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Elucidating TOR Signaling and Rapamycin Action: Lessons from *Saccharomyces cerevisiae*

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INTRODUCTION

The immunosuppressive and anticancer drug rapamycin acts by binding the highly conserved immunophilin FKBP12 (FK506-binding protein of 12 kDa, encoded by the *FPR1* gene in *Saccharomyces cerevisiae*), and the FKBP12-rapamycin complex then binds and inhibits the kinase TOR (target of rapamycin, encoded by the homologous *TOR1* and *TOR2* genes in *S. cerevisiae*) (63, 64, 135) (Fig. 1). This mechanism of action is conserved from yeasts to humans. Indeed, a number of important discoveries that contributed to elucidating rapamycin's mode of action, including the discovery of TOR, were made in *S. cerevisiae*. Since its discovery, TOR has been widely investigated and has been recognized as a central controller of cell growth in eukaryotes (52, 130). *S. cerevisiae* also played a significant part in elucidating the cellular role of TOR and in defining TOR signaling pathways. In this review, we discuss the contributions made by *S. cerevisiae* to understanding rapamycin action and TOR function.

RAPAMYCIN ACTION

Rapamycin is a potent antibiotic produced by a strain of *Streptomyces hygroscopicus* isolated from a soil sample collected in Rapa-Nui (Easter Island) (156). The pharmaceutical potential of rapamycin was originally discovered in a screen for novel antifungal agents. Much later, rapamycin was found to exhibit immunosuppressive activity due to its capacity to block the growth and proliferation of T cells (136, 142). More recently, rapamycin has been found to display anticancer properties (57, 70, 73).

Rapamycin binds 12-kDa FK506-binding protein (FKBP12) with high affinity. FKBP12 was first identified in vitro as a receptor of FK506, an immunosuppressant structurally related to rapamycin, and was later shown to be a cytoplasmic peptidylprolyl rotamase (62, 135). The observations that *S. cerevisiae* mutants lacking FKBP12 are viable and resistant to rapamycin toxicity and that rapamycin analogs still bind and inhibit FKBP12 rotamase activity but do not immunosuppress indicated that FKBP12 is not the target through which rapamycin blocks growth (14, 63, 64, 90, 135, 164). Rather, an FKBP12-rapamycin complex is the toxic agent that then acts on another target to inhibit cell growth.

The findings that dominant mutations in either *TOR1* (*TOR1-1*, Ser1972Arg) or *TOR2* (*TOR2-1*, Ser1975Ile) confer complete resistance to the growth-inhibitory properties of rapamycin and that such mutations prevent the binding of FKBP12-rapamycin to TOR demonstrated that TOR is the relevant target through which rapamycin blocks cell growth (19, 63, 101, 145, 171). Finally, the observation that loss of TOR function in *S. cerevisiae* mimics rapamycin treatment indicated that FKBP12-rapamycin inhibits TOR function (92). More details are given below concerning the mode of action of rapamycin and the evolutionary conservation of this mode of action.

TOR IS CONSERVED IN LOWER AND HIGHER EUKARYOTES

After the original identification of TOR in the yeast *Saccharomyces cerevisiae*, TOR was identified in fungi, mammals, flies, worms, and plants, suggesting that TOR is conserved in all

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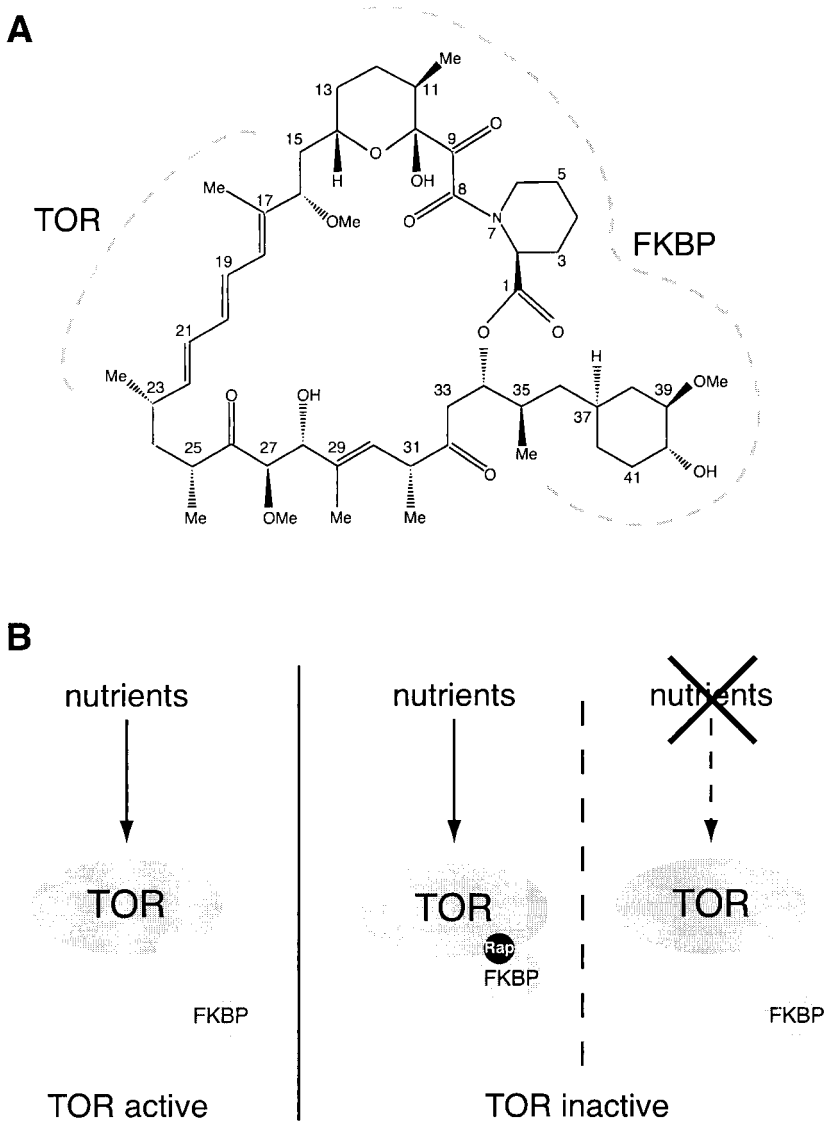

FIG. 1. Rapamycin-FKBP complex binds and inhibits TOR. (A) Chemical structure of rapamycin. The TOR- and FKBP-interacting regions of rapamycin (30) are indicated by dashed lines. (B) TOR is active in the presence of nutrients and inactive upon nutrient limitation or FKBP-rapamycin (Rap) binding.

eukaryotic life forms. In the opportunistic fungal pathogen *Cryptococcus neoformans,* a *TOR1* homologue was cloned by degenerate PCR, and a TOR2 homologue was detected by sequencing expressed sequence tags (36). Moreover, a *C. neoformans* FKBP12 homologue was cloned based on its ability to interact with the FKBP12-rapamycin binding domain in TOR1 (36). These findings revealed that the antifungal activity of rapamycin in *C. neoformans* is mediated via conserved complexes involving FKBP12 and TOR homologues. More recently, a TOR1 homologue has also been identified and characterized in the fungal pathogen *Candida albicans* (37), and TOR1 and TOR2 homologues have been identified and characterized in the fission yeast *Schizosaccharomyces pombe* (161, 162).

