster-migrating form of *Ure2*, concomitant with the disappearance of the slower-migrating form of *Ure2* (Fig. 3A,B). The effect of rapamycin on the mobility of *Ure2* was blocked in an *fpr1* rapamycin-resistant mutant (Fig. 3A,B). When cells growing in rich medium were shifted to SLAD medium limiting for ammonium, a similar and rapid increase in the electrophoretic mobility of *Ure2* was observed (Fig. 3A). When protein extracts from cells grown in rich medium in the absence of rapamycin were treated with alkaline phosphatase, the mobility of the *Ure2* protein was increased to an extent similar to that observed with rapamycin treatment or nutrient limitation (Fig. 3B). We conclude that *Ure2* is a phosphoprotein that is rapidly de-

phosphorylated following exposure to rapamycin or nitrogen limitation.

Because the *Tor* kinase is known to regulate association of the *Tap42* regulatory subunit with protein phosphatase 2A (Di Como and Arndt 1996; Jiang and Broach 1999), we addressed whether *Tor* regulates *Ure2* via *Tap42*. When a strain expressing a *Tap42-11* conditional mutant was shifted from 24°C to 37°C, analysis of gene expression by hybridization to whole genome arrays revealed no induction of the nitrogen utilization genes regulated by the *Tor* pathway (not shown). The *tap42-11* mutation confers partial resistance to rapamycin (Di Como and Arndt 1996). However, by Northern blot analysis, rapamycin still induced expression of the *GAP1* and *MEP2* genes, and repressed expression of the *RPS26* ribosomal protein gene, in a *tap42-11* mutant strain compared with the isogenic *TAP42* wild-type strain at the permissive growth temperature (not shown). These observations suggest that the *Tor* pathway may not regulate *Ure2* and *Gln3* via the *Tap42* regulatory subunit of PP2A.

Discussion

The TOR kinases are the targets of rapamycin in complex with the cellular protein FKBP12, and both TOR and FKBP12 are conserved from yeast to man. The TOR kinases regulate translation via divergent mechanisms in mammalian cells (PHAS-I, p70 S6 kinase) and yeast cells (eIF4G stability). Recent studies have revealed that the TOR kinase pathway also regulates cellular responses to nutrients. Here, we examined whether the *Tor* pathway regulates gene expression in yeast cells.

Table 2. Rapamycin represses expression of ribosomal proteins

Gene	Fold change	Gene product
Ribosomal proteins		
<i>RPL9A</i>	-43.2	ribosomal protein YL11
<i>RPS26A</i>	-22.1	ribosomal protein S26
<i>RPL6B</i>	-16.6	ribosomal protein YL8B
<i>RPL10E</i>	-6.8	60S ribosomal protein P0
<i>CRY2</i>	-6.5	ribosomal protein CRY1 (rp59)
<i>RPS18B</i>	-6.1	ribosomal protein S18
<i>URP1</i>	-5.4	ribosomal protein URP1
<i>RPS28B</i>	-5.2	ribosomal protein rp28
<i>RPS5</i>	-4.6	ribosomal protein S5
<i>RPS3</i>	-3.7	ribosomal protein RPS3
<i>RPS26B</i>	-3.4	ribosomal protein S26
<i>RPS28A</i>	-3.2	ribosomal protein S28
<i>RPS25</i>	-2.5	ribosomal protein S21
<i>RPL37A</i>	-2.4	ribosomal protein L25
<i>RPS33B</i>	0.0	ribosomal protein S33 (YL27)
<i>RPL4B</i>	9.9	ribosomal protein L4B (YL5)
Mitochondrial ribosomal proteins		
<i>MRPL27</i>	0.0	MRPL27
<i>MRPL39</i>	1.5	MRPL39
<i>MRPL33</i>	1.6	MRPL33
<i>MRPL36</i>	1.8	MRPL36

