TOR2 Is Part of Two Related Signaling Pathways Coordinating Cell Growth in *Saccharomyces cerevisiae*

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ABSTRACT

The *Saccharomyces cerevisiae* genes *TOR1* and *TOR2* encode phosphatidylinositol kinase homologs. TOR2 has two essential functions. One function overlaps with TOR1 and mediates protein synthesis and cell cycle progression. The second essential function of TOR2 is unique to TOR2 and mediates the cell-cycle-dependent organization of the actin cytoskeleton. We have isolated temperature-sensitive mutants that are defective for either one or both of the two TOR2 functions. The three classes of mutants were as follows. Class A mutants, lacking only the TOR2-unique function, are defective in actin cytoskeleton organization and arrest within two to three generations as small-budded cells in the G2/M phase of the cell cycle. Class B mutants, lacking only the TOR-shared function, and class C mutants, lacking both functions, exhibit a rapid loss of protein synthesis and a G1 arrest within one generation. To define further the two functions of TOR2, we isolated multicopy suppressors that rescue the class A or B mutants. Overexpression of *MSS4*, *PKC1*, *PLC1*, *RHO2*, *ROM2*, or *SUR1* suppressed the growth defect of a class A mutant. Surprisingly, overexpression of *PLC1* and *MSS4* also suppressed the growth defect of a class B mutant. These genes encode proteins that are involved in phosphoinositide metabolism and signaling. Thus, the two functions (readouts) of TOR2 appear to involve two related signaling pathways controlling cell growth.

TOR1 and TOR2 are phosphatidylinositol (PI) kinase homologs originally identified genetically by dominant mutations that conferred rapamycin resistance in *Saccharomyces cerevisiae* (Heitman *et al.* 1991; Cafferkey *et al.* 1993; Kunz *et al.* 1993; Helliwell *et al.* 1994). We have previously demonstrated that loss of both TOR1 and TOR2 causes a first-generation arrest in the early G1 phase of the cell cycle, as does rapamycin treatment (Barbet *et al.* 1996). The arrested cells exhibit characteristics of starved cells in stationary phase (G0), including glycogen accumulation and a decrease in protein synthesis. The block in protein synthesis occurs at the level of initiation and is the cause of the cell cycle arrest. Thus, it appears that TOR1 and TOR2 are part of a novel signal transduction pathway involved in regulating translation, cell growth, and progression through the G1 phase of the cell cycle in response to nutrients (Barbet *et al.* 1996; Di Como and Arndt 1996; Thomas and Hall 1997). In mammalian cells, the TOR counterpart (FRAP/RAFT/mTOR) is part of a general mitogenic signaling pathway regulating translation and G1 progression (Brown *et al.* 1994, 1995; Chiu *et al.* 1994; Sabatini *et al.* 1994; Sabers *et al.* 1995; Beretta *et al.* 1996; Thomas and Hall 1997)

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TOR2 has two functions. In contrast to cells lacking TOR1 and TOR2, cells lacking only TOR2 do not display a G0 arrest phenotype, but instead undergo a few cell divisions before arresting randomly in the cell cycle (Kunz *et al.* 1993). Importantly, even overexpression of TOR1 cannot suppress the loss of TOR2. Cells lacking only TOR1 exhibit only mild growth defects (Helliwell *et al.* 1994). These phenotypes suggest that, in addition to having a redundant function with TOR1 in controlling G1 progression, TOR2 also has an essential, unique function that is not directly related to the cell cycle (Kunz *et al.* 1993; Helliwell *et al.* 1994; Zheng *et al.* 1995). Recent results have demonstrated that the TOR2-unique function is required for organization of the actin cytoskeleton. Mutants defective only in TOR2 do not exhibit the normal polarized distribution of the actin cytoskeleton and are rescued by overexpression of TCP20, an actin-specific chaperone (Schmidt *et al.* 1996).

