Cell, Vol. 82, 121–130, July 14, 1995, Copyright © 1995 by Cell Press

TOR Kinase Domains Are Required for Two Distinct Functions, Only One of Which Is Inhibited by Rapamycin

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Summary

The rapamycin-sensitive signaling pathway is required to transduce specific mitogenic signals to the cell cycle machinery responsible for G1 progression. Genetic studies in yeast identified two related genes on this pathway, *TOR1* and *TOR2*, thought to encode novel phosphatidylinositol kinases. We now show that an intact kinase domain is required for the G1 cell cycle functions of both proteins, for the ability of a mutation in a neighboring FKBP12-rapamycin-binding domain of the TOR1 protein to inhibit the growth of yeast cells when overexpressed, and for the essential function of the TOR2 protein. The G1 function of both TOR proteins is sensitive to rapamycin, but the essential function of TOR2 is not. Thus, FKBP12-rapamycin does not appear to inhibit the kinase activity of TOR proteins in a general way; instead, it may interfere selectively with TOR protein binding to or phosphorylation of G1 effectors.

Introduction

The bacterially derived immunosuppressants FK506 and rapamycin have proved to be useful tools for dissecting the signaling pathways that result in resting T cells making a commitment to divide (Schreiber, 1992). This process requires two distinct signals delivered in tandem. Signals emanating from the T cell receptor (TCR) render the cell competent for cell division by establishing a second, autocrine signaling pathway involving interleukin-2 (IL-2) and the IL-2 receptor (IL-2R). Signals from the IL-2R commit the cell to a program of cell division by communicating with components of the cell cycle machinery necessary for DNA replication. Although both FK506 and rapamycin inhibit the ability of T cells to divide by binding to the same immunophilin receptor (FKBP12), the resulting complexes target different signaling pathways. FKBP12–FK506 inhibits the TCR signaling pathway by binding to and inhibiting the phosphatase activity of calcineurin, one of the components of the TCR pathway; a second immunosuppressant–immunophilin complex, cyclosporin–cyclophilin, also inhibits calcineurin (Liu et al., 1991). In contrast, FKBP12–rapamycin inhibits the IL-2R signaling pathway by binding to a 289 kDa kinase named FRAP in humans and RAFT in rats (Brown et al., 1994; Sabatini et al., 1994; Chiu et al., 1994). In effect, the immunosuppressants have two protein-binding surfaces that mediate the heterodimerization of target proteins (immunophilins and the signaling proteins calcineurin and FRAP) and can, thus, be considered as analogous to proteins such as Grb2, which heterodimerize their targets.

Although the rapamycin-sensitive target was first examined in T cells, it is used by many eukaryotic cells to communicate changes in cell growth circumstances to the cell cycle machinery necessary for DNA replication. For example, low concentrations of rapamycin induce G1 cell cycle arrest in yeast. Genetic studies in the yeast Saccharomyces cerevisiae identified two related genes, *TOR1* and *TOR2*, containing apparent open reading frames encoding 2470 amino acids and 2474 amino acids, respectively (Heitman et al., 1991; Cafferkey et al., 1993; Kunz et al., 1993; Helliwell et al., 1994). The highest degree of similarity, with over 60% amino acid identity, resides in their C-termini, which are related to the catalytic domains of phosphatidylinositol (PI) kinases. This C-terminal kinase domain is interchangeable between *TOR1* and *TOR2*, suggesting they have similar functions (Helliwell et al., 1994). That the proteins themselves have distinct functions is evident from studies of the *tor1* null mutant, which has only a partial growth defect under normal growth conditions, and the *tor2* null, which has a lethal phenotype with cells arrested randomly in the cell cycle. Like treatment with rapamycin, the *tor1 tor2* double disruption results in G1 arrest (Kunz et al., 1993; Helliwell et al., 1994). When the conserved serine residues, Ser-1972 in *TOR1* and Ser-1975 in *TOR2*, are changed to isoleucine or arginine, either gene confers dominant rapamycin resistance.

The C-terminal kinase domains in FRAP and TOR are more than 60% identical. The substrate(s) of these domains has not yet been identified, despite their apparent similarity to the PI3-kinase (PI3K) and PI4-kinases (PI4K). The two cloned PI3Ks, the p110 PI3K in mammals and Vps34 in yeast, also have protein kinase activity (Dhand et al., 1994; Stack and Emr, 1994). Although the consequences of the two kinase activities are currently unknown, roles have been established for these proteins in several signaling pathways, including mitogenic pathways (for reviews see Carpenter and Cantley, 1990; Divecha and Irvine, 1995).

In this study, we show that TOR1 binds directly to FKBP12–rapamycin and that a small region surrounding Ser-1972, the FKBP12–rapamycin-binding (FRB) domain, is sufficient for mediating this interaction. Substitution of Ser-1972 with residues larger than serine abolish this interaction both in vivo and in vitro and confer dominant rapamycin resistance, although substitution with alanine does not. Genetic analyses indicate that the kinase domains in both TOR proteins are required for their rapamycin-sensitive G1 functions. Point mutations that inactivate

known PI and protein kinases (kinase-dead mutations) were found to abolish the ability of the TOR mutants at Ser-1972 (TOR1) and Ser-1975 (TOR2) to allow growth in the presence of rapamycin and to abolish the ability of one of these, TOR1 (Ser-1972→Ile), to produce a unique, dominant cell growth inhibitory phenotype when overproduced. Furthermore, overproduction of a TOR kinase-dead mutant causes G1 cell cycle arrest. These results establish that the kinase domains of TOR proteins are essential for G1 cell cycle progression in yeast. We also show that a function of TOR2 that is required for cell viability, the essential function, is dependent on a functional TOR kinase domain. This essential function is nonetheless resistant to the actions of rapamycin, in contrast with its G1 function. The results suggest that FKBP12–rapamycin may interfere with the binding or phosphorylation of G1 effectors only, rather than inhibit the kinase activity of TOR proteins in a general way. These studies indicate that the TOR/FRAP proteins define a novel family of kinases and that the kinase domains of these proteins are essential for their cellular functions.

Results

TOR1 Binds to Yeast FKBP12-Rapamycin Both In Vivo and In Vitro

Two mammalian homologs of TOR proteins, FRAP and RAFT, have recently been purified using FKBP12–rapamycin affinity matrices (Brown et al., 1994; Sabatini et al., 1994). We have used a similar strategy to examine whether TOR1 and TOR2 proteins bind directly to the yeast FKBP12 (yFKBP12)–rapamycin (using glutathione S-transferase [GST]–yFKBP12 fusion protein complexed to rapamycin; yGFK–rapamycin) complex. A single, apparently low abundance polypeptide of M_r 220,000 from crude yeast lysates was specifically retained on a yGFK–rapamycin column, but not to yGFK–FK506 or yGFK alone (Figure 1A). The gel mobility of the yeast protein is similar to that of human FRAP (Brown et al., 1994), suggesting the proteins are related. Indeed, the 220 kDa protein was recognized specifically by an anti-TOR1 immune serum, but not the preimmune serum on a Western blot (Figure 1B). We generated yeast mutants carrying the chromosomal mutations Ser-1972→Arg in *TOR1* or Ser-1975→Arg in *TOR2* by homologous recombination. The Ser-1972 mutation in TOR1 abolished the binding of this protein to yGFK–rapamycin in two independent mutant strains, while no effect was observed with the TOR2 mutant strains (Figure 1C). Thus, the 220 kDa protein purified by yGFK–rapamycin appears to be TOR1, and mutation at Ser-1972 prevents its binding to yFKBP12–rapamycin. Since a TOR2 fragment has been shown to bind to FKBP12–rapamycin in a yeast two-hybrid assay (Stan et al., 1994), TOR2 most likely also binds; our inability to detect such binding in the TOR1 mutant strains may be due to its low level of expression.