Unlike most lower eukaryotes, which contain two *TOR* genes, higher eukaryotes appear to possess only one *TOR* gene. The first TOR identified in a higher eukaryote was mammalian TOR (mTOR; also known as FRAP, RAFT, and RAPT). mTOR was discovered based on its ability to interact in vitro with the FKBP12-rapamycin complex (18, 127, 128) or by a two-hybrid screen (29). The subsequent demonstration that an mTOR variant constructed to contain a mutation (Ser2035Ile) analogous to the previously identified yeast *TOR2-1* mutation (Ser1975Ile) confers rapamycin resistance in mammalian cells indicated that mTOR is the in vivo target of FKBP12-rapamycin and that rapamycin action is conserved from yeasts to mammals (19). More recently, the isolation of *Drosophila melanogaster* mutants and the sequencing of the *Caenorhabditis elegans* and *Arabidopsis thaliana* genomes allowed the cloning and characterization of *TOR* genes (*dTOR*,

TABLE 1. TOR is evolutionarily conserved[a]

TOR protein	% Identity								
	ScTOR1	ScTOR2	Sptor1	Sptor2	CnTOR1	CeTOR	AtTOR	dTOR	mTOR
ScTOR1	—	67	42	47	39	28	36	37	38
ScTOR2		—	43	48	40	28	38	38	40
SpTOR1			—	52	42	28	38	40	42
SpTOR2				—	44	29	42	42	44
CnTOR1					—	26	35	38	39
CeTOR						—	28	32	35
AtTOR							—	38	40
dTOR								—	53
mTOR									—

[a] The table shows the identity (expressed as a percentage) between the TOR proteins so far identified in eukaryotes. ScTOR1, *Saccharomyces cerevisiae* TOR1; ScTOR2, *Saccharomyces cerevisiae* TOR2; SpTOR1, *Schizosaccharomyces pombe* TOR1; SpTOR2, *Schizosaccharomyces pombe* TOR2; CnTOR1, *Cryptococcus neoformans* TOR1; CeTOR, *Caenorhabditis elegans* TOR; AtTOR, *Arabidopsis thaliana* TOR; dTOR, *Drosophila* TOR; mTOR, mammalian TOR (human in this case).

CeTOR, and *AtTOR*, respectively) from these organisms (106, 114, 169; J. Avruch and F. Mueller, personal communication for *CeTOR*).

The *S. cerevisiae TOR1* and *TOR2* genes encode two large (approximately 280 kDa) and highly homologous (67% identical) TOR1 and TOR2 proteins. TOR1 and TOR2, as suggested by their similarity, are functionally redundant. However, TOR2 has an additional function that TOR1 is unable to perform (see below). In *S. pombe*, the two TOR proteins Tor1 and Tor2 are 52% identical to each other and 42 to 48% identical to the *S. cerevisiae* TOR1 and TOR2 proteins (161). mTOR, dTOR, CeTOR, and AtTOR show approximately 38%, 37%, 28%, and 36% identity with the *S. cerevisiae* TOR proteins, respectively (114, 169) (Table 1).

DOMAIN STRUCTURE OF TOR

A feature common to all TOR proteins is a conserved C-terminal region with strong homology to the related catalytic domains of phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 4-kinase (Fig. 2). The presence of this characteristic phosphatidylinositol kinase (PIK) homology domain has defined a TOR-related family of kinases termed the PIK-related kinases. In addition to TOR, this family includes the mammalian ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia related), DNA-dependent protein kinase, the *Drosophila* mei-41, and the yeast MEC1, RAD53, and TEL1 proteins (for a recent review, see reference 2). All these proteins are involved in diverse cellular functions, such as control of cell growth, regulation of cell cycle progression, a DNA damage checkpoint, recombination, and maintenance of telomere length. Despite significant homology to lipid kinases, no lipid kinase activity has been demonstrated for any of the PIK-related kinases. However, yeast and mammalian TORs have been reported to exhibit Ser/Thr protein kinase activity (see below). Genetic studies in *S. cerevisiae* have revealed that the integrity of the TOR kinase domain is essential for TOR function (3, 67, 92, 171).

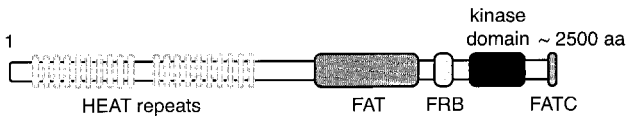

FIG. 2. Structure of the TOR kinases. Functional domains conserved in TOR proteins are depicted, including the N-terminal HEAT repeats, the central FAT domain, and the C-terminal FKBP-rapamycin binding (FRB), kinase, and FATC domains. aa, amino acids.

N-terminal to the kinase domain, the TORs contain a potential regulatory region of approximately 100 amino acids, the FKBP12-rapamycin binding (FRB) domain (Fig. 2). The location of the FRB domain in TOR was initially identified in *S. cerevisiae* by *TOR1* and *TOR2* point mutations that prevent FKBP12-rapamycin binding and confer dominant rapamycin resistance (22, 67). The FRB domain, the minimal protein domain capable of binding FKBP12-rapamycin, was then defined by deletion analysis of the region in *mTOR* corresponding to the region in *S. cerevisiae TOR* containing the rapamycin resistance-conferring mutations (28). Analysis of the original rapamycin resistance-conferring *TOR* alleles revealed that a Ser residue (Ser^{1972} in TOR1 and Ser^{1975} in TOR2) is important for the interaction of TOR with the FKBP12-rapamycin complex (22, 67, 145). The yeast $Ser^{1972/1975}$ is conserved in mTOR (18, 127). Subsequent genetic studies identified two additional residues (Trp^{2042} and Phe^{2049} in TOR2) also conserved in mTOR that play a critical role in FKBP12-rapamycin binding to the yeast TOR proteins (101).