Here we describe the isolation and characterization of different classes of *TOR* mutants that indicate that TOR2 indeed has two functions (or readouts). Suppressor analyses of representative mutants of these phenotypic classes suggest that the TOR2 functions are carried out by related signaling pathways.

MATERIALS AND METHODS

Strains, plasmids, and media: The yeast strains used in this study are described in Table 1. *Escherischia coli* strain MH4

is Δ*(lac)X74 hsr⁻ hsm⁺ strA leuB600 galE galK*. Plasmid pJK4 (YCplac111::*TOR2*) (Kunz *et al.* 1993) was constructed as follows. The 8 kb *Sma*I fragment of pJK3-3 (pSEY18::*TOR2*) (Kunz *et al.* 1993) was cloned into the *Sma*I site of YCplac111 (*CEN LEU2*), and the resultant plasmid was cut with *Bam*HI and *Pst*I and ligated with the *Bam*HI-*Pst*I fragment of pJK3-3. Plasmid YCplac111::*tor2-21* was constructed as follows. Because the mutation in the *tor2-21* allele (Ser-233 to Phe), originally isolated on YEplac181, eliminates the *Sac*I site within the open reading frame (ORF), *tor2-21* could be isolated as a single *Sac*I-*Pst*I fragment (T. Beck, personal communication). This *Sac*I-*Pst*I fragment was ligated into the multicloning site of YCplac111. Plasmid pSH22 is pSEY18 (2μ *URA3*) containing a 4.1 kb *Bam*HI-*Xho*I fragment including *MSS4*. Plasmid pSH24 is pSEY18 with a 4.3 *Sph*I fragment containing *PKC1*. pSH25 is pSEY18 containing a 3.4 kb *Bam*HI fragment containing *PLC1*. pSH27 is a double-deletion construct of pSH121.30, an original suppressor, such that a 1.9 kb *Sma*I-*Sph*I fragment remains containing *SUR1*. pEMY105 (gift of S. Emr) is pRS426 (2μ *URA3*) containing *FAB1*. YEp352-STT4 (YEp352-PSTB20) (gift of J. Thorner) is 2μ *URA3 STT4*. pGB1 is pSEY18 containing *PIK1*. pC-186 (2μ *URA3 RHO2*) and pAS30 (2μ *URA3 ROM2*) have been described previously (Schmidt *et al.* 1997). Media were prepared as described (Sherman 1991). Rich media contained 2% glucose (YPD), 2% galactose (YPG), 2% raffinose (YPR), or 3% ethanol and 3% glycerol (YPE/glycerol). Defined medium (SD) contained 2% glucose as the carbon source and, unless otherwise indicated, ammonium (0.01 M) as the nitrogen source. SD medium with proline (1 mg/ml) or glutamate (1 mg/ml) as the nitrogen source contained only tryptophan, histidine, and leucine as amino acid supplements (Vandenbol *et al.* 1987); these three amino acids were required to satisfy the auxotrophies of our strains and cannot be used as a nitrogen source. SD-complete medium contains a complete complement of amino acids.

Hydroxylamine mutagenesis, plasmid shuffling, and screening for *tor2ts* mutants: Mutagenesis was performed on YCplac111::*TOR2* (*CEN LEU2*) (pJK4) plasmid DNA as described (Sikorski and Boeke 1991). Samples were incubated at 75° for 12.5, 15, and 17.5 min in 0.1 m hydroxylamine, and then desalted with a Sephadex G-50 spin column. Aliquots from the three plasmid banks were transformed into the *E. coli* strain MH4, which is a leucine auxotroph. The percentage of Leu⁻ transformants indicated that between 2% and 5% of the plasmids in the three banks had lost the *LEU2* marker by mutagenesis. These plasmid banks were then transformed into NB4-6a (*tor1 tor2*) or MH346-1a (*TOR1 tor2*). Transformants were plated at a density of approximately 500 per plate. After 2–3 days at 24°, colonies were replica plated onto medium containing 5-fluoroorotic acid (5-FOA) and incubated at 24° to select for cells that could lose the *URA3*-marked *TOR2*-containing plasmid pJK3-3. These were then replica plated (1) to SD-LEU and incubated at 24° and (2) to YPD and incubated at 37°. For colonies that appeared to have a growth defect on YPD at 37°, the corresponding colony on the master plate was restreaked for further analysis. Plasmid DNA was isolated from transformants that displayed temperature-sensitive growth, and these plasmids were retransformed into the original host strains and subjected to the plasmid shuffle procedure once again. Plasmids that conferred temperature-sensitive growth after this were deemed to contain *tor2ts* alleles and were kept for further study. Allele *tor2-156ts* was isolated from among (8000) screened transformants of MH346-1a (*TOR1 tor2*). The remaining 28 alleles were obtained from 30,000 transformants of NB4-6a (*tor1 tor2*).