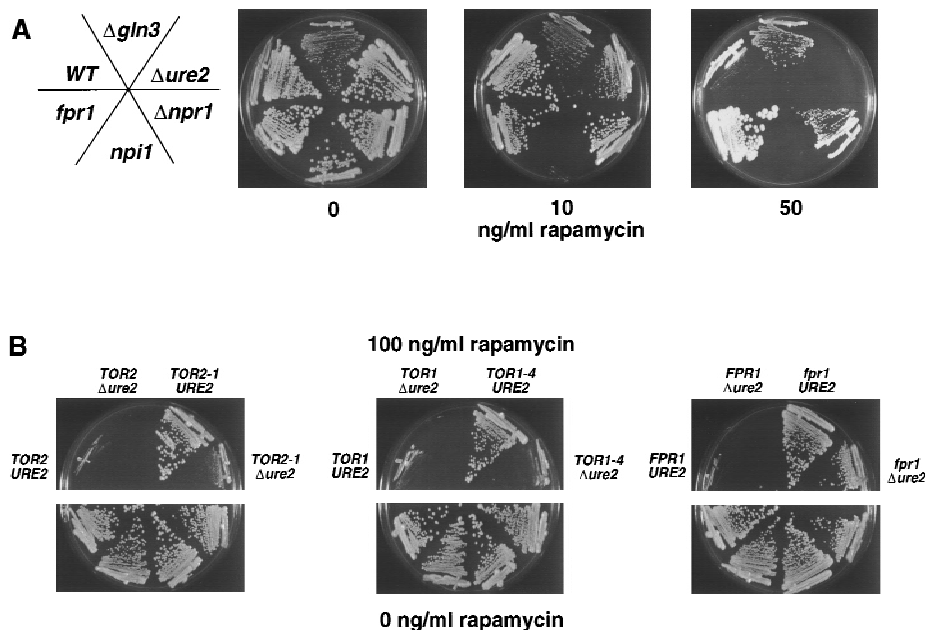


Figure 2. Mutations affecting nitrogen-source utilization alter rapamycin toxicity. (A) Isogenic wild-type (MLY41) and $\Delta gln3$ (MLY139), $\Delta ure2$ (MLY140a), $\Delta npr1$ (MLY54a), $npi1$ (MLY141a), and $fpr1$ (MLY88) mutant strains were grown on YPD medium containing 0, 10, or 50 ng/ml rapamycin and incubated for 3 days at 30°C. (B) Isogenic wild-type (MLY41) and $\Delta ure2$ (MLY140a), $TOR2-1$ (MLY152 α), $TOR2-1 \Delta ure2$ (MLY158 α), $TOR1-4$ (MLY90-1), $TOR1-4 \Delta ure2$ (MLY148 α), $fpr1$ (MLY88), and $fpr1 \Delta ure2$ (MLY146a) mutant strains were grown on YPD medium containing 0 or 100 ng/ml rapamycin and incubated for 3 days at 30°C.

Whole-genome array analysis revealed that inhibiting the Tor kinase with rapamycin has profound effects on the transcription of many yeast genes. Rapamycin rapidly induces expression of nitrogen-source utilization genes (nitrogen catabolite-repressed genes) known to be regulated by the repressor Ure2 and the transcription factor Gln3. In contrast, another protein synthesis inhibitor, cycloheximide, does not induce the nitrogen catabolite-repressed genes (Fig. 1). Rapamycin also globally represses expression of ribosomal proteins, in accord with recent reports that rapamycin inhibits ribosome biogenesis and rRNA and tRNA gene expression (Zaragoza et al. 1998; Powers and Walter 1999).

We have found that nitrogen-source regulators play a general role in rapamycin toxicity in yeast. Mutations in the gene encoding the Ure2 repressor, the Gln3 transcriptional activator, and the Npr1 kinase and Npi1 ubiquitin ligase confer either rapamycin resistance ($gln3$, $npr1$) or hypersensitivity ($ure2$, $npi1$). We show that the Ure2 protein is a phosphoprotein. Ure2 is rapidly dephosphorylated following nutrient limitation or inhibition of the TOR kinases with rapamycin, with a time course similar to induction of the Ure2-repressed genes by rapamycin. As outlined in Figure 4, our findings support a model in which the TOR kinase pathway responds to nutrients, and regulates nitrogen-utilization gene expression by regulating the phosphorylation of Ure2 and thereby modulating interaction with, and inhibition of, Gln3. Such a model is analogous to the role of Tor in regulating phosphorylation and interaction of Tap42 with PP2A in yeast cells, of the Tap42 homolog $\alpha 4$ regulator of PP2A in mammalian cells, and of the PHAS-I

inhibitor of eIF4E in mammalian cells. Recent studies have revealed a similar role for the Tor kinases in regulating nutrient permease stabilization and degradation by the Npr1 kinase (Schmidt et al. 1998). Mutations in Gln3 and Npr1 nitrogen-source regulators result in relative, but not absolute, rapamycin resistance, indicating that these mutations likely represent two of several independent signaling branches downstream of the TOR kinase (see Fig. 4), such that mutation of any one branch confers only partial drug resistance.