The crystal structure of FKBP12-rapamycin bound to the FRB domain of mTOR has been determined (30). This structure revealed that FKBP12 and the FRB domain of mTOR interact primarily via rapamycin. Rapamycin simultaneously occupies a hydrophobic binding pocket in FKBP12 and a hydrophobic pocket in the FRB domain and thus "glues" FKBP12 and mTOR together (30). The residues that form the rapamycin-binding pocket of mTOR are conserved in the *S. cerevisiae* TOR1 and TOR2 proteins, and thus all three proteins are likely to contain a hydrophobic pocket with similar architecture (30). The protein-protein contacts between FKBP12-rapamycin and mTOR, although a minor contribution to the overall interaction between FKBP12-rapamycin and mTOR, may explain why rapamycin by itself cannot interact with TOR. The mechanism by which FKBP12-rapamycin inhibits TOR function is unknown. The FKBP12-rapamycin complex may inhibit TOR kinase activity directly or, for example, may block access to substrates or partner proteins (46, 117). mTOR kinase activity has been shown to be inhibited by

the FKBP12-rapamycin complex in vitro (19, 20, 137), but this inhibition remains controversial (21, 117).

In addition to the catalytic and FRB domains, TOR proteins also contain up to 20 tandemly repeated HEAT motifs at their N termini (5) (Fig. 2). The term HEAT motif is derived from the four proteins where this domain was originally identified: huntingtin, elongation factor 3, the A subunit of type 2A protein phosphatase (PP2A), and TOR. Each HEAT repeat consists of an antiparallel α-helical motif of approximately 40 amino acids (56, 68) and is thought to mediate protein-protein interactions. Recently, it has been proposed that HEAT repeats anchor *S. cerevisiae* TOR2 to the plasma membrane, possibly by mediating an interaction with a membrane-associated protein (93). The N-terminal part of mTOR has been shown to interact with gephyrin, a protein involved in the postsynaptic clustering of glycine receptors in spinal cord neurons (126).

Additional domains found in TOR and in other members of the PIK-related kinase family are the FAT and FATC domains (Fig. 2). The FAT domain, spanning approximately 500 amino acids N-terminal to the FRB and catalytic domains in TOR, is found only in members of the PIK-related kinase family (3, 17). Although the function of the FAT domain remains to be elucidated, it has been proposed that this domain could serve as a scaffold or as a protein-protein interaction domain, similar to the HEAT repeats (17). Finally, the FATC domain, a 35-amino-acid sequence in the extreme C terminus, occurs only in combination with the FAT domain and may be important for catalytic activity of PIK-related kinases (17, 87).

TOR SIGNALING IN YEASTS AND HIGHER EUKARYOTES

Organization of the Actin Cytoskeleton

S. cerevisiae TOR2 has two essential signaling functions. One function is shared with TOR1 and is required for activation of translation initiation and early G_1 progression in response to nutrients (see below) (7, 44). The second essential function is unique to TOR2 and mediates the cell cycle-dependent polarization of the actin cytoskeleton (132, 134). Polarization of the actin cytoskeleton is essential for targeting secretion to the bud site and thus for establishing and maintaining cell polarity (133). The shared function of TOR2 is sensitive to rapamycin, whereas the unique TOR2 function is not (134, 171). The molecular basis for the selective inhibition of only one of the two TOR2 functions by rapamycin is unknown.

TOR2 signaling to the actin cytoskeleton is mediated by activation of the small GTPase RHO1 via the exchange factor ROM2 (132, 133). RHO1, in turn, signals to the actin cytoskeleton via its direct effector protein kinase C1 (PKC1) and a PKC1-activated mitogen-activated protein (MAP) kinase cascade (65, 66). Although the PKC1-controlled MAP kinase cascade maintains cell integrity by activating the transcription of genes required for cell wall synthesis (74, 170), it remains to be determined whether control of the actin cytoskeleton by this MAP kinase cascade also occurs at the transcriptional level or in a more direct fashion. To date, it is not known if the rapamycin-insensitive, TOR2-unique function is conserved in other organisms. Rapamycin-insensitive TOR signaling has not been detected in mammalian cells, possibly because studies on TOR signaling in mammalian cells have relied exclusively on rapamycin to inhibit mTOR.

Translation Initiation

Initiation of protein synthesis in eukaryotes is a highly regulated process mediated by several polypeptide initiation factors (for a review, see Hershey and Merrick [69]). The heterotrimeric initiation factor 4F (eIF4F) mediates translation initiation by facilitating ribosome binding to the 5′ cap (m^7GpppN, where m is a methyl group and N is any nucleotide) structure of the mRNA. In *S. cerevisiae*, the eIF4F complex is composed of the cap-binding subunit eIF4E (encoded by *CDC33*), eIF4G (*TIF4631* and *TIF4632*, encoding two similar proteins termed eIF4G1 and eIF4G2, respectively), and eIF4A (encoded by *TIF1* and *TIF2*).

The loss of TOR function in yeast cells results in an early and severe inhibition of translation initiation (7). It is as a consequence of this translation defect that TOR-inhibited cells arrest in the G_1 phase of the cell cycle. The mechanism by which TOR1 and TOR2 activate translation initiation is uncertain, although the most plausible hypothesis is that the TOR pathway positively controls translation initiation through activation of eIF4E (7). Several observations suggest that TOR may control translation at the level of eIF4E. First, *cdc33* and *tor* mutants display remarkably similar phenotypes (7, 39). Second, the EAP1 protein has recently been identified in *S. cerevisiae* based on its capacity to interact with eIF4E (33). EAP1 blocks cap-dependent translation via competition with eIF4G, and disruption of the *EAP1* gene confers partial resistance to rapamycin, suggesting a role for EAP1 similar to the one described for mammalian eIF4E-binding protein 1 (4E-BP1; see below).

Translational inhibition upon TOR inactivation may involve degradation of the initiation factor eIF4G because degradation of eIF4G protein has been reported in rapamycin-treated cells (12). However, it is unknown whether the degradation of eIF4G is a primary cause of translation inhibition or a secondary effect of the translational downregulation caused by TOR inhibition.

Several lines of evidence indicate that the G_1 cell cycle arrest observed in rapamycin-treated cells is, at least in part, a consequence of the inhibited translation of *CLN3* (7, 44), a cyclin involved in G_1 progression (111, 154). The abundance of CLN3 depends on its relative rates of synthesis and degradation (166) and therefore, regulation of CLN3 synthesis or stability is a critical step in controlling progression through the cell cycle (7, 39, 48, 119). The finding that the G_1 arrest in response to TOR inactivation is suppressed by cap-independent expression of CLN3 supports a model in which TOR stimulates cap-dependent translation initiation, including translation of *CLN3* and other G_1 cyclins, to drive cells through G_1 and into S phase (7).