Deletional analyses using an in vitro transcription–translation system (rabbit reticulocyte lysates) indicate that TOR1 with a C-terminal truncation up to amino acid 2157

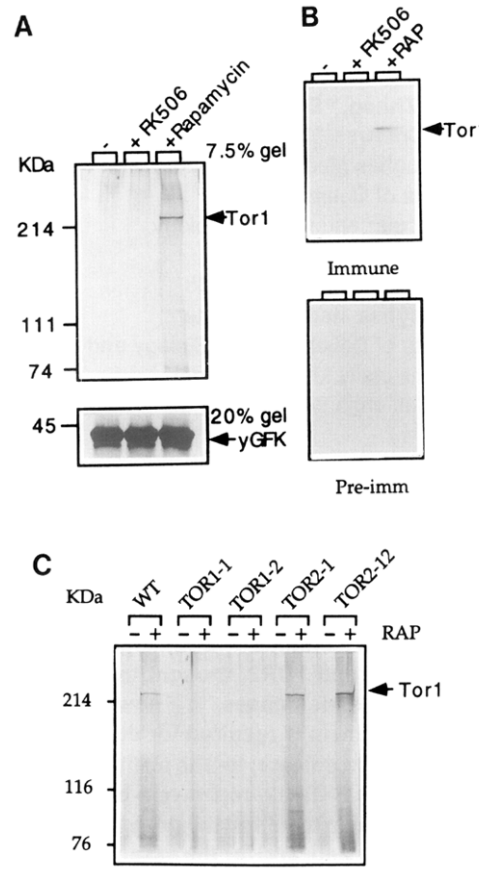


Figure 1. TOR1 Binds Directly to FKBP12–Rapamycin Complex

(A and B) S. cerevisiae yeast lysates were applied to a Mono Q column. After extensive washing, samples were eluted with 1 M NaCl. The eluants were incubated with 0.7 μM yGFK alone, yGFK–FK506, or yGFK–rapamycin and were allowed to bind to a glutathione S-Sepharose (GTS) column. The GTS-bound material was washed, eluted into SDS gel sample buffer, separated by 7.5% (A, top, and B) or 20% (A, bottom) SDS–polyacrylamide gel electrphoresis, and analyzed by silver staining (A), by immunoblot with anti-TOR1 immune serum (B, top) or with preimmune serum (B, bottom) as a control.

(C) TOR1 (Ser-1972→Arg) does not bind to an FKBP12–rapamycin complex. The following haploid yeast strains were used for the yGFK–rapamycin binding assay: wild type (MATa, *TOR1*, *TOR2*), two independent clones (*TOR1-1* and *TOR1-2*) of the mutant (MATa, *TOR1* [Ser-1972→Arg], *TOR2*), and two independent clones (*TOR2-1* and *TOR2-12*) of the mutant (MATa, *TOR1*, *TOR2* [Ser-1975→Arg]). The purification procedure is the same as Figure 1A. The final yGFK- or yGFK–rapamycin-bound materials were analyzed by silver staining.

is able to bind to yGFK–rapamycin. A further removal of 21 amino acids abolishes this binding (Figure 2A). We found a 45 kDa fragment of TOR1 (TOR1-45, amino acids 1775–2157) containing Ser-1972 was sufficient to bind to yGFK–rapamycin. Two naturally selected dominant rapamycin-resistant mutations at Ser-1972, arginine and isoleucine, disrupt yGFK–rapamycin interaction with TOR1-45 completely. Similarly, glutamic acid, a residue potentially analogous to phosphoserine, also abolishes the ability of TOR1-45 to bind yGFK–rapamycin. In contrast, alanine, a potential mimic of unphosphorylated serine, does not affect yGFK–rapamycin binding (Figure 2B).

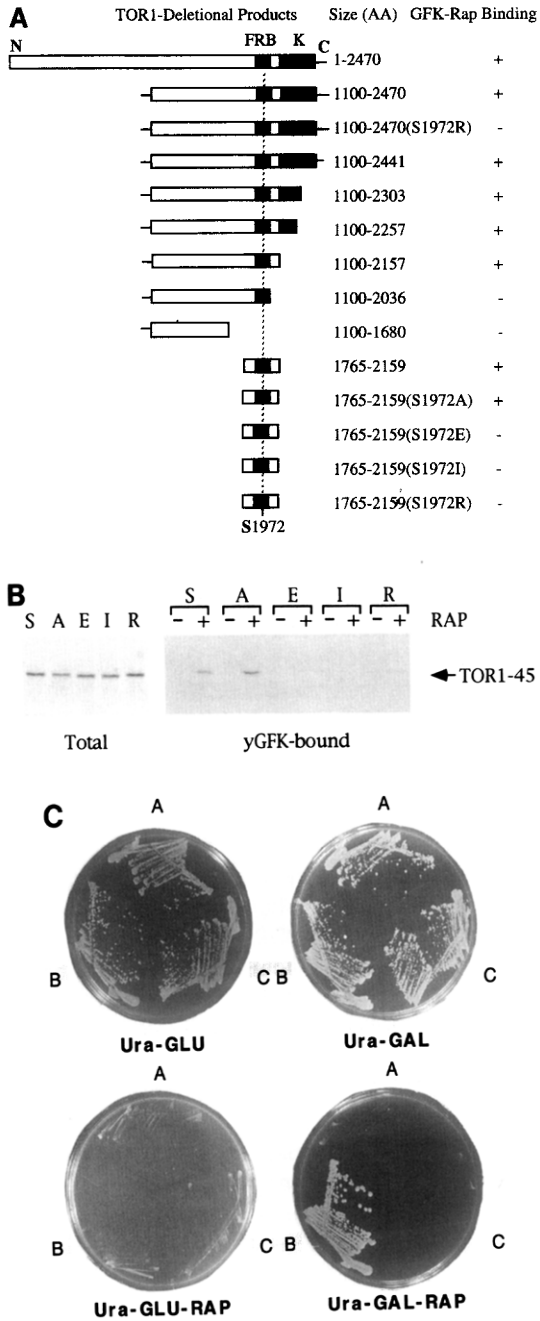


Figure 2. Mapping FRB Domain in TOR1

(A) A series of ^{35}S-labeled TOR1 deletional mutants was generated in vitro, incubated with 0.7 μM yGFK or yGFK–rapamycin, and purified onto GTS columns. The GTS-bound materials were eluted into SDS protein gel sample buffer, separated on SDS protein gels, and analyzed by X-ray autoradiography. Bars indicate different truncated TOR1s. Plus indicates TOR1 fragments that bind to yGFK–rapamycin; minus indicates TOR1 domains that do not bind to yGFK–rapamycin.

(B) Assay for the effects of substitutions at Ser-1972 on the binding of TOR1 to FKBP12–rapamycin. The ability of ^{35}S-labeled wild-type TOR1-45, or Ser-1972→Ala, Ser-1972→Glu, Ser-1972→Ile, and Ser-1972→Arg mutant TOR1-45 (FRB domain) to bind yGFK or yGFK–rapamycin was assayed similarly to (A). Total indicates total ^{35}S-labeled TOR1-45 FRB used. S, wild-type Ser-1972; A, Ala-1972; E, Glu-1972; I, Ile-1972; R, Arg-1972.

(C) Overexpression of FRB domain of TOR1 allows yeast to resist rapamycin. S. cerevisiae EGY48 (Gyuris et al., 1993) expressing the TOR1-45 FRB domain was assayed for rapamycin sensitivity. TOR1-45 (FRB) expression is under the control of the *GAL1* promoter (GAL, 2 μM, *URA*). Yeast cells were streaked onto uracil-dropout plates containing glucose or galactose supplemented with or without 100 nM rapamycin, and incubated at 30°C for 3 days. Rapamycin resistance was scored by the ability of the cells to form colonies. A, control; B, wild-type TOR1-45 (FRB); C, Ser-1972→Arg mutant TOR1-45 (FRB).