Plasmids bearing each of the *ts* alleles were transformed into JK9-3d**a** (*TOR1 TOR2*) and NB17-3d (*tor1 TOR2*) to determine whether these alleles were recessive to the genomic copy of *TOR2*. As expected, none of the plasmid-borne alleles exhibited any dominant effects in JK9-3d**a** (*TOR1 TOR2*) as judged by a normal growth rate of transformants at 37° on solid minimal medium lacking leucine. However, the alleles *tor2-21ts* and *tor2-156ts* caused a reduction in growth rate at 37° when expressd in NB17-3d (*tor1 TOR2*), and additionally, the plasmid-borne *tor2-156ts* slightly inhibited the growth of NB17-3d (*tor1 TOR2*) at 24° (data not shown). This suggests that in the absence of the genomic copy of *TOR1*, these *CEN* plasmid-based mutant alleles can have a dominant effect over the single genomic copy of *TOR2*.

Growth curves and FACS analysis: Cultures were grown to log phase at 24° in SD medium, then diluted to give an OD_{600} of approximately 0.05 in prewarmed media, and shaken at 37°. The OD_{600} was then determined at the times indicated. To easily assess the number of generations each strain went through, we divided all the resultant OD readings by the OD_{600} at the time of shift for any particular strain. Samples for fluorescence-activated cell sorting (FACS) were prepared and analyzed as described previously (Helliwell *et al.* 1994)

^{35}S-methionine incorporation: Strains were grown to log phase in SD-complete medium and diluted to an OD_{600} of 0.05 in 4.5 ml of fresh prewarmed SD-complete medium; 1 ml was removed to determine the starting OD_{600} and, to the remaining 3.5 ml, ^{35}S-methionine was added (in 100 μl SD-complete medium) to a final concentration of 10 μM (the concentration of cold methionine in SD-complete medium is 130 μM). The zero timepoint was taken immediately and the cultures were incubated at 37°. During the incubation, samples were taken at several timepoints. For each timepoint, 300 μl aliquots were taken and added directly to 700 μl of 10% trichloroacetic acid (TCA) on ice. A 20 μl aliquot was added directly to a glass fiber filter to act as a control for the total number of counts available in each labeling reaction. At the end of the experiment, the samples were heated at 95° for 4 min to deacylate tRNAs and were then filtered through TCA-soaked glass fiber filters, which were washed twice with ice-cold 10% TCA and once with acetone. The filters were then counted by liquid scintillation. Values for each strain were calculated by dividing the number of counts (1) by the starting OD_{600} of the culture and (2) by the counts obtained from the control filters. Values were then plotted as normalized against a wild-type control strain.

Morphological observations: Cells were stained with rhodamine phalloidin (stain for actin filaments) and 4′,6′-diamidino-2-phenylindole (DAPI) (stain for DNA) as described (Pringle *et al.* 1989). Cells were fixed and treated 8 hr after shift to the nonpermissive temperature, as described (Schmidt *et al.* 1997). Microscopy was performed with a Zeiss Axiophot II fluorescence microscope.