The eIF4F complex is highly conserved. Formation of the mammalian eIF4F complex is regulated by the 4E-BP family of translational repressors (11, 100, 108, 120). The 4E-BPs compete with the eIF-4G proteins for binding to eIF4E, and binding of 4E-BPs to eIF4E is regulated by the phosphorylation state of 4E-BPs (124). Low phosphorylation of 4E-BP promotes the formation of a 4E-BP–eIF4E complex, whereas high

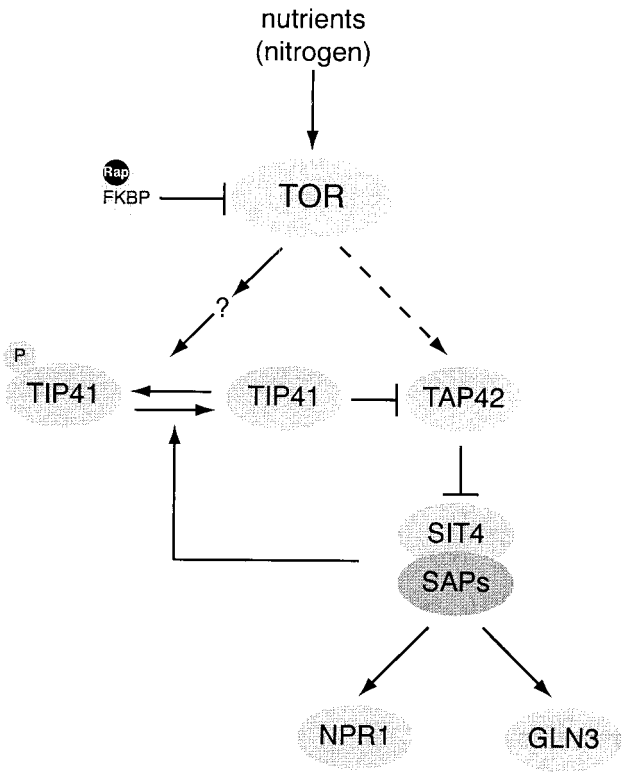

FIG. 3. TOR controls phosphatases in *S. cerevisiae*. Under good nutrient (nitrogen) conditions, TOR inhibits the phosphatase SIT4 by promoting the association of SIT4 with TAP42. Two different models have been proposed for the mechanism by which TOR controls the SIT4-TAP42 complex. Jiang and Broach (81) proposed that TOR controls the interaction between SIT4 and TAP42 by phosphorylating TAP42 directly (indicated by a dashed arrow). Jacinto et al. (79) suggested that the association of SIT4 and TAP42 is controlled primarily by the TAP42 interactor TIP41. TOR may phosphorylate and inactivate TIP41 by an unknown mechanism. Dephosphorylated TIP41 positively regulates SIT4 by binding and inhibiting TAP42. The association of TIP41 with TAP42 enhances SIT4 phosphatase activity, allowing free SIT4 subunits to associate with SAPs and activate target phosphoproteins such as NPR1 and GLN3. Dephosphorylation and activation of TIP41 are mediated by SIT4, indicating that TIP41 is part of a feedback loop that amplifies SIT4 activity. Arrows indicate activation; bars indicate inhibition. Adapted from Jacinto et al. (79). Rap, rapamycin.

phosphorylation of 4E-BP inhibits this interaction. mTOR, in conjunction with the PI3K signaling pathway, modulates 4E-BP phosphorylation. mTOR immunoprecipitates phosphorylate 4E-BP1 in vitro (21, 50), although it remains to be determined whether mTOR phosphorylates all or some of the phosphorylated sites in 4E-BP1 (51). mTOR and PI3K signaling may also contribute to control translation initiation in response to amino acids or insulin by regulating the phosphorylation state of eIF-4GI (123).

The 40S ribosomal phosphoprotein S6 has been proposed to affect translation initiation of a group of mRNAs possessing a 5′-terminal oligopyrimidine tract (5′ TOP) in mammals and *Drosophila melanogaster* (reviewed by Meyuhas and Hornstein [107]). Most 5′ TOP mRNAs encode components of the translational apparatus, such as ribosomal proteins, elongation factors, and the poly(A)-binding protein. mTOR, in the presence of amino acids, promotes phosphorylation of S6 through activation of p70 S6 kinase ($p70^{S6k}$). Phosphorylation of S6, in turn, results in the upregulation of translation initiation. mTOR phosphorylates $p70^{S6k}$ in vitro, suggesting that mTOR may act on this protein directly (21, 78). $p70^{S6k}$ phosphorylation is also controlled by the PI3K signaling pathway in response to growth factors such as insulin (152). Interestingly, the mTOR and PI3K inputs on $p70^{S6k}$ can be separated (43, 104). Thus, mTOR and PI3K also control translation initiation by regulating $p70^{S6k}$ activity.

Early on, mTOR was known to control translation only via $p70^{S6k}$ and S6 phosphorylation (31, 47, 94, 122). However, the observation that phosphorylation of the yeast equivalent of S6 (S10) is not important for growth led to the model in *S. cerevisiae* that TOR controls translation via eIF-4E and cap-dependent translation (7, 82). This model from *S. cerevisiae*, in turn, led to the finding that mTOR controls translation via eIF-4E (and 4E-BP) in addition to $p70^{S6k}$ (10). 4E-BP phosphorylation was originally thought to be controlled by MAP kinase independently of mTOR (100, 115) rather than by mTOR independently of MAP kinase (10, 157).

Ribosome Biogenesis

The production of ribosomes is an energetically very costly process that requires the action of all three RNA polymerases and involves more than 100 gene products (160). Thus, to couple the rate of protein synthesis to the energetic and metabolic demands of the cell, the abundance of the components of the translation machinery must be finely regulated according to growth conditions and nutrient availability. In *S. cerevisiae*, TOR signaling regulates ribosome biogenesis at both the transcriptional and translational levels and plays an important role in coupling nutrient availability to the transcription of genes involved in the formation of ribosomes. Inhibition of the TOR pathway by rapamycin treatment or nutrient starvation leads to a downregulation of transcription of ribosomal protein mRNAs by polymerase II (24, 60, 121) as well as transcription of rRNA and tRNA by polymerase I and polymerase III (121, 168). TOR also controls processing of at least the 35S precursor rRNA (121).