Cells expressing the Ser-1972→Arg, Ser-1972→Ile, and the Ser-1972→Glu mutants, which fail to bind to FKBP12–rapamycin, were able to resist rapamycin (Table 1). In contrast, the Ser-1972→Ala mutant, which retains the ability to bind FKBP12–rapamycin, was unable to confer resistance to rapamycin. When the FRB domain-containing TOR1-45 is overproduced, yeast cells were able to grow normally in 100 nM rapamycin, a concentration at which the growth of normal yeast cells is completely inhibited (Figure 2C). In contrast, cells expressing TOR1-45 (Ser-1972→Arg), a mutant that does not bind yGFK–rapamycin in vitro, are sensitive to rapamycin (Figure 2C). Thus, these in vivo assays for the interaction between FKBP12–rapamycin and TOR1 confirm the results obtained in the in vitro biochemical experiments and suggest that TOR1 binding to FKBP12–rapamycin occurs in vivo.

The Kinase Domains in TOR1 and TOR2 Are Essential for Their G1 Functions

Because of their sequence similarity with other PI kinases, proteins of the TOR/FRAP family were anticipated to have PI kinase activities. Thus far, we have tested in vitro kinase activities of recombinant TOR1 proteins produced in yeast and E. coli and FRAP proteins expressed in E. coli and mammalian and Sf9 cells toward PI, PI phosphate derivatives, and several other potential lipid substrates. We have been unable to demonstrate any such activity with TOR1 or FRAP proteins. We also carried out HPLC analyses of total cellular PI phosphate products, using in vivo [^{3}H]inositol labeling, following rapamycin treatment or overexpression of TOR1 proteins. No significant alterations in PI phosphate products were observed (X.-F. Z., P. A. Beal, and S. L. S., unpublished data). Since PI3K and Vps34 were previously shown to exhibit protein kinase activity, we also examined whether TOR1 and FRAP have intrinsic protein kinase activity. To facilitate this analysis, we introduced two kinase inactivation substitutions, Arg-2276→Pro and Asp-2294→Glu, into the wild-type *TOR1* and Ser-1972 *TOR1* dominant rapamycin-resistant mutants. The analogous kinase-dead mutations were shown previously to abolish the activities of PI3K and Vps34, respectively (Schu et al., 1993; Dhand et al., 1994), and we have shown that similar mutations in FRAP abolish a serine-autophosphorylation activity in wild-type FRAP and its Ser-2035 (corresponding to Ser-1972 in TOR1) mutant expressed in mammalian (E. J. Brown, P. A. Beal, J. Chen, T.-B. Shin, and S. L. S., unpublished data) and Sf9 cells (P. A. Beal, C. Keith, and S. L. S., unpublished data). However, we have not been able to obtain conclusive biochemical evidence for a protein kinase activity in wild-type TOR1 expressed in yeast cells. It is possible that the autophos-

Table 1. Effect of Mutations on TOR1

Mutation	GFK–Rap Binding	Rapamycin Resistance	Complementation of Temperature Sensitivity of Δ*tor1*
Ser-1972 (wt)	Yes	No	Yes
Ser-1972 Arg-2276→Pro	ND	No	ND
Ser-1972 Asp-2294→Glu	ND	No	ND
Ser-1972→Ala	Yes	No	Yes
Ser-1972→Arg	No	Yes	ND
Ser-1972→Arg/Arg-2276→Pro	No	No	ND
Ser-1972→Arg/Asp-2294→Glu	No	No	ND
Ser-1972→Glu	No	Yes	ND
Ser-1972→Glu/Arg-2276→Pro	No	No	ND
Ser-1972→Glu/Asp-2294→Glu	No	No	ND
Ser-1972→Ile	No	Yes	Yes
Ser-1972→Ile/Arg-2276→Pro	No	No	ND
Ser-1972→Ile/Asp-2294→Glu	No	No	ND

ND, not determined.

phorylation site on TOR1 does not exist or that the conditions for yeast cell lysis are too harsh to preserve this activity. By taking a genetic approach, we have succeeded in revealing the importance of the kinase domains in TOR proteins, and we find that the cellular effect of the mutations discussed above correlate precisely with their effect on the protein kinase activity observed with FRAP. Both TOR1 kinase-dead mutations abolish the ability of the Ser-1972→Ile mutant to confer dominant rapamycin resistance when the double mutants (Ser-1972→Ile/Arg-2276→Pro and Ser-1972→Ile/Asp-2294→Glu) were expressed at low levels in glucose medium (Figure 3A). Similar effects were also seen with the Ser-1972→Glu and the Ser-1972→Arg double mutants (Table 1). It was previously established that the C-terminus of both *TOR1* and *TOR2* are functionally interchangeable (Helliwell et al., 1994). We engineered chimeric TOR proteins comprising the N-terminus of TOR2 and C-terminus of TOR1. The wild-type chimeric TOR2–TOR1 protein (CEN4, LEU) complements the *tor2* null strain, showing that it is an active and functional protein and confirming the earlier report (Helliwell et al., 1994; see Figure 6). However, the two kinase-dead mutations abolish the ability of the serine TOR2 mutant to resist the actions of rapamycin (Figure 3B).

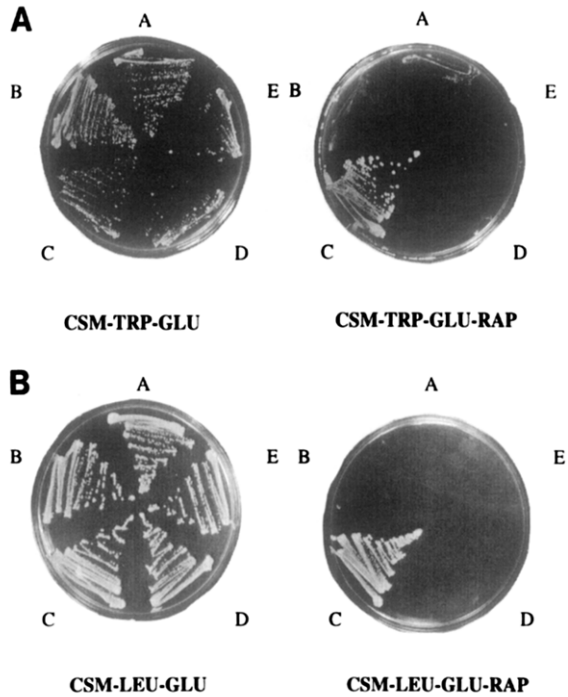


Figure 3. Kinase Domains Are Essential for Rapamycin-Sensitive G1 Functions of TOR1 and TOR2

(A) S. cerevisiae BJ5459 expressing wild-type or mutant TOR1 proteins under the control of the galactose-inducible promoter (GAL, 2 μM, *TRP*) were assayed for their sensitivity to 100 nM rapamycin as in Figure 2C. A, control; B, wild-type TOR1; C, TOR1 (Ser-1972→Ile); D, TOR1 (Ser-1972→Ile/Arg-2276→Pro); E, TOR1 (Ser-1972→Ile/Asp-2294→Glu).

(B) BJ5459 expressing *TOR2* or *TOR2–TOR1* wild-type and mutant genes (*ARS/CEN*, *LEU*) were assayed for their sensitivity to 100 nM rapamycin. A, TOR2; B, TOR2–TOR1; C, TOR2–TOR1 (Ser-1972→Ile); D, TOR2–TOR1 (Ser-1972→Ile/Arg-2276→Pro); E, TOR2–TOR1 (Ser-1972→Ile/Asp-2294→Glu).

Kinase-dead mutants of protein kinases often exhibit a dominant negative effect on growth by interacting in a nonproductive way with effector proteins normally associating with the wild-type kinase. We found that the Arg-2276→Pro and the Asp-2294→Glu *TOR1* mutants, when overproduced following galactose induction, inhibit growth of yeast cells cultured on solid (but not liquid) medium as shown in Figure 4; flow cytometric analysis revealed these cells undergo G1 cell cycle arrest (data not shown). This observation indicates that the kinase-dead TOR1 proteins exhibit a dominant negative effect on TOR1 function. Since the *tor1* null mutation alone does not cause G1 arrest, the dominant negative *TOR1* mutants must interfere with the G1 functions of both TOR proteins.