Multicopy suppressor isolation: Strains SH121 (class A) and SH229 (class B) were used to isolate high-copy suppressors of unique and shared function defects, respectively. Class D mutant SH129 (*tor2-29ts*) grows slower at nonpermissive temperature than class D mutant SH130 (*tor2-30ts*), indicating that the unique function encoded by *tor2-29ts* is not completely intact, but still sufficient for growth. We did not use class B mutants SH230 or SH2119 to isolate suppressors because they had high reversion rates. The isolation of the multicopy suppressors was performed using a 2μ based library derived from strain R1 (Helliwell *et al.* 1994). Cells were allowed to recover from the transformation in liquid YPD at the permissive temperature for 5 hr before being plated on selective media at the nonpermissive temperature. Transformants growing at 37° were passaged through 5-FOA plates to demonstrate that the suppression was plasmid linked. Plasmids conferring suppression were rescued and retransformed

into the parental strain to recheck suppression. Deletions and subclones were made using standard techniques to ascertain which ORF on each clone was responsible for suppression.

RESULTS

Isolation of temperature sensitive *tor2* mutants: A strain disrupted in *TOR2* arrests after three to four generations with no previously observed cell cycle phenotype. In contrast, strains disrupted in *TOR1* and *TOR2* arrest rapidly, within one cell cycle, and in early G1 (Kunz *et al.* 1993; Helliwell *et al.* 1994; Barbet *et al.* 1996). Furthermore, overexpression of TOR1 cannot suppress a *tor2*-null or *tor2^{ts}* mutation (Helliwell *et al.* 1994; T. Schmelzle and M. N. Hall, unpublished results). These differing arrest phenotypes suggest that TOR2 has at least two distinct essential functions. The first is involved in G1 progression and can be provided by *TOR1*; the second is not involved in cell cycle control and cannot be provided by *TOR1*. We refer to these two functions throughout this study as the TOR-shared and the TOR2-unique functions, respectively. The TOR-shared function is required for the activation of translation initiation. The TOR2-unique function is required for the organization of the actin cytoskeleton.

To define further the two functions of TOR2, we attempted to isolate temperature-sensitive *tor2* mutants that were defective for only one of the two TOR2 functions. We isolated 29 independent plasmid-borne *tor2^{ts}* alleles using a screen based on *in vitro* mutagenesis and plasmid shuffling (see materials and methods; Sikorski and Boeke 1991). All plasmids containing a temperature-senstive allele and the starting plasmid used for the mutagenesis (YCplac111::*TOR2*) were transformed into both MH346-1a (*TOR1 tor2* / pSEY18::*TOR2*) and NB4-6a (*tor1 tor2* / pSEY18::*TOR2*), and the pSEY18-borne wild-type *TOR2* gene was eliminated using 5-FOA (see materials and methods). Our nomenclature convention for the resulting *TOR1 tor2^{ts}* and *tor1 tor2^{ts}* strains is as follows. MH346-1a carrying substitute plasmid YCplac111::*TOR2* (relevant genotype *TOR1 tor2* / p*TOR2*) is named SH100. The same host strain carrying a *tor2^{ts}* plasmid instead of the *TOR2* wild-type plasmid is named SH1x, where x is the number of the *ts* allele; *e.g.*, the *TOR1 tor2* strain carrying *tor2-21^{ts}* is SH121. NB4-6a (*tor1 tor2*) kept viable with YCplac111::*TOR2* is called SH200; when its plasmid-borne *TOR2* allele is replaced with a *tor2^{ts}* allele, it is named SH2x, where x is again the number of the *ts* allele (Table 1).