All the above findings point to TOR signaling as an essential pathway in the control of ribosome biogenesis. However, the mechanism by which TOR controls the synthesis of ribosomes remains largely unknown. TAP42, an essential phosphoprotein that interacts with the catalytic subunits of protein phosphatase 2A (PP2A) and PP2A-related phosphatases, is under control of the TOR pathway (see below) (44, 81). The finding that a mutation in *TAP42* inhibits polyribosome formation suggests that TAP42/protein phosphatase functions upstream of translational initiation (44). Furthermore, a role for TAP42/protein phosphatase in regulating ribosomal protein and rRNA gene expression has also been proposed (121).

In mammalian cells, mTOR controls the synthesis of rRNA in a process that involves activation of $p70^{S6k}$ (96, 103). mTOR also regulates the abundance of ribosomal proteins and other components of the translation machinery, such as the poly(A)-binding protein, by promoting translation of 5′ TOP mRNAs (80) (see above). Thus, like yeast TOR, mTOR controls ribo-

some biogenesis at both the transcriptional and translational levels.

Control of Phosphatases by TOR

The regulation of phosphatase activity constitutes a particularly important branch in TOR signaling (130). Genetic screens in *S. cerevisiae* have identified TAP42 (a phosphatase-associated protein of 42 kDa), the type 2A phosphatase (PP2A) catalytic subunits PPH21 and PPH22, and the type 2A-related phosphatase SIT4 as components of the TOR signaling pathway (44). TAP42 is an essential, conserved protein that independently associates with SIT4 and with the catalytic subunits of PP2A in response to nutrient availability and TOR activity. In the presence of a good nitrogen source such as ammonium or glutamine, TOR keeps SIT4 inactive by promoting the binding of TAP42 to SIT4 (Fig. 3). Upon nitrogen starvation or rapamycin treatment (conditions that inactivate TOR), SIT4 is released from TAP42 and thereby activated (8, 44). As described below, activated SIT4 dephosphorylates target proteins such as NPR1 and GLN3. Interestingly, TAP42-associated SIT4 may not be inactive for substrates other than NPR1 and GLN3 (38).

TOR may control the association of TAP42 with the PP2A catalytic subunits and with SIT4 by phosphorylating TAP42 directly (81). Phosphorylated TAP42 effectively competes with the PP2A regulatory subunits CDC55 and TPD3 for binding to the PP2A catalytic subunits, whereas dephosphorylated TAP42 does not compete for binding (81). However, although rapamycin treatment causes dephosphorylation of TAP42 in vivo (81), it is unlikely that the phosphorylation state of TAP42 plays a major role in SIT4 regulation (79). Upon rapamycin treatment, dephosphorylation of TAP42 occurs much more slowly than dissociation of the SIT4-TAP42 complex (44) and also much more slowly than SIT4 appears to be activated (8, 131). Thus, another protein(s) may participate in the regulation of SIT4. Recently, a conserved TAP42-interacting phosphoprotein, TIP41, was identified in *S. cerevisiae* (79). TIP41 positively regulates SIT4 by binding to and inhibiting TAP42 (79). The binding of TIP41 to TAP42 is stimulated by rapamycin treatment via SIT4-dependent dephosphorylation of TIP41, suggesting that TIP41 is part of a feedback loop that rapidly amplifies SIT4 phosphatase activity under TOR-inactivating conditions (79) (Fig. 3). Whether TOR phosphorylates TIP41 directly (or indirectly) remains to be determined.

TAP42 is conserved in mammals and plants (61, 109), suggesting that a regulation of PP2A activity similar to that found in *S. cerevisiae* may exist in higher eukaryotes. Indeed, the murine α4 phosphoprotein, the mammalian homologue of TAP42, binds directly to the catalytic subunits of PP2A (77, 109), PP4, and PP6 (27, 110). However, there is substantial controversy regarding the rapamycin sensitivity of the α4-phosphatase interaction (27, 109). Although it is unclear which component(s) of the α4-phosphatase complex is sensitive to rapamycin, inactivation of mTOR by rapamycin causes rapid dephosphorylation of the ribosomal S6 kinase $p70^{s6k}$ by PP2A (41, 118), suggesting that TOR also negatively controls phosphatase activity in mammals.

Regulation of Amino Acid Permeases

Yeast cells can utilize a wide variety of compounds as nitrogen or carbon sources, including sugars, amino acids, and peptides. This versatility is determined by the ability of cells to transport these nutrients across the plasma membrane, followed either by their direct utilization or by conversion to metabolites required by the cell. Amino acids are essential for cell growth because they constitute the building blocks for protein synthesis. However, in *S. cerevisiae*, some amino acids (such as glutamine, glutamate, and asparagine) are also important because they serve as nitrogen sources. Therefore, amino acid permeases play an important role in cell growth and viability.

Based on their function and regulation, yeast amino acid permeases can be divided into two classes (144). Permeases of one class, including the general amino acid permease GAP1, are regulated in response to the available nitrogen source. In the presence of a good nitrogen source, such as ammonium or glutamine, the uptake activity of these permeases is low, whereas in medium containing a poor nitrogen source, such as proline or urea, transport activity is strongly induced. The second class of amino acid permeases consists of transporters that are specific for single amino acids or a small set of structurally related amino acids. The histidine permease HIP1 and the tryptophan permease TAT2 belong to this group of specific amino acid permeases.

Studies in *S. cerevisiae* have revealed that the TOR pathway plays a prominent role in regulation of amino acid permease activity. Inhibition of TOR function by rapamycin or nitrogen starvation induces ubiquitination and degradation of TAT2 and, as a consequence, leads to a decrease in tryptophan import (9, 131). Starvation-induced downregulation of amino acid permeases also applies to HIP1 and possibly to all specific amino acid permeases (9). In contrast to TAT2 and HIP1, rapamycin treatment causes a significant increase in GAP1 protein (9, 131). Thus, TOR proteins appear to regulate inversely the high-specificity permeases, such as TAT2 and HIP1, and the broad-specificity permease GAP1 in response to nutrient availability.

Upregulation of GAP1 upon nitrogen starvation is mediated by the Ser/Thr nitrogen permease reactivator kinase NPR1 (40, 54, 55, 155). In the presence of a poor nitrogen source, NPR1 promotes GAP1 function (54, 55), probably by phosphorylating and protecting GAP1 from degradation (40, 146). In agreement with the opposite regulation of GAP1 and TAT2, NPR1 has been proposed to function as a negative regulator of TAT2. Indeed, tryptophan import decreases upon NPR1 overexpression (131). How does the cell modulate NPR1 to inversely regulate GAP1 and TAT2 in response to the nitrogen source? NPR1 is a phosphoprotein whose phosphorylation state is controlled by the TOR signaling pathway in response to the nitrogen source (131). In response to a good nitrogen source, TOR keeps NPR1 phosphorylated and in an inactive form that is unable to protect GAP1 from ubiquitination. Under poor nitrogen conditions, NPR1 becomes dephosphorylated and activated in a SIT4- and TIP41-dependent manner (79). Activation of NPR1 leads to GAP1 protection and to TAT2 ubiquitination and degradation (131). It is unknown whether NPR1 directly phosphorylates GAP1 or TAT2.