An Intact Kinase Domain Is Also Required for the Ser-1972→Ile Mutation in TOR1 to Inhibit Cell Growth Dominantly

When tested for rapamycin sensitivity under galactose induction, the TOR1 (Ser-1972→Ile) mutant prevented growth of yeast cells in a rapamycin-independent manner, even

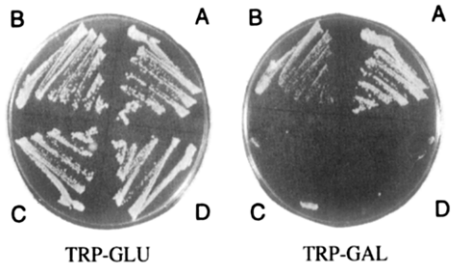


Figure 4. Overexpression of TOR1 Kinase-Dead Mutants Cause G1 Cell Cycle Arrest

S. cerevisiae BJ5459 expressing wild-type or mutant TOR1 proteins under the control of the *GAL1* promoter (GAL, 2 μM, *TRP*) were assayed for their ability to grow on glucose or galactose media. A, control; B, the wild-type TOR1; C, TOR1 (Arg-2276→Pro); D, TOR1 (Asp-2294→Glu).

though its low level of expression in the presence of glucose is sufficient to resist rapamycin. In liquid culture, yeast cells overexpressing the wild-type TOR1 protein or its Ser-1972→Ala mutant have a normal growth rate that is comparable to that of yeast cells carrying a control vector (Figure 5). In contrast, the growth of yeast cells overexpressing the TOR1 (Ser-1972→Ile) protein was significantly diminished. This dominant growth-arresting effect is dependent upon the existance of an intact kinase domain. When the kinase-inactivating mutations Arg-2276→Pro or Asp-2294→Glu were introduced into TOR1 (Ser-1972→Ile), the dominant growth inhibitory effect of the Ser-1972→Ile mutation was lost (Figure 5). This difference in growth inhibition is not due to a higher level of TOR protein expression by the TOR1 (Ser-1972→Ile)–expressing cells, as judged by Western blots (data not shown). This growth-arresting phenotype of the *TOR1* (Ser-1972→Ile) allele is clearly different from the dominant negative effects seen with kinase-dead TOR1 mutants. The *TOR1* (Ser-1972→Ile) mutant allele appears to preserve all the normal function of wild-type *TOR*, as evidenced by its ability to resist the actions of rapamycin and by its ability to complement the *tor2* null as a chimeric protein with a *TOR2* N-terminus. In addition, the dominant negative effect of the kinase-dead TOR1 mutants is seen only on solid medium. In contrast, overexpression of TOR1 (Ser-1972→Ile) causes cell growth arrest in both liquid (Figure 5) and solid (data not shown) cultures. The effects of overexpression of TOR1 (Ser-1972→Ile) mutant on cell cycle progression and checkpoints are currently under investigation.

TOR2 Has an Essential Function That Is Not Inhibitable by FKBP12-Rapamycin

To investigate whether the function of TOR2 is sensitive to the actions of rapamycin, we engineered a *tor1* null strain by inserting a *LEU2* marker into the BamHI site of *TOR1* gene in the kinase domain (Figure 6A). This insertion has been shown previously to knock out the function of the *TOR1* gene (Kunz et al., 1993; Helliwell et al., 1994). The *Δtor1* strain was found to undergo G1 cell cycle arrest when treated with rapamycin, indicating that the TOR2 protein is a target of the actions of rapamycin in vivo (Figure 6A). Despite the fact that a *tor2* disruption in a wild-type

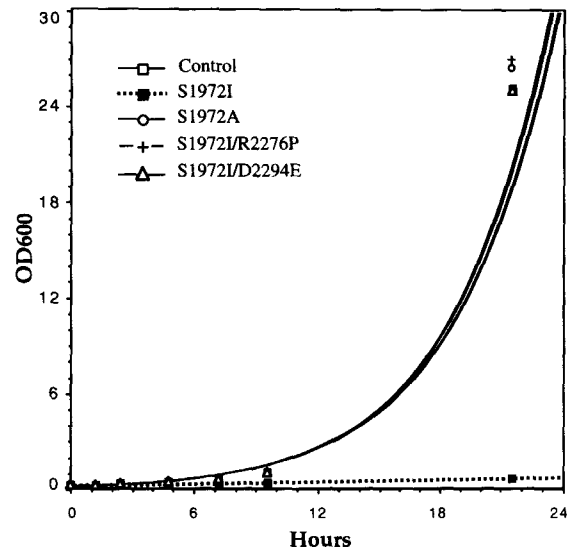
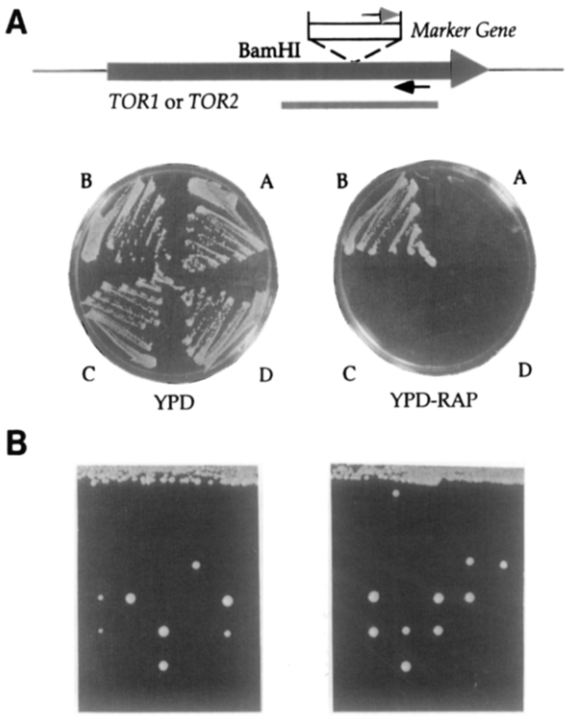


Figure 5. Dominant Growth Arrest by Overexpression of TOR1 (Ser-1972→Ile) Is Dependent on an Intact Kinase Domain

Yeast cells expressing wild-type or mutant TOR1 proteins under the control of galactose-inducible promoter (GAL, 2 μM, *TRP*) were assayed for their ability to grow under conditions of galactose induction. The overnight yeast cultures in glucose were diluted into sucrose media at OD_{600} = 0.25 and were incubated for 4 hr at 30°C. The cells were harvested by centrifugation, resuspended into galactose medium at OD_{600} = 0.25, and incubated at 30°C. Aliquots of cultures were withdrawn at different times for OD measurement at 600 nM.

TOR1 background is lethal, the *TOR1* (Ser-1972→Arg) mutant allele allows yeast to grow in the presence of rapamycin. These observations suggest that if rapamycin inhibits all functions of TOR2, then the TOR1 (Ser-1972→Arg) mutant, but not the wild-type TOR1, causes a gain of TOR1 function that should complement the *tor2* null lethal phenotype. We tested this by disrupting the *TOR2* gene either in a wild-type *TOR1* or a *TOR1* (Ser-1972→Arg) mutant background (Figure 6B). First, diploid heterozygous *TOR2/tor2* mutants in either a wild-type *TOR1/TOR1* or mutant homozygous $TOR1^{SR}$/$TOR1^{SR}$ background were generated by inserting a *LEU2* marker into the BamHI site in the kinase domain previously shown to inactivate the *TOR2* gene (Helliwell et al., 1994). Tetrad analyses showed that in both genetic backgrounds, only two or less viable spores (versus four) were obtained (Figure 6B). None of the viable spores were found to cosegregate with the *LEU2* marker, indicating that in either genetic background, the *tor2* disruption is lethal. This result demonstrates that the TOR1 (Ser-1972→Arg) mutation does not complement the loss of the TOR2 essential function. Given the dominant rapamycin-resistant phenotype of this TOR1 mutation and the sensitivity of TOR2 to rapamycin, we conclude that the essential function of TOR2, in contrast with its G1 function, is not inhibited by FKBP12–rapamycin. This is consistent with the observation that treating the *tor1* null strain described above with rapamycin resulted in G1 cell cycle arrest, which is in contrast with the random cell cycle arrest phenotype observed with the *tor2* null strain.