The growth of the *TOR1 tor2^{ts}* strains (SH1x series) and *tor1 tor2^{ts}* strains (SH2x series) at 24° and 37° on solid rich medium containing glucose as the carbon source (YPD) was assessed. Table 2 summarizes these results, from which we have classified the mutant strains into four classes, A through D, based on whether a mutant was defective for the unique, the shared, or both functions of TOR2 (Table 3). Class A mutants, represented by strains SH121 (*tor2-21^{ts}*) and SH1156 (*tor2-156^{ts}*), did not form colonies after 3 days at 37° and were thus temperature sensitive for the TOR2-unique function (Figure 1). The shared function is provided in these mutants by the chromosomal wild-type *TOR1* gene. Interestingly, we never obtained a class A mutant in which the shared function is provided by *TOR2*, *i.e.*, a *TOR2* allele that is defective only for the unique function. Class B mutants, represented by strains SH230 (*tor1 tor2-30^{ts}*) and SH2119 (*tor1 tor2-119^{ts}*), also arrested growth at 37° and were temperature sensitive only for the shared function (see class D below). Class C mutants, represented by strains SH221 (*tor1 tor2-21^{ts}*) and SH2156 (*tor1 tor2-156^{ts}*), were temperature sensitive for both TOR2 functions. Class D consists of pseudomutants and is represented by strains SH130 (*tor2-30^{ts}*) and SH1119 (*tor2-119^{ts}*). These pseudomutants formed normal-sized colonies at all temperatures on solid medium, indicating that the class B mutants, which contain the same *TOR2* alleles but in a *tor1* background, were defective only in the shared function. Some class D mutants had a partial

TABLE 1

Yeast strains used in this study

Strain	Genotype	Refs.
JK9-3d**a**	*MAT***a** *leu2-3, 112 trp1 ura3 rme1 his4 HML***a**	1
MH346-1a	JK9-3d**a** *ade2 tor2::ADE2* / pSEY18::*TOR2*	1
NB4-6a	MH346-1a *his3 HIS4 tor1::HIS3* / pSEY18::*TOR2*	2
SH100	JK9-3d**a** *ade2 tor2::ADE2* / YCplac111::*TOR2*	3
SH200	JK9-3da *ade2 his3 HIS4 tor1::HIS3 tor2::ADE2* / YCplac111::*TOR2*	3
SH1x	JK9-3d**a** *ade2 tor2::ADE2* / YCplac111::*tor2*-x (x = allele number)	3
SH2x	JK9-3d**a** *ade2 his3 HIS4 tor1::HIS3 tor2::ADE2* / YCplac111::*tor2*-x (x = allele number)	3
MH349-3d	JK9-3d**a** *tor1::LEU2-4*	4

References: 1, Kunz *et al.* (1993); 2, Barbet *et al.* (1996); 3, this study; 4, Helliwell *et al.* (1994). See Table 2 for complete list of SH1x and SH2x strains.

TABLE 2

Growth of *tor*^{ts} mutants on YPD

Strain	Genotype	Temperature 24°	Temperature 37°
SH100	*TOR2*	++++	++++
	Class A		
SH121	*tor2-21*	++++	–
SH1156	*tor2-156*	++++	–
	Class B		
SH214	*tor1 tor2-14*	++++	–
SH225	*tor1 tor2-25*	++++	–
SH229	*tor1 tor2-29*	++++	–
SH230	*tor1 tor2-30*	+++	–
SH232	*tor1 tor2-32*	++++	–
SH251	*tor1 tor2-51*	++++	–
SH259	*tor1 tor2-59*	++++	–
SH269	*tor1 tor2-69*	++++	–
SH276	*tor1 tor2-76*	+++	–
SH277	*tor1 tor2-77*	+++	–
SH278	*tor1 tor2-78*	++++	–
SH279	*tor1 tor2-79*	+++	–
SH281	*tor1 tor2-81*	+++	–
SH298	*tor1 tor2-98*	++++	–
SH2103	*tor1 tor2-103*	++++	–
SH2119	*tor1 tor2-119*	+++	–
SH2128	*tor1 tor2-128*	++++	–
SH2132	*tor1 tor2-132*	++++	–
SH2135	*tor1 tor2-135*	+++	–
SH2136	*tor1 tor2-136*	+++	–
SH2140	*tor1 tor2-140*	++++	–
SH2143	*tor1 tor2-143*	++++	–
SH2146	*tor1 tor2-146*	++++	–
SH2147	*tor1 tor2-147*	++++	–
SH2148	*tor1 tor2-148*	++++	–
SH2150	*tor1 tor2-150*	++++	–
SH2152	*tor1 tor2-152*	++++	–
	Class C		
SH221	*tor1 tor2-21*	++++	–
SH2156	*tor1 tor2-156*	+++	–
	Class D		
SH114	*tor2-14*	++++	+
SH125	*tor2-25*	++++	++
SH129	*tor2-29*	++++	+++
SH130	*tor2-30*	++++	++++
SH132	*tor2-32*	++++	++
SH151	*tor2-51*	++++	++
SH159	*tor2-59*	++++	++
SH169	*tor2-69*	++++	++
SH176	*tor2-76*	++++	+++
SH177	*tor2-77*	++++	+++
SH178	*tor2-78*	++++	++
SH179	*tor2-79*	++++	+++
SH181	*tor2-81*	++++	+++
SH198	*tor2-98*	++++	+++
SH1103	*tor2-103*	++++	+++
SH1119	*tor2-119*	++++	++++
SH1128	*tor2-128*	++++	++
SH1132	*tor2-132*	++++	+
SH1135	*tor2-135*	++++	+
SH1136	*tor2-136*	++++	++
SH1140	*tor2-140*	++++	++
SH1143	*tor2-143*	++++	+
SH1146	*tor2-146*	++++	++
SH1147	*tor2-147*	++++	+++
SH1148	*tor2-148*	++++	+++
SH1150	*tor2-150*	++++	++
SH1152	*tor2-152*	++++	++