At present, it is not known whether TOR plays a role in regulating the traffic of nutrient permeases through the membrane in higher eukaryotes. However, in a recent report, it was proposed that the mTOR pathway may be involved in a process that rapidly mobilizes the glucose transporter GLUT4 to a highly insulin-responsive compartment upon insulin stimulation (16). mTOR has also been proposed to play an important role in the stimulation of another glucose transporter, GLUT1, by insulin, although this effect appears to be at the level of translation (148).

Autophagy

In response to nitrogen or carbon limitation, yeast cells undergo a catabolic membrane-trafficking process known as autophagy (for recent reviews, see references 1 and 89). During this process, a large number of cytoplasmic components are nonselectively enclosed within a double-membrane structure (autophagosome) and delivered to the vacuole for degradation. Although autophagy was first described in developing kidney cells (32), the most important advances in the understanding of autophagy were obtained much later with the discovery of autophagy in *S. cerevisiae* and the isolation of the first autophagy mutants (149, 153).

Inactivation of TOR function by rapamycin induces autophagy even in rich nutrient conditions, indicating that TOR inhibits autophagy (113). The mechanism by which TOR inhibits autophagy is being elucidated by Ohsumi and coworkers. Kamada et al. (85) reported that the protein kinase APG1 is essential for autophagy and plays a pivotal role in the control of autophagy by TOR. APG1 associates with APG13 and APG17 to form the APG1 protein complex (105). The role of TOR in the regulation of autophagy is to maintain APG13 in a phosphorylated form with low affinity for APG1 and thereby to inhibit APG1 activity (85) (Fig. 4). Inactivation of TOR by rapamycin treatment or nutrient starvation causes rapid dephosphorylation of APG13, which increases the affinity of this protein for APG1 and enhances APG1 kinase activity (85). How TOR promotes APG13 phosphorylation is currently unknown. The finding that mutations in the TOR-controlled protein TAP42 have no effect on either APG1 activity or autophagy induction suggests that the APG1-APG13 interplay comprises a novel TOR signaling pathway regulating autophagy (85).

Autophagy also occurs in animal cells that are serum starved or challenged with specific hormonal stimuli (for recent reviews, see references 1 and 147). A number of yeast autophagy genes, such as *APG1*, *APG5*, *APG7*, *APG12*, and *AUT7*, have homologues in mammals (83, 129, 150), but only some of these homologues have been demonstrated to participate in autophagy in mammalian cells. This is the case for the mammalian gene *beclin-1*, a homologue of yeast *APG6* that promotes autophagy in autophagy-defective *S. cerevisiae* (97). Interestingly, the mTOR signaling pathway may play a role in the regulation of autophagy, as rapamycin addition to mammalian cells in culture induces autophagy even in nutrient-rich medium (15, 141). Inhibition of autophagy by mTOR may involve the $p70^{S6k}$ signaling branch (15, 141).

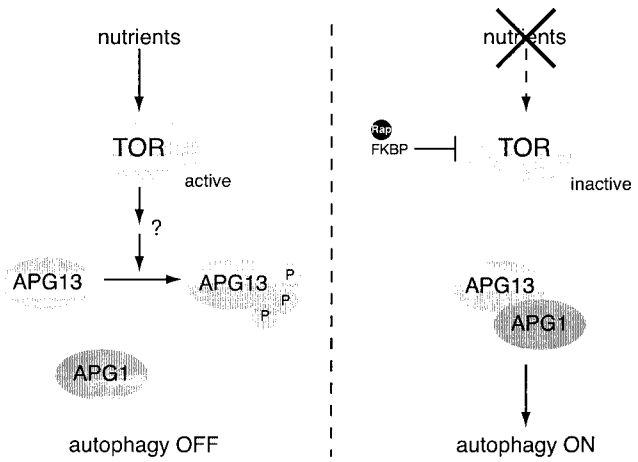

FIG. 4. TOR inhibits autophagy in *S. cerevisiae*. Under good nutrient conditions, TOR inhibits autophagy by promoting phosphorylation (P) of APG13 and thereby preventing the formation of an APG1-APG13 complex, which is essential for the induction of autophagy. Inactivation of TOR by rapamycin (Rap) treatment or nutrient deprivation results in rapid dephosphorylation of APG13. Dephosphorylated APG13 associates with APG1, and the active APG13-APG1 complex induces autophagy. Arrows indicate activation; bars indicate inhibition.

Transcriptional Control of Nutrient Metabolism

Control of gene expression at the level of transcription represents an important branch of TOR signaling. Genome-wide expression analysis of yeast cells treated with rapamycin or of cells shifted from a rich to a poor nitrogen or carbon source revealed a prominent role of TOR in the coordination of transcription of nutrient-regulated genes (24, 60, 91, 140). Rapamycin rapidly and strongly modulates the expression of several hundred genes involved in various metabolic pathways, including nitrogen metabolism, the glycolytic pathway, and the tricarboxylic acid cycle. The most striking set of genes affected by rapamycin treatment, however, are those involved in the uptake and assimilation of different nitrogen sources. Specifically, rapamycin causes a decrease in the expression of genes participating in the uptake and metabolism of preferred nitrogen sources (glutamine and ammonium) and a pronounced increase in the expression of genes involved in the uptake and use of poor nitrogen sources (urea and proline). Thus, rapamycin induces a nitrogen starvation response.