C

Plasmid-born *TOR*	# of *LEU2*$^+$	# of *URA*$^+$	Rescue of Δ*tor2* ?
TOR2	40	20	Yes
TOR2-TOR1	40	20	Yes
TOR2-TOR1(DE)	38	0	No
TOR2-TOR1(RP)	40	0	No
TOR2-TOR1(SI)	24	12	Yes
TOR2-TOR1(SI/DE)	30	0	No

Figure 6. TOR2 Has an Essential Function That Is Not Sensitive to Rapamycin

TOR1 or TOR2 gene was disrupted by insertion of a marker gene into the conserved BamHI site. The small arrows indicate PCR primers for analyzing the disruption results. The small bar shows the *TOR1* or *TOR2* sequence used for making the disruption constructs.

(A) Yeast cells lacking *TOR1* are still sensitive to rapamycin. Haploid wild-type or *tor1* null yeast cells were assayed for sensitivity to 100 nM rapamycin. A, wild-type yeast (MATa, *TOR1, TOR2*); B, mutant yeast (MATa, *TOR1* [Ser-1972→Arg], *TOR2*); C and D, two clones of *tor1* disrupted strains (MATa, *tor1::LEU, TOR2*).

(B) *TOR1* (Ser-1972→Arg) does not complement lethality associated with *TOR2* disruption. Diploid yeast heterozygous for *TOR2* disruption in a wild-type *TOR1* background (MATa/α, *TOR1/TOR1, TOR2/tor2Δ::LEU*) or a Ser-1972→Arg mutant *TOR1* background (MATa/α, *TOR1* [Ser-1972→Arg]/*TOR1* [Ser-1972→Arg], *TOR2/tor2::LEU*) were allowed to sporulate. Tetrads were dissected, and viable spores were grown into colonies. In either genetic background, tetrad analyses always show a 2:2 or a 1:3 segregation of viable versus nonviable spores.

(C) The kinase domain is required for the essential function of *TOR2*. Diploid yeast cells carrying a heterozygous *TOR2* disruption (*TOR2/tor2::HISG-URA-HISG*) and plasmid-born wild-type and mutant *TOR2* or *TOR2-TOR1* fusion genes (*LEU2, ARS/CEN*) under the endogenous *TOR2* promoter were sporulated. Dissected spores were grown on YPD plates, from which replica *LEU-* or *URA*-dropout plates were made. Viable spores were counted with *URA* marker from a total of viable spores carrying *LEU2*. Rescue of the lethality caused by *TOR2* disruption was determined by analyzing the cosegregation of the two markers.

Since the specificity of the essential function of TOR2 is determined by its N-terminus (Helliwell et al., 1994), we asked whether this essential function requires a functional kinase domain. We established diploid yeast strains with a heterozygous insertion of a *URA* marker into the BamHI site of the *TOR2* gene (*TOR2/tor2*). Plasmids expressing extrachromosomal (*CEN4, LEU2*) *TOR2*, *TOR2-TOR1*, *TOR2-TOR1(SI)*, *TOR2-TOR1(DE)*, *TOR2-TOR1(SI/DE)* were then introduced into the heterozygotes. After sporulation, we examined how the *LEU2* and *URA* markers associate in the viable spores. Of the ~40 viable spores showing *LEU2* prototrophy for each strain, half were found to have the *URA* marker for cells expressing *TOR2*, *TOR2-TOR1*, and *TOR2-TOR1(SI)*, but none were found to have the *URA* marker with cells expressing the kinase-dead TOR proteins (Figure 6C). Thus, the essential function of TOR2 requires a functional kinase domain.

Discussion

TOR1 (or *DRR1*) and *TOR2* (or *DRR2*) genes were originally isolated as mutations at a conserved residue, Ser-1972 in TOR1 or Ser-1975 in TOR2, that confer a dominant rapamycin-resistant phenotype in yeast (Cafferkey et al., 1993; Helliwell et al., 1994). Our results demonstrate that TOR proteins are direct targets of FKBP12–rapamycin. TOR1 and TOR2 (this study) and their mammalian homologs FRAP (Brown et al., 1994) and RAFT (Sabatini et al., 1994) bind directly to the FKBP12–rapamycin complex during in vitro purification or in a two-hybrid yeast screening (Chiu et al., 1994). Isorapamycin, a rapamycin derivative that binds FKBP12 with high affinity but is a poor inhibitor of cell cycle progression, binds less efficiently to both FRAP (Brown et al., 1994) and TOR1 (X.-F. Z. and S. L. S., unpublished data). Mutational analyses indicate that the conserved residues, Ser-1972 in TOR1 and Ser-1975 in TOR2, are essential for interaction with FKBP12–rapamycin in both our in vivo and in vitro binding studies. The steric requirements for this residue are highly stringent. Serine and alanine are permissive for the binding of FKBP12–rapamycin, whereas larger residues abolish this interaction. These binding results correlate well with the ability of mutant TOR proteins to resist the inhibitory actions of rapamycin. Mutations that disrupt binding of FKBP12–rapamycin to TOR proteins confer rapamycin resistance in an in vivo assay. In contrast, the Ser-1972→Ala mutant, which binds FKBP12–rapamycin, is as sensitive to rapamycin as wild-type TOR1. On the other hand, the gene encoding the Ser-1972→Ala mutant appears to have normal *TOR1* function, as shown by its ability to complement the temperature sensitivity of a *tor1* mutant strain (Table 1). A small FRB domain of TOR1 surrounding Ser-1972 is sufficient for binding to the complex. When overex-

pressed, this domain also allows cells to resist the actions of rapamycin, indicating that such binding occurs in vivo. In contrast, when the domain containing the Ser-1972→Arg mutation that disrupts binding to FKBP12–rapamycin in vitro is overexpressed, it does not produce the rapamycin-resistant phenotype. Together, these results establish that binding to the FRB domain is essential for the G1 inhibitory function of FKBP12–rapamycin and that the conserved serine residue is critical for this interaction.

PI kinases and their phospholipid-derived products have been shown to be important in a variety of cellular processes, including mitogenic signaling pathways (for reviews see Carpenter and Cantley, 1990; Divecha and Irvine, 1995). The kinase domain among TOR-related proteins from yeast to humans is highly conserved, showing over 60% identity between TOR1 and human FRAP in this region. However, a phylogenetic analysis reveals that *FRAP*, *TOR1*, *TOR2*, *MEC1/ESR1*, and *e2787* (*TEL1*) appear to constitute a third family of "lipid" kinases that have clearly diverged from the PI3K and PI4K families, which themselves are phylogenetically distinct from each other (C. Keith and S. L. S., unpublished data). Consistent with this analysis, we have not been able to demonstrate TOR- or FRAP-catalyzed phosphorylation of a variety of potential lipid substrates, including PI and PI phosphates, and lipid extracts from cells. In addition, we have not detected any change in the concentration of cellular PI phosphates following rapamycin treatment of yeast cells labeled in vivo with [^{3}H]inositol, or following the overexpression of the TOR1 protein. Although we have not been able to obtain conclusive biochemical evidence for a protein kinase activity in wild-type TOR1 expressed in yeast, possibly due to the harsh conditions required for the lysis of yeast cells, we have been able to demonstrate a serine autophosphorylation kinase activity in wild-type FRAP and Ser-2035 mutants of FRAP expressed in mammalian (E. J. Brown, P. A. Beal, J. Chen, T.-B. Shin, and S. L. S., unpublished data) and Sf9 cells (P. A. Beal, C. Keith, and S. L. S., unpublished data). It is therefore likely that TOR proteins and their analogous Ser-1972/Ser-1975 mutants also have protein kinase activity.