(Continued)

TABLE 2

Continued

Pluses and minuses indicate growth (colony size) after 2.5 days at 37°.

growth defect indicating that, in these strains, the TOR2-unique function was partly defective.

Suppression of growth defects by nutrient source or medium supplements: Because the TORs have previously been implicated in nutrient sensing (Barbet *et al.* 1996; Di Como and Arndt 1996), we examined the effect of altering the carbon or nitrogen source on growth of various mutants. Mutants from all four classes were assessed for growth at 37° on rich medium containing galactose (YPG), raffinose (YPR), or ethanol/glycerol (YPE/glycerol) as the sole carbon source; growth was assessed previously (Table 2) on YPD medium, containing glucose as the carbon source. The growth defect of class A mutants, strains SH121 (*tor2-21*^{ts}) and SH1156 (*tor2-156*^{ts}), was fully suppressed on the raffinose medium (data not shown), was partly suppressed on the galactose medium (Table 4), and was not suppressed on the ethanol/glycerol medium (data not shown). The growth defect of class B and C mutants was not suppressed by any carbon source, except for strains SH214 (*tor1 tor2-14*^{ts}), SH298 (*tor1 tor2-98*^{ts}), SH2140 (*tor1 tor2-140*^{ts}), SH2143 (*tor1 tor2-143*^{ts}), SH2150 (*tor1 tor2-150*^{ts}), and SH2152 (*tor1 tor2-152*^{ts}). These mutants eventually formed very small colonies

TABLE 3

TOR function in different classes of *tor*^{ts} mutants

Mutant	TOR shared	TOR2 unique
Class A	+	–
Class B	–	+
Class C	–	–
Class D	+	+

Symbols: plus, function provided; minus, function not provided.