TOR controls the expression of nutrient-regulated genes by sequestering several nutrient-responsive transcription factors in the cytoplasm (8). The expression of most of the nitrogen-responsive genes is regulated by the GATA transcription factors GLN3 and GAT1 and their cytoplasmic repressor URE2 (102). Under good nitrogen conditions, GLN3 is retained in the cytoplasm by URE2. The binding of GLN3 to URE2 requires TOR-dependent phosphorylation of GLN3 by a mechanism that involves TAP42-mediated inhibition of the phosphatase SIT4 (8) (Fig. 5). Rapamycin treatment or nitrogen starvation causes GLN3 to become dephosphorylated and to dissociate from URE2. GLN3 then translocates into the nucleus to activate its target genes (8) (Fig. 5). GLN3 importin and exportin have recently been identified as SRP1 and

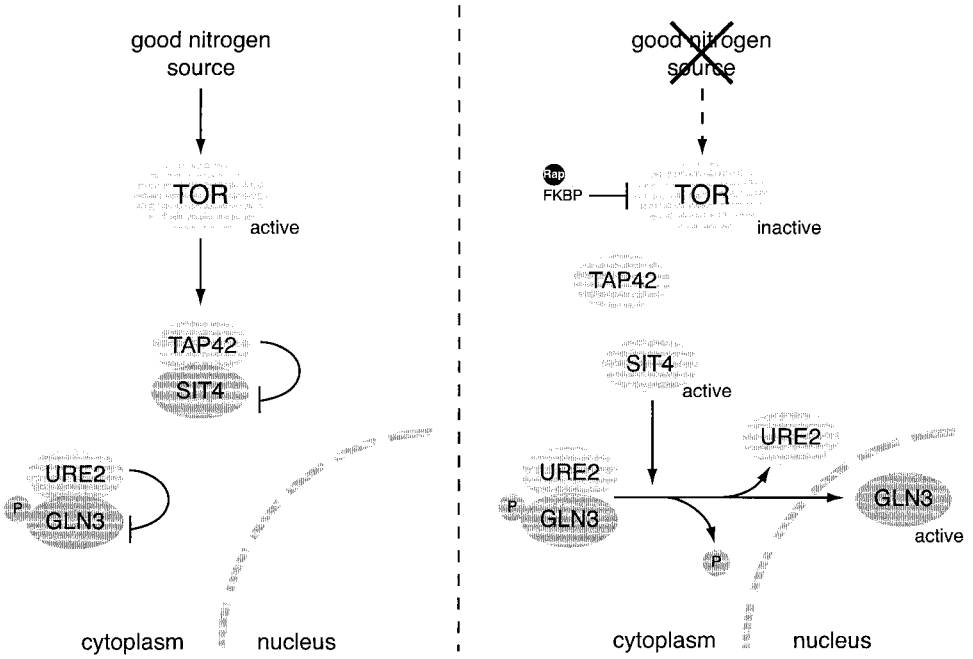

FIG. 5. TOR prevents nuclear accumulation of the nitrogen-regulated transcription activator GLN3 via TAP42-mediated inhibition of the phosphatase SIT4. Under good nitrogen conditions, GLN3 is phosphorylated and retained in the cytoplasm by URE2. Upon nitrogen starvation or rapamycin treatment, SIT4 is released from TAP42 and activated. Activated SIT4 dephosphorylates the GATA transcription factor GLN3. Dephosphorylated GLN3 dissociates from URE2 and translocates into the nucleus, where it activates transcription of target genes. Arrows indicate activation; bars indicate inhibition.

CRM1, respectively (25). URE2 is also phosphorylated in a rapamycin-sensitive manner (24, 60), but the regulation of URE2 phosphorylation is TAP42 independent. TOR also prevents the access of GAT1 to the nucleus (8), but, although GAT1 inhibition might be mediated by URE2, a direct interaction between GAT1 and URE2 has not been shown. Several lines of evidences suggest that GLN3 and GAT1 are regulated in response to different stimuli (35, 95).

Inactivation of TOR by rapamycin also activates the partially redundant Zn^{2+} finger transcription factors MSN2 and MSN4 (8), both of which respond to different types of cellular stress, including carbon source limitation (53, 143, 151). TOR may inhibit MSNs (MSN2 and MSN4) in response to nutrients by promoting the association of MSNs with the abundant 14-3-3 proteins BMH1 and/or BMH2 (8). In agreement with this model, BMH1 and BMH2 have been shown to positively regulate rapamycin-sensitive signaling (13). Glucose withdrawal or rapamycin treatment causes a release of MSN from BMH2. Contrary to what was observed for GLN3, this process is independent of TAP42 and SIT4 (8), suggesting that another, unknown TOR pathway regulates MSN.

TOR also controls the heterodimeric bHLH/Zip transcription factor composed of RTG1 and RTG3 (91). *RTG1* and *RTG3* were originally identified as genes required under conditions in which mitochondrial respiratory function is impaired (98, 99). RTG1 and RTG3 regulate the expression of tricarboxylic acid and glyoxylate cycle genes that participate in the synthesis of intermediates, primarily α-ketoglutarate, required for de novo synthesis of some amino acids, such as glutamine and glutamate. Similar to GLN3 regulation, inhibition of TOR by rapamycin or nitrogen starvation results in both rapid nuclear accumulation of RTG1 and RTG3 and induction of their target genes (91). RTG2, a positive regulator of RTG1 and RTG3, is essential for the TOR- and nitrogen-inhibited nuclear accumulation of RTG1 and RTG3 (91).

How TOR maintains RTG1 and RTG3 in the cytoplasm is not well understood. It has been reported that RTG3 is phosphorylated upon rapamycin-mediated inactivation of TOR (91). Whether RTG3 phosphorylation is influenced by TAP42 and/or SIT4 (PP2A) remains to be determined. Recently, it was reported that MKS1, a phosphoprotein controlled by TOR, is a negative regulator of RTG1 and RTG3, suggesting a high degree of complexity in regulation of the RTG branch of TOR signaling (45, 138).

mTOR signaling also controls transcription in mammalian cells. Recently, it was reported that mTOR phosphorylates the transcriptional activator STAT3 (167). Activation of STAT3 occurs in response to the neuropoietic cytokine ciliary neurotrophic factor and requires phosphorylation on tyrosine and serine residues (163). While members of the Jun-associated kinase/Tyk family of tyrosine kinases mediate phosphorylation of STAT3 on Tyr705, mTOR appears to phosphorylate STAT3 on Ser727 directly.

The TOR signaling pathway in yeast and mammalian cells controls gene expression via mRNA stability in addition to mRNA synthesis. Inhibiting TOR signaling, through either nutrient limitation or rapamycin treatment, causes the accelerated turnover of a subset of mRNAs (4, 6). Moreover, the inhibition of TOR appears to destabilize mRNAs by multiple mechanisms (4).