Activation of TOR and implications for rapamycin action in mammalian cells

Our studies implicate a role for the TOR-signaling cascade in sensing nitrogen sources in yeast cells. How the TOR kinases are activated is not understood, but recent findings in both yeast and mammalian cells suggest possible models involving the sensing of amino acids. Rapamycin has been thought to inhibit signaling pathways required for mammalian cells to respond to growth factors, such as interleukin-2 and insulin (Kuo et al. 1992; Price et al. 1992). However, rapamycin also inhibits response to nutrients, such as amino acids, which may activate a TOR-signaling cascade that functions in parallel with growth factor-signaling cascades to promote cell proliferation. For example, the p70 S6 kinase is activated by the mTOR-signaling pathway in response to either glutamine or leucine (Fox et al. 1998; Hara et al. 1998; Xu et al. 1998; Kimball et al. 1999). How these amino acids are sensed by mammalian cells is not yet

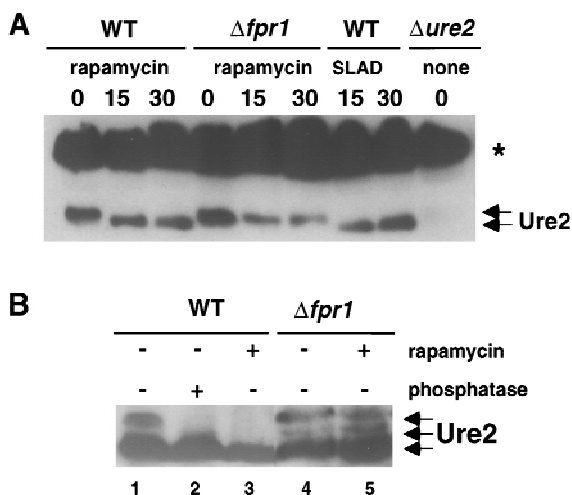


Figure 3. Tor and nutrients regulate the phosphorylation state of Ure2. (A) The isogenic wild-type (WT, strain MLY41) and $\Delta fpr1$ mutant strain (strain MLY88) were grown to early exponential phase ($OD_{600} = 0.4$) in YPD medium and treated with 0.2 $\mu\text{g/ml}$ rapamycin for 0, 15, and 30 min. Alternatively, the isogenic wild-type strain MLY41 was grown as above, washed with SLAD ammonium limiting medium, and then incubated in SLAD medium for 15 and 30 min. Total cell extracts were prepared and 100 μg of protein analyzed by SDS-PAGE (12.5%) and Western blot with Ure2 antibodies. Cell extract from a $\Delta ure2$ mutant strain (strain MLY140a) was included as a control for antisera specificity. (Arrows) The migration position of phosphorylated isoforms of Ure2; (*) a protein that crossreacts non-specifically with the anti-Ure2 antisera. (B) Protein cell extract from wild-type strain MLY41 was incubated with 4 units of alkaline phosphatase for 30 min at 37°C. Protein extracts from rapamycin treated or untreated wild-type strain (MLY41) or the $\Delta fpr1$ mutant strain (MLY88), as indicated, were mock incubated and loaded in lanes 1, 3, 4, and 5 to compare the mobility of phosphorylated and dephosphorylated Ure2. Note that cell extracts in A were analyzed in 12.5% SDS-polyacrylamide gels; the extracts in B were fractionated in 10% SDS-PAGE for a longer period of time, which could explain the greater separation of phosphorylated and dephosphorylated Ure2 observed in B.

known. Activation of p70 S6 kinase by the Tor pathway is inhibited in cells expressing conditional tRNA synthetase mutants, or in which tRNA synthetases are inhibited with amino acid alcohols. Thus, charged tRNAs may activate mTOR, or uncharged tRNAs might inhibit mTOR (Iiboshi et al. 1999b).