In this study, we have taken a chemical/genetic approach to study the role of the kinase domains in mediating TOR functions. Rapamycin, which causes G1 cell cycle arrest in yeast, has been used as a chemical equivalent of a loss-of-function conditional allele of TOR proteins: its inclusion in functional experiments results in the loss-of-function of endogenous TOR proteins. Since the Ser-1972/Ser-1975 TOR mutants described above do not bind FKBP12–rapamycin, they have been used in the presence of rapamycin to assess the functional role of TOR kinase domains. We find that kinase-dead mutations analogous to those that inactivate the lipid and protein kinase activity of PI kinases, and the protein kinase activity of FRAP, abolish the ability of the dominant rapamycin-resistant mutations in both TOR1 and TOR2 to resist the actions of rapamycin. These experiments with double mutants establish that an intact kinase domain is essential for the G1 functions of both TOR proteins. In addition, when overexpressed, the kinase-dead single mutants of TOR1 mentioned above show a dominant negative phenotype, resulting in G1 cell cycle arrest of yeast growing on a solid medium. Since TOR1 and TOR2 appear to have redundant G1 functions, the kinase-dead TOR1 protein may bind to the G1 effectors common to TOR1 and TOR2, inhibiting the action of both wild-type proteins.

In experiments that do not rely upon the actions of rapamycin, we found that overproduced TOR1 (Ser-1972→Ile), but not the wild-type or TOR1 (Ser-1972→Ala) mutant proteins, dominantly inhibits cell proliferation by a mechanism that is also dependent upon its kinase domain (Figure 5). Although we have not fully characterized the resulting phenotype, preliminary experiments suggest that overexpression of this Ser-1972 mutant may interfere with the function of a cell cycle checkpoint (X.-F. Z. and S. L. S., unpublished data). The TOR1 (Ser-1972→Ile) mutant appears to preserve the normal functions of the wild-type protein, as it allows cells to resist rapamycin dominantly, it suppresses the temperature sensitivity of a *tor1* mutant (Table 1), and its C-terminal domain (including the kinase and mutant FRB domains) provides the essential TOR2 function when fused to the N-terminal domain of TOR2. Thus, growth inhibition is not due to a dominant negative effect. The growth inhibitory phenotype of the TOR1 (Ser-1972→Ile) mutant may be due to the constitutive modification of a TOR1 effector, possibly due to an activating influence of the isoleucine group on the kinase of TOR1. Changes in the conserved serine residue may provide a conformational basis for kinase activation. Ser-1972 and its flanking sequences are found in the kinase domain of FRAP and TOR proteins, but not in the kinase domains of the known PI kinases. This sequence may be analogous to ones found in the regulatory domains of the serine/threonine kinases PKG and PKC (these domains are also immediately N-terminal to the kinase domains) and the homologous regulatory subunits RI and RII of PKA. Studies of these protein kinases indicate that the putatively analogous sequences act as inhibitory pseudosubstrates, interacting directly with the kinase active site. A striking similarity in the kinases of the FRAP/TOR family and the cAMP-dependent protein kinase PKA is that in both instances the key serine residue in the regulatory domain can be mutated to alanine without removing the binding properties of the domains, while mutation to larger residues results in potent inhibition of binding (Buechler and Taylor, 1991). Our observations suggest that Ser-1972 and its surrounding FRB domain may be behaving in a way that is analogous to the RII subunit of PKA and the N-terminal regulatory subunit of PKG.

The TOR poteins are functionally and structurally related, yet they also have distinguishing features. The C-terminal kinase domains of the TOR proteins can be interchanged without loss of function (Helliwell et al., 1994), and certain mutations at the conserved serine in their FRB domains in either protein allow yeast to resist the actions of rapamycin dominantly. Double disruption of *TOR1* and *TOR2* results in G1 cell cycle arrest, and as shown above, in both cases their G1 function requires an active kinase

domain. In addition, the dominant negative TOR1 kinase-dead mutant arrests the cell cycle in G1, as does rapamycin. These results suggest that the kinase domains of both proteins share a common G1 function. But, although the loss of *TOR1* only slightly affects yeast cell growth, the loss of *TOR2* is lethal and results in random cell cycle arrest (Kunz et al., 1993). The dominant nature of the FRB domain mutations in TOR1, which allow yeast to proliferate even though both endogenous TORs are inhibited by rapamycin, suggests that the mutations may lead to a gain of TOR1 function that complements the functions of TOR2. Although rapamycin inhibits at least the G1 function of the TOR2 protein as evidenced by the rapamycin sensitivity of a *tor1* null strain (see above), and *TOR2* is an essential gene, rapamycin-treated yeast harboring the TOR1 (Ser-1972→Arg) mutation do not show the *tor2* null lethal phenotype, suggesting specifically that the *TOR1* mutation may suppress this phenotype. An alternative explanation is that rapamycin might inhibit only a subset of the functions of TOR2 and that in the presence of rapamycin its essential function is still intact. This is indeed suggested by the finding that the rapamycin-treated *tor1* null strain is observed to arrest specifically in G1, suggesting that rapamycin might target only the G1 function of the TOR2 protein and not its essential function. To test this, we studied whether a dominant rapamycin-resistant *TOR1* mutant could suppress the lethality of a *tor2* null mutation. Tetrad analyses with diploid yeast containing a heterozygous *TOR2* allele (*TOR2/Δtor2*) in either a wild-type *TOR1/TOR1* or homozygous mutant *TOR1* (Ser-1972→Arg)/*TOR1* (Ser-1972→Arg) background revealed that in either genetic background, the *tor2* null is lethal. Thus, although rapamycin inhibits the G1 function of the TOR2 protein, it does not inhibit a second, essential function of the protein. We tested whether this TOR2 essential function requires an intact kinase domain by complementing the *tor2* null with plasmid-born wild-type or kinase-dead mutant *TOR2–TOR1* fusion genes. Only those genes with an intact kinase domain were able to rescue the lethality associated with the *TOR2* disruption. Thus, this essential TOR2 function also requires a functional kinase domain. Since both rapamycin-sensitive and -insensitive functions of TOR2 require a functional kinase domain, FKBP12–rapamycin does not appear to inhibit directly the kinase activity of TOR proteins.

A model that explains the dual functions of the kinase domain in TOR2 is shown in Figure 7. Distinct effectors for the G1 and essential functions of TOR2 may associate with separate domains of TOR2. We propose that the G1 effector binds to the C-terminal conserved domain, while the effector for the essential function binds to the unique N-terminal domain. Presumably, the G1 effector is able to bind to the same region of the TOR1 protein. The effectors may be involved in localization, where a thus far hidden TOR2 lipid kinase activity could be envisioned, may be modulated by a phospholipid and/or adenine nucleotide complex involving the kinase domain, in analogy to guanine nucleotide complexes of guanine nucleotide-binding proteins, or most simply may be a substrate for a protein kinase activity in TOR2. Since FKBP12–rapamycin does not appear to inhibit the catalytic activity of the kinase of TOR2 in a general way, it may instead selectively prevent the G1 effector from binding to or being phosphorylated by TOR2.

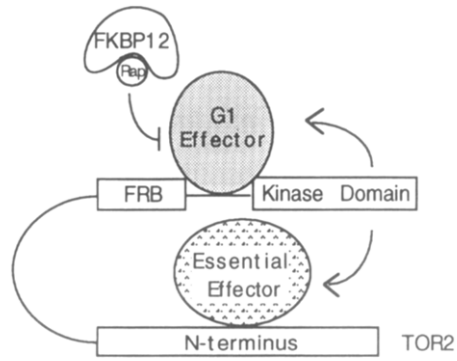


Figure 7. A Model of TOR Proteins and Their Functions

The TOR2 protein has at least two functions that require an intact kinase domain. Only its G1 function is sensitive to rapamycin. We propose that FKBP12–rapamycin interferes with the association or phosphorylation of a G1 effector(s) of both TOR1 and TOR2, but not an effector of the essential function of TOR2.