TABLE 4

Effects of carbon source and media supplements on growth of *tor*^{ts} mutants

Strain	Glucose	Galactose	1 m Sorbitol	0.5 m NaCl	0.1 m $CaCl_2$
SH100	++++	++++	++++	++++	++++
			Class A		
SH121	−	++	++++	++++	++
SH1156	−	++	++++	++++	++
			Class B		
SH214	−	+	++++	+++	+
SH225	−	−	+++	+	+
SH229	−	−	+++	+	+
SH230	−	−	++	−	+
SH232	−	−	−	−	−
SH251	−	−	+++	++	+
SH259	−	−	+++	++	+
SH269	−	−	+++	+	+
SH276	−	−	−	−	−
SH277	−	−	−	−	−
SH278	−	−	+++	−	+
SH279	−	−	+	−	−
SH281	−	−	−	−	−
SH298	−	+	+	++	+
SH2103	−	−	+	++	−
SH2119	−	−	++	++	+++
SH2128	−	−	++	+	−
SH2132	−	−	++	++	+
SH2135	−	−	++	+	−
SH2136	−	−	+	+	−
SH2140	−	+	+	+	−
SH2143	−	+	+	++	+
SH2146	−	−	+	+	−
SH2147	−	−	+	−	+
SH2148	−	−	+	−	−
SH2150	−	−	++	−	+
SH2152	−	−	++	−	−
			Class C		
SH221	−	−	−	−	−
SH2156	−	−	−	−	−
			Class D		
SH114	+	++++	++++	++++	++++
SH125	+	++++	++++	++++	++++
SH129	+	++++	++++	++++	++++
SH130	+	++++	++++	++++	++++
SH132	++	++++	++++	++++	++++
SH151	++	++++	++++	++++	++++
SH159	++	++++	++++	++++	++++
SH169	++	++++	++++	++++	++++
SH176	++	++++	++++	++++	++++
SH177	++	++++	++++	++++	++++
SH178	++	++++	++++	++++	++++
SH179	++	++++	++++	++++	++++
SH181	++	++++	++++	++++	++++
SH198	++	++++	++++	++++	++++
SH1103	++	+++	++++	++++	++++
SH1119	++	+++	++++	++++	++
SH1128	+++	++++	++++	++++	++++
SH1132	+++	++++	++++	++++	++++
SH1135	+++	++++	++++	++++	++++
SH1136	+++	++++	++++	++++	++++
SH1140	+++	++++	++++	++++	++++
SH1143	+++	++++	++++	++++	++++
SH1146	+++	++++	++++	++++	++++
SH1147	+++	++++	++++	++++	++++
SH1148	+++	++++	++++	++++	++++
SH1150	++++	++++	++++	++++	++++
SH1152	++++	++++	++++	++++	++++

The pluses indicate growth (colony size) at 37°; minuses indicate no growth.