Rapamycin may have novel clinical indications as a chemotherapy agent for rapamycin-sensitive tumors, which include glioblastoma and prostate carcinoma (Dilling et al. 1994; Shi et al. 1995; Hosoi et al. 1999; Louro et al. 1999). The spectrum of rapamycin action might also be extended by applying insights derived from basic studies on rapamycin-sensitive signaling cascades. For example, the enzyme asparaginase is used to treat acute lymphoblastic leukemia, and exogenous asparaginase sensitizes cultured mammalian cells to rapamycin, possibly by reducing extracellular levels of glutamine and asparagine and thereby impairing TOR-dependent

cell proliferation (Iiboshi et al. 1999a). Clearly, much still remains to be learned about TOR regulation of nutrient signaling, but studies in yeast continue to provide insights into conserved pathways as targets for therapy.

Materials and methods

RNA isolation and Northern analysis

Strain MLY41a was grown to an OD_{600} of 0.4 and treated or not treated with 0.2 $\mu\text{g/ml}$ rapamycin for various periods of time as indicated in figure legends. Cells were harvested by centrifugation, washed with DEPC-treated water, and immediately frozen in an ethanol dry-ice bath. Total RNA was isolated from 50 OD_{600} units of cells by use of the RNeasy Mini kit from Qiagen, according to the manufacturer's instructions. RNA was quantified by spectrophotometry, actin mRNA level was detected by autoradiography in an initial gel, and equal amounts of RNA normalized to the level of the actin message were loaded on 5% formaldehyde and 1.2% agarose gels. Gels were electrophoresed in 20 mM MOPS (pH 7.0), 5 mM NaOAc, and 1 mM EDTA. RNA was transferred to Nytran Plus membranes (Schleicher & Schuell) and cross-linked by UV irradiation. The membranes were then incubated for 2 hr at 42°C in prehybridization solution (5 \times SSC, 5 \times Denhardt's solution, 50% formamide, 0.2% SDS, 10 mg/ml salmon sperm DNA) and the ^{32}P -labeled radioactive probe was added and hybridized overnight at 42°C. The membranes were washed twice in 2 \times SSC at 42°C and four times at 62°C in 2 \times SSC, 0.2% SDS. RNA was visualized by autoradiography. Northern probes were labeled with [α - ^{32}P]dCTP with the random primer labeling kit from Stratagene. Quantitation of Northern blots was performed on a Molecular Dynamics Storm 860 PhosphorImager.

Gene chip hybridization and analysis

Total RNA preparation for gene array analysis was scaled up for 200 OD_{600} of cells and performed as above. Poly(A) mRNA was isolated from total RNA with a mini-oligo (dT) cellulose spin column kit from 5 prime-3 prime Inc. (Boulder, CO). Poly(A) mRNA was used to prepare cDNA with the Superscript Choice System (GIBCO BRL) and biotinylated cRNA was synthesized

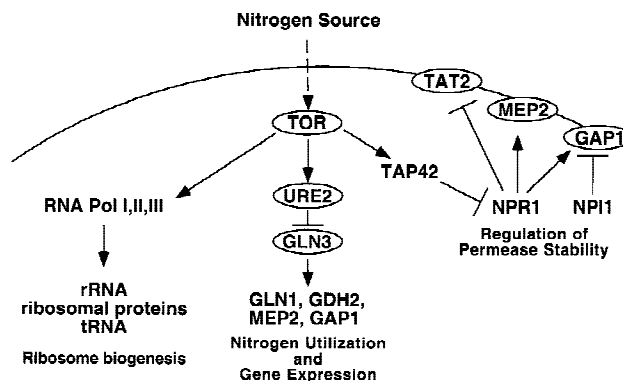


Figure 4. The TOR signaling cascade regulates gene expression in response to nutrients. The Tor proteins are activated by nutrients, and regulate the expression of genes involved in the utilization of nitrogen sources via Ure2 and Gln3, ribosome biogenesis by RNA polymerases, and amino acid permease stability.

with Biotin-11-CTP and the Megascript T7 Kit (Ambion). Biotinylated cRNA was fragmented by incubation at 94°C for 35 min in 40 mM Tris acetate (pH 8.1), 100 mM KOAc, and 30 mM MgOAc. Free unincorporated Biotin nucleotides were eliminated with the RNeasy Mini Kit (Qiagen). Biotinylated cRNA was hybridized to the gene chip arrays at 45°C overnight. Hybridization, washing, and streptavidin staining were performed in the Affymetrix Gene Chip fluidics station 400. Gene chips were scanned in a Hewlett-Packard G2500A Gene array scanner and expression data was analyzed with the Affymetrix Gene Chip analysis suite version 3.1.