TOR proteins and their mammalian FRAP homologs mediate mitogenic signaling pathways distinct from the Ras–MAPK and, in mammalian cells, Jak–Stat pathways. The present work reveals that TOR1 and TOR2 are complex, multidomainal proteins that use their kinase activities to effect multiple cell cycle functions, and it provides insights into the mechanism by which FKBP12–rapamycin inhibits G1 cell cycle progression.

Experimental Procedures

Yeast Strains and Genetic Methods

Standard procedures for culturing and mating strains, diploid isolation, sporulation, and tetrad analyses were used (Guthrie and Fink, 1991). S. cerevisiae strains used in this study are listed in Table 2. The two yeast haploid strains carrying the Ser-1972→Arg mutation in *TOR1* and Ser-1975→Arg mutation in *TOR2*, respectively, were derived from YFK007 (Brizuela et al., 1991) by integrating an approximately 800 bp PCR products containing either Ser-1972→Arg (AGC to AGG) in *TOR1* or Ser-1975→Arg (AGT to AGG) in *TOR2*. These were confirmed by sequencing. An *HO* gene in pOL16 was used to construct wild-type or homozygous mutant diploid YFK007 yeast strains from corresponding haploid yeast strains.

Assay for TOR Interactions with FKBP12-Rapamycin

To purify yeast FRB proteins from yeast S. cerevisiae, approximately 0.5 lb of baker's yeast was lysed with glass beads in TBSN+ (50 mM Tris–HCl, 150 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml trypsin inhibitor, 1 mM PMSF, 1 mM DTT, 1% NP-40 [pH 7.5]). Lysates were cleared twice by centrifugation for 30 min at 25,000 × g at 4°C. The pH of the supernatant was adjusted to 7.5 with 1 N NaOH. The lysate was then loaded onto a Mono Q column. After extensive washing with TBSN+, the bound material was eluted with 1 M NaCl TBSN+ and the eluant was equally divided into three fractions, each of which was incubated with 0.7 μM yGFK alone, yGFK–FK506, or yGFK–rapamycin for 15 min at 4°C and loaded onto glutathione S-Sepharose column (GTS, Boehringer). After washed with 1 M NaCl TBSN+ extensively, the GTS-bound material was eluted into 2 × protein gel sample buffer, separated on SDS–polyacrylamide

Table 2. S. cerevisiae Strains

Strain	Genotype	Source
BJ5459	*MATa ura3-52 trp1 lys2-801 leu2Δ1 his3Δ20 can1 pep4::his3 prb1Δ1.6R*	B. Jones
EGY48	*MATa ura3 his3 trp3 LEXAop-leu2*	E. Golemis
NKY260	*MATa/α ura3/ura3 leu2/leu2*	N. Kleckner
YFK007	*MATa ade-101 leu-Δ1 lys2-801 trp-Δ1 ura3-52*	S. Parent
YFK1SR	*MATa ade-101 leu-Δ1 lys2-801 trp-Δ1 ura3-52 TOR1* (Ser-1972→Arg)	This study
YFK2SR	*MATa ade-101 leu-Δ1 lys2-801 trp-Δ1 ura3-52 TOR2* (Ser-1975→Arg)	This study
dYFK007	*MATa/α ade-101 leu-Δ1 lys2-801 trp-Δ1 ura3-52 TOR1/TOR1*	This study
dYFK1SR	*MATa/α ade-101 leu-Δ1 lys2-801 trp-Δ1 ura3-52 TOR1* (Ser-1972→Arg)/*TOR1* (Ser-1972→Arg)	This study
YFK*tor1*Δ	*MATa ade-101 leu-Δ1 lys2-801 trp-Δ1 ura3-52 tor1Δ::LEU2*	This study
dYFK*tor2*Δ	*MATa/α ade-101 leu-Δ1 lys2-801 trp-Δ1 ura3-52 TOR1/TOR1 tor2Δ/TOR2*	This study
dYFK1SR*tor2*Δ	*MATa/α ade-101 leu-Δ1 lys2-801 trp-Δ1 ura3-52 TOR1* (Ser-1972→Arg)/*TOR1* (Ser-1972→Arg) *tor2Δ/TOR2*	This study
YMW1	*MATα, ade2-1 ade-3(Δ22) his3-11,15 leu2-3,112 trp1-1 can-100 wra3-1*	M. Walhberg

gel, and analyzed by silver staining and by immunoblotting with anti-TOR1 antiserum or preimmune serum. For analyses of mutant TOR proteins, 2 liters of YFK007, YFK1SR, and YFK2SR, respectively, were grown to saturation in YPD. Cells were harvested, and the binding assays were performed as described above.

To map regions of TOR1 necessary for binding to FKBP12–rapamycin, *TOR1* cDNA corresponding to residues 1100–2470 was subcloned into pBluescript SK-EE vector, in which *TOR1* is fused in frame to a Glu–Glu epitope. The resulting plasmid was digested with the following restriction enzymes (the number indicates the last amino acid residue of the translation products): SmaI (2470), AatII (2441), AflII (2303), BglII (2257), EcoRV (2157), HindIII (2036), BamHI (1680), and was used directly for in vitro transcription/translation in rabbit reticulocyte lysates to prepare ^{35}S-labeled TOR1 deletion mutant proteins. The ^{35}S-labeled TOR1 proteins were incubated with 0.7 μM yGFK alone, yGFK–FK506, or yGFK–rapamycin for 15 min at 4°C, diluted 50 times into ice-cold TN buffer (50 mM Tris, 1 M NaCl [pH 8.0]), and loaded onto a GTS column. After washing with ice-cold TN buffer extensively, the GTS-bound materials were eluted into 2× protein gel sample buffer, separated on SDS–polyacrylamide gel, and analyzed by autoradiography. TOR1-45 (residues 1765–2159) was generated with the following pair of primers by PCR with PFU DNA polymerase (Stratagene) from plasmids containing wild-type or *TOR1* mutants at Ser-1972 as templates: 5′-CG GGA TCC **GCC** ATG GTT CAG GAA GAG ACT-3′ and 5′-C CGA ATT CTA GAT ATC CAA ATG TCT CTT GAA-3′. The amplified DNAs were subcloned into pET5a. This plasmid was then linearized with EcoRI and used for in vitro transcription.

Isolation of *TOR1* and *TOR2* Genes from S. cerevisiae

A genomic DNA library was constructed in pRS315 from a spontaneous dominant rapamycin-resistant yeast mutant and was introduced into a wild-type, rapamycin-sensitive yeast strain. A plasmid that conferred resistance to 100 nM rapamycin was recovered and found to be identical to the *TOR1* gene, except with a mutation converting Ser-1972 to isoleucine. The corresponding wild-type *TOR1* gene was isolated by gap repair of BglII-cut plasmid in wild-type yeast. The *TOR2* genes were isolated similarly. To introduce a convenient restriction site for subcloning, the wild-type and mutant *TOR1* plasmids were replaced at the 5′ end with a SacI site followed by the second amino acid of *TOR1* using PCR with the following pair of primers: 5′-GAG CTC TTG AAC CGC ATG AGG AGC AGA TTT GG-3′ and 5′-ACG GGG CCC AAG GGA ACT GCC-3′. The PCR product was digested with SacI–SpeI and was used to replace the same restriction fragment of the original genomic clones.