TOR RESPONDS TO NUTRIENTS

TOR inactivation by rapamycin treatment results in a nutrient starvation response, suggesting that TOR responds to nutrient availability (see above) (7). In *S. cerevisiae*, TOR signaling has been proposed to respond to nitrogen and possibly carbon sources (8, 35, 95, 131, 140). However, the specific nitrogen or carbon metabolites that act upstream of TOR are unknown. Recent evidence argues that TOR signaling, or at least a specific subset of the TOR pathways, responds to the amino acid glutamine, suggesting that glutamine is a particularly important indicator of nutrient status (35). Indeed, glutamine is a preferred nitrogen source and controls carbon metabolism via the tricarboxylic acid cycle (35, 102). Glutamine depletion activates the TOR-controlled transcription factors GLN3, RTG1, and RTG3. However, other TOR readouts, such as localization of MSN2/4 and GAT1 and downregulation of ribosomal protein mRNAs, are not activated under the same glutamine starvation conditions, suggesting that TOR must also respond to other yet-to-be identified nutrients (35). Schreiber and coworkers have proposed that TOR may act as a multichannel processor that can elicit different responses to distinct nutrient signals (95, 140).

Glutamine may play a particularly important role in TOR signaling in both yeast and mammalian cells. TOR in *S. cerevisiae* (35), as described above, and in mammalian cells (75) appears to respond to glutamine. Furthermore, a recent transcriptional profiling of mammalian cells revealed that rapamycin treatment mimics glutamine or leucine starvation more than glucose starvation (116). Interestingly, a decrease in blood glutamine levels causes immunosuppression in humans and mice similar to that caused by rapamycin treatment (23, 84).

In mammalian cells, amino acid deprivation causes rapid dephosphorylation and activation of the eIF-4E binding protein (4E-BP) and dephosphorylation and inhibition of $p70^{S6k}$ (59, 158, 165). Both of these processes are mediated by mTOR, indicating that amino acids signal to 4E-BP and $p70^{S6k}$ via mTOR. It has been proposed that the aminoacylation state of tRNA may be responsible for the regulation of $p70^{S6k}$ phosphorylation (76). In yeast cells, the GCN2 kinase senses intracellular amino acid availability through a tRNA-binding domain that exhibits similarity to histidyl-tRNA synthetase. Upon amino acid limitation, levels of uncharged tRNA increase significantly and activate the kinase domain of GCN2 kinase, leading to activation of GCN4, a transcriptional activator of several genes involved in amino acid biosynthesis (reviewed by Hinnebusch [71, 72]). Although the GCN2 pathway is conserved in mammals (88) and despite the similarity of the mechanism of amino acid sensing proposed for GCN2 and mTOR, a connection between these two signaling pathways has never been demonstrated. Dennis et al. (42) argue that amino acid pools rather than the amount of aminoacylated tRNA are important for mTOR signaling. Moreover, sensing of the intracellular level of glutamine in *S. cerevisiae* seems to be independent of the aminoacylated state of glutaminyl-tRNA (our unpublished data). Thus, how TOR may sense amino acid pools is unknown.

In mammals, both TOR and PI3K signaling are required for regulation of common downstream effectors such as 4E-BP and $p70^{S6k}$ (52, 130). Although the nature of the link between mTOR and the PI3K signaling pathway is controversial, most evidence suggests that mTOR activity is not regulated by PI3K. Whereas some studies have presented evidence that protein kinase B (PKB), a downstream effector of PI3K, stimulates phosphorylation and activity of mTOR in response to insulin and that mTOR is a direct substrate of PKB (112, 137, 139), other studies have failed to detect significant alteration of mTOR kinase activity in response to amino acids or insulin (58, 59). Furthermore, mTOR mutants bearing Ala substitutions at two PKB-dependent phosphorylation sites (Ser^{2448} and Thr^{2446}) indicate that PKB-dependent phosphorylation of mTOR is not essential for mTOR function (139). Finally, cells deficient in the PI3K effector PDK1 fail to activate PKB yet display normal mTOR activity, indicating that PKB activity is not necessary for mTOR activity (159). Thus, mTOR and PI3K, although connected by common targets, may respond separately to amino acids and growth factors, respectively.

Recently, two different, novel mechanisms have been proposed for regulation of mTOR. Dennis et al. (42) have shown that the mTOR pathway is influenced by the intracellular concentration of ATP. Although the mechanism by which ATP acts on TOR is unknown, it appears to be different from that used by amino acids, as both ATP and amino acids are required for mTOR signaling (42). Fang et al. (46) reported that phosphatidic acid, which accumulates in mammalian cells upon mitogenic stimulation, is required for activation of mTOR downstream effectors. Phosphatidic acid interacts directly with the FRB domain in mTOR, and this rapamycin-sensitive interaction correlates with the ability of mTOR to activate downstream effectors (46). The finding that phosphatidic acid has no effect on mTOR kinase activity (and the effect of rapamycin or insulin on mTOR kinase activity is controversial, as discussed above) suggests that the inhibitory effect of rapamycin on mTOR may derive from its competition with phosphatidic acid for binding to the FRB, independent of an effect on intrinsic mTOR kinase activity (46). In other words, displacement of phosphatidic acid by rapamycin may affect the interaction of mTOR with a substrate rather than intrinsic mTOR kinase activity.

TOR AND STRESS

TOR promotes cell growth in response to nutrient availability (125, 130). Several lines of evidence suggest that TOR also plays a role in cell growth under stress conditions other than nutrient limitation. First, TOR controls the transcription factors MSN2 and MSN4 (8), which activate expression of genes in response to several different environmental stress conditions, including heat shock and H_2O_2 treatment (26, 49). As described above, TOR controls cellular localization of MSN2 and MSN4 by preventing their access to the nucleus (8). The specific environmental conditions in response to which TOR controls MSN remains to be determined. Second, *S. pombe* cells lacking TOR1 are sensitive to osmotic stress, oxidative stress, high external pH, and high or low temperature (86, 161). Third, *tor1* mutants of *S. cerevisiae* are sensitive to high concentrations of salt, suggesting that TOR1 is necessary for the proper cellular response to saline stress (34). Although the role of TOR in salt stress signaling is at present unknown, a connection between the TOR-controlled transcription factors

GLN3 and GAT1 and the lithium and sodium extrusion pump ENA1, which is essential for survival under saline stress conditions, has been shown (34). GLN3 and GAT1 mediate induction of *ENA1* transcription upon rapamycin treatment and, like TOR1, are required for growth under salt stress (34).

Why does TOR respond to environmental stress? One plausible explanation is that TOR, as a central controller of cell growth, may respond to several different types of stress to ensure that growth occurs only when overall conditions are favorable.

CONCLUSION

TOR appears to activate a number of different effector pathways in response to a wide variety of stimuli, including the nitrogen source, amino acids, ATP, phosphatidic acid, and stress. The mechanisms by which TOR responds to and integrates different inputs remain to be determined. However, the answers to these open questions will most likely come, at least in part, from *S. cerevisiae*.

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