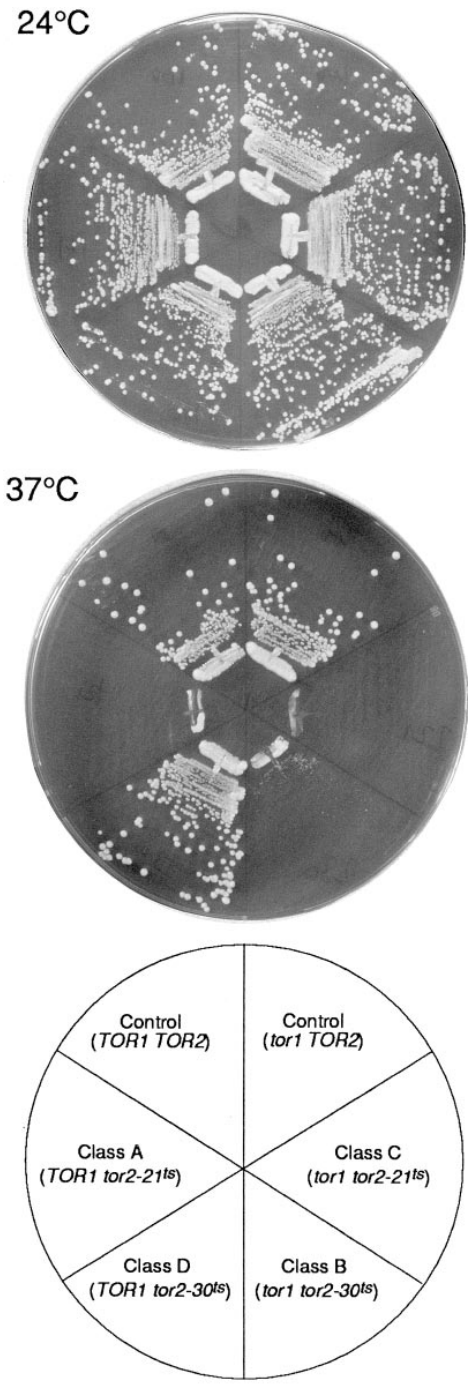

Figure 1.—Growth of class A through D mutants. Mutant and control strains were streaked on rich medium (YPD) and incubated at 24° for 3 days or 37° for 2.5 days. Strains are as follows: control (*TOR1 TOR2*) is SH100; control (*tor1 TOR2*) is SH200; class A (*TOR1 tor2-21^{ts}*) is SH121; class B (*tor1 tor2-30^{ts}*) is SH230; class C (*tor1 tor2-21^{ts}*) is SH221; class D (*TOR1 tor2-30^{ts}*) is SH130.

after 3 days, but only on the galactose medium (Table 4). Class D mutants that exhibited a partial growth defect all grew like wild type on galactose and raffinose medium, but ethanol/glycerol medium was not able to suppress the slower growth of these mutants. Mutants from all four classes were also assessed for growth at 37° on defined medium containing glucose as the carbon source and either proline, glutamate, or ammonium as the nitrogen source. Proline, glutamate, and ammonium are poor, intermediate, and good nitrogen sources, respectively. Unlike the carbon source, the nitrogen source had no effect on the growth of any of the mutants; none of the nitrogen sources suppressed the growth defect of any mutant.

Growth at 37° was also assessed on YPD medium supplemented with 1 m sorbitol, 0.5 m NaCl or 0.1 m $CaCl_2$. The growth defect of Class A mutants was fully suppressed by 1 m sorbitol or 0.5 m NaCl, and was partly suppressed by 0.1 m $CaCl_2$. Allele-specific effects were seen for class B mutants grown on medium containing one of the three supplements, ranging from complete suppression to no suppression (Table 4). The growth defect of class C mutants was not suppressed by 1 m sorbitol, 0.5 m NaCl, or 0.1 m $CaCl_2$. Class D slow-growing mutants all grew like wild type on medium supplemented with 1 m sorbitol, 0.5 m NaCl, or 0.1 m $CaCl_2$, with the exception of SH1152 (*TOR1 tor2-152^{ts}*), for which $CaCl_2$ had no effect. Thus, of all the mutants, class A mutants were most susceptible to suppression by either the carbon source or media supplements.

Class A mutants arrest in G2/M, and class B and C mutants arrest in G1: We characterized further the growth and cell cycle phenotypes of the class A, B, and C mutants. The growth and cell cycle arrests of the mutants were analyzed by determining growth curves (OD_{600}) and by FACS analysis. Class A mutants SH121 (*tor2-21^{ts}*) and SH1156 (*tor2-156^{ts}*) ceased to grow after 7–8 hr at 37°, after having completed two to three divisions (Figure 3 and data not shown). Most of the arrested cells (>80%) contained a 2n DNA content, indicating a block in the G2/M phase of the replicative cycle (Figure 2 and data not shown). Scoring the cells morphologically for cell cycle distribution showed that these strains arrested with an increased number of cells in different stages of budding, as described in greater detail below (Table 5 and data not shown). This postreplicative budded-cell arrest upon shifting a *tor2^{ts}* mutant to nonpermissive temperature differs from an apparently random arrest that was observed when TOR2 was depleted by down-regulating its transcription using the regulatable *GAL1* promoter (Kunz *et al.* 1993; Helliwell *et al.* 1994). At present, we cannot provide an explanation for this apparent discrepancy. However, it may be related to the fact that depleting TOR2 by down-regulating its transcription is a much more gradual depletion than that obtained by shifting the temperature-sensitive mutants to high temperature; cells containing *TOR2* under control of the glucose-repressed galactose promoter arrest growth, at 30°, only after approximately 14 hr in glucose medium.

In contrast to class A mutants, all the class B and C mutants arrested within one generation (within 3–4 hr)