Media and strains

Yeast media were prepared as described previously (Sherman 1991; Gimeno et al. 1992). Rapamycin and cycloheximide were added to the media from concentrated stock solutions in 90% ethanol/10% Tween 20 and water, respectively. Yeast transformations were performed by the lithium acetate method (Schiestl et al. 1993). Unless noted otherwise, mutant yeast strains were constructed by PCR-mediated gene disruptions with the G418-resistance gene cassette derived from template plasmid pFA6-kanMX2 as described (Table 3; Wach et al. 1994). MLY88 α and MLY90-1 were generated as spontaneous resistant mutants of strain MLY40 on YPD medium containing 0.2 μ g/ml rapamycin. The rapamycin-resistant mutation in strain MLY88 α was complemented by the introduction of a plasmid bearing the wild-type *FPR1* gene (pYJH23). Strain MLY90-1 was shown to harbor the *TOR1-4* mutation by meiotic segregation of the rapamycin-resistance phenotype opposite a *tor1::LEU2* deletion mutation following a genetic cross. MLY152 *a/a* was created by PCR mutagenesis of strain MLY40 α and the presence of the Ser-1975-Ile mutation in the *TOR2* gene confirmed by DNA sequencing. Strains MLY158 α , MLY148 α , and MLY146 α were constructed by mating strain MLY140 α with strains MLY152 α , MLY90-1, and MLY88 α , respectively, followed by sporulation and dissection of tetrads.

Protein extracts, alkaline phosphatase treatment, and Western analysis

Cells harvested and frozen as indicated above were resuspended in ice-cold lysis buffer (20 mM KPO₄ at pH 7.2, 1 mM EDTA, 1

mM EGTA, 0.5% Triton X-100, 25 mM β -glycerophosphate, 25 mM NaF, 100 μ M Na₃VO₄, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1% aprotinin, 1 μ g/ml pepstatin, 100 μ M leupeptin). Cells were lysed at 4°C with glass beads by five strokes of 1 min each in a bead beater (Biospec) with cooling for 1 min between strokes. Following centrifugation, protein was quantitated by Bradford assay (Amersham) and 100 μ g of protein extract was loaded onto SDS-polyacrylamide gels. Proteins were detected by Western blot analysis with rabbit polyclonal antisera against Ure2 (Wickner 1994) and ECL plus detection (Amersham). For alkaline phosphatase treatment, 100 μ g of protein extract were adjusted to 2 mM MgCl₂ and 4 units of alkaline phosphatase (Boehringer Mannheim) were added and samples incubated at 37°C for 30 min. Extracts were mixed with protein sample buffer boiled for 4 min and analyzed by Western blot as indicated above.

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Table 3. Strain list

Strain	Genotype	Source
MLY41	Σ 1278b <i>MATa ura3-52</i>	Lorenz (1997)
MLY40	Σ 1278b <i>MATα ura3-52</i>	Lorenz (1997)
MLY88	Σ 1278b <i>MATα ura3-52 fpr1</i>	this study
MLY139a	Σ 1278b <i>MATa ura3-52 Δgln3::G418</i>	Lorenz (1998)
MLY140a	Σ 1278b <i>MATa ura3-52 Δure2::G418</i>	Lorenz (1998)
MLY54a	Σ 1278b <i>MATa ura3-52 Δleu2::hisG Δnpr1::LEU2</i>	Lorenz (1998)
MLY141a	Σ 1278b <i>MATa ura3-52 npil</i>	Lorenz (1998)
MLY152 α	Σ 1278b <i>MATα ura3-52 TOR2-1 (Ser-1975-Ile)</i>	this study
MLY158 α	Σ 1278b <i>MATα ura3-52 TOR2-1 Δure2::G418</i>	this study
MLY90-1	Σ 1278b <i>MATα ura3-52 TOR1-4</i>	this study
MLY148 α	Σ 1278b <i>MATα ura3-52 TOR1-4 Δure2::G418</i>	this study
MLY146 α	Σ 1278b <i>MATα ura3-52 fpr1 Δure2::G418</i>	this study

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