Immunological Techniques

To generate TOR1-specific antiserum, the GST–TOR1 (1223–2381) was expressed as inclusion bodies, purified conveniently by washing the cell pellets after lysis, and injected into rabbits. After boosting twice with antigens, the antisera tested positive for TOR1 on a Western blot. Western blot analyses were carried out as decribed previously (Harlow and Lane, 1988). Antigens were detected using the ECL method (Amersham). The anti-TOR1 serum was used at a dilution of 1:1000. The anti-flu hemaglutinin monoclonal antibody, MAb 12CA5, was used at 1:300 dilution. For immunoprecipitation, cell lysates were incubated with MAb 12CA5 antibody at a dilution of 1:300 for 1 hr at 4°C. The immunocomplexes were captured with protein G–conjugated Sepharose by incubating for another hour at 4°C, washed three times with TBSN+, and three times with TBS plus 1 mM DTT. The final immunoprecipitates were either directly used for in vitro kinase assays, or were eluted into SDS protein sample buffer for Western blot analyses.

TOR1 Expression Plasmid Constructs and In Vitro Mutagenesis

To create a galactose-inducible yeast expression vector, the *G6PD* promoter region of pG-1 (2 μM, *TRP*) (Schena et al., 1991) was inserted into the BamHI site with the following oligo sequence encoding an 5′ HA epitope: 5′-T ACC ATG GGA TAC CCA TAC GAT GTT CCA GAT TAC GCT TGG TAC CAT GGA GCT CGA GTC GAC GGA C-3′ and 5′-C GTC GAC TCG AGC TCC ATG GTA CCA AGC GTA ATC TGG AAC ATC GTA TGG GTA TCC CAT GGT AGA TC-3′. The SmaI–SacI fragment containing the *G6PD* promoter of the HA epitope–modified pG1 was replaced with a XhoI (blunted)–SacI fragment containing the *GAL10* promoter from YCPIF15. The full-length *TOR1* was then cloned into this expression vector with a HA tag on its immediate 5′ end.

To introduce mutations at Ser-1972, the SacII–KpnI fragment of *TOR1* was subcloned into pBluescript SK(+) (Stratagene) (pBluescript TOR1SK). In vitro phagemid mutagenesis was carried out with the following primers: Ser-1972→Ala, 5′-ATGTTCAACGAAAAATTGGCG-GGCCGCATCTTCCAGTCCTTCA-3′; Ser-1972→Glu, 5′-ATGTTCAA-CGAAAAATTGGCGTTCCGCATCTTCCAGTCCTTC-3′; Ser-1972→Arg, 5′-GTTCAACGAAAAATTGGCGCCTCGCATCTTCCAGTCCT-3′. The mutant DNA was inserted back into the full-length *TOR1* gene by subcloning the NcoI–KpnI mutant fragment into the HA-tagged TOR1 construct. To introduce kinase inactivation mutations into *TOR1*, the KpnI–SalI fragment of *TOR1* was subcloned into pBluescript KS(+). In vitro mutagenesis was carried out as above with the following primers: Arg-2276→Pro, 5′-CTGGGACTAGGTGATCCCCATCCAAGCAA-CCTG-3′; Asp-2294→Glu, 5′-GTTATCCACATTGAATTCGGCGATTG-TTTTGA-3′. To reconstruct the full-length Arg-2276 and the Asp-2294 mutant *TOR1* gene, the KpnI–SalI DNA fragments from the *TOR1* mutant mutagenesis constructs were excised, purified, and subcloned back into the HA-tagged wild-type or mutant *TOR1*. All mutations were confirmed by DNA sequencing.

To construct *TOR2–TOR1* mutants, the BamHI–PstI fragment of wild-type and mutant *TOR1*, which contains codons 1681–2470 and the 3′-noncoding sequences of *TOR1*, were excised from the above *TOR1* expression construct and fused with a *TOR2* genomic DNA containing the upstream promoter region and codons 1–1688 in pRS315.

The *TOR1-45* wild-type and mutant DNA fragments were subcloned into the BamHI–EcoRI sites of pYES (2 μM, *GAL1*) (Invitrogen).

Testing Growth Properties of Yeast Strains

To test the growth of yeast strains on plates, freshly grown yeast colonies were streaked onto selection plates containing either glucose or galactose in the presence or absence of 100 nM rapamycin and incubated for 3–4 days at 30°C. To test the growth of yeast strains

in liquid culture, yeast cells were first grown overnight in glucose selection medium, diluted into YP-sucrose medium at OD_{600} = 0.25, and incubated for 2 hr at 30°C. Cells were pelleted and resuspended into YP-galactose medium. Cells were incubated at 30°C with shaking. Aliquots of 1 ml culture were drawn at different times for OD measurements at 600 nM.

TOR1 and *TOR2* Disruptions

The *TOR1* disruption vector was constructed by inserting the BglII–BglII fragment from YEp13 containing the *LEU2* selection marker gene into the BamHI site of *TOR1* in pBluescript TOR1SK. The gene disruption construct was linearized and transformed into haploid YKF007 strain selecting for leucine (*tor1::LEU2*) prototrophy. The disruption of the *TOR1* locus was confirmed by PCR analyses of genomic DNA prepared from these *LEU2*$^+$ clones with one *TOR1* primer (5′-AAC GCC AGA GAT TTG CTT GTG-3′) and with one *LEU2* primer (5′-GAT CTT CTT AGG GGC AGA CAT-3′).

The SphI–SmaI fragment of *TOR2* was subcloned into pTZ18 (Bio-Rad) vector to give pTZ-TOR2SS. The *TOR2* disruption vector was constructed by inserting either the same BglII–BglII fragment of a *LEU2* marker gene as above, or a BglII–BamHI fragment that contains a *HISG–URA–HISG* marker gene cassette from PNKY51 into the BamHI site of *TOR2*. The *LEU2* disruption plasmid was linearized and transformed into diploid wild-type YFK007 (MATa/α *TOR1/TOR1*) or homozygous *TOR1* (Ser-1972→Arg) mutant (MATa/α *TOR1* [Ser-1972→Arg]/*TOR1* [Ser-1972→Arg]) selecting for leucine or uracil prototrophies. The *HISG–URA–HISG* disruption construct was linearized and introduced into a diploid strain NKY260. The heterozygous disruption of the *TOR2* locus was confirmed by PCR analyses of the *TOR2* disruption with one *TOR2* primer (5′-GAG GTA GAA TTC TTA GAA GAG-3′), one *LEU2* primer (5′-GAT CTT CTT AGG GGC AGA CAT-3′), or one *HISG* primer (5′-TAA GCG GGT GGT GTC TAA CAT-3′). Diploid strains heterozygous for *TOR2* disruption were sporulated by patching yeast cells onto sporulation medium and incubating at room temperature and were dissected on YPD. After viable spores formed colonies on YPD plates, replica plates were made with synthetic medium for testing cosegregation of the selective markers.

Complementation of the Temperature Sensitivity of a *tor1* Mutant Strain

A XhoI–BamHI fragment (nucleotides 1289–2473) and SacII–BamHI fragment (nucleotides 3901–5348) of TOR1 were cloned in a three part ligation into pRS304 (Sikorski and Hieter, 1989) to create pYDF79. pYDF79 was linearized with BamHI and transformed into YMW1 (M. Wahlberg, University of Texas Southwestern). A transformant was picked for Southern blot analysis to confirm that it does contain a *tor1* deletion. This mutant grows about 80% as fast as the wild-type strain at 24°C and 30°C, but is arrested at 37°C.

Acknowledgments

We are grateful to Liuzhong Xu for discussions and reagents, Eric Brown for technical assistance, Peter A. Beal for participation in the TOR lipid kinase assays, and Eric Brown, Peter A. Beal, Tae-Bum Shin, and Curtis Keith for sharing unpublished information. This work was supported by grants (CA-39612 to G. R. C.; GM-38627 to S. L. S.) from the National Institutes of Health. X.-F. Z. is supported by the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation Fellowship DRG-1278. J. C. is supported by a fellowship from the Irvington Institute. G. R. C. and S. L. S. are Investigators at the Howard Hughes Medical Institute.

Received March 17, 1995; revised May 19, 1995.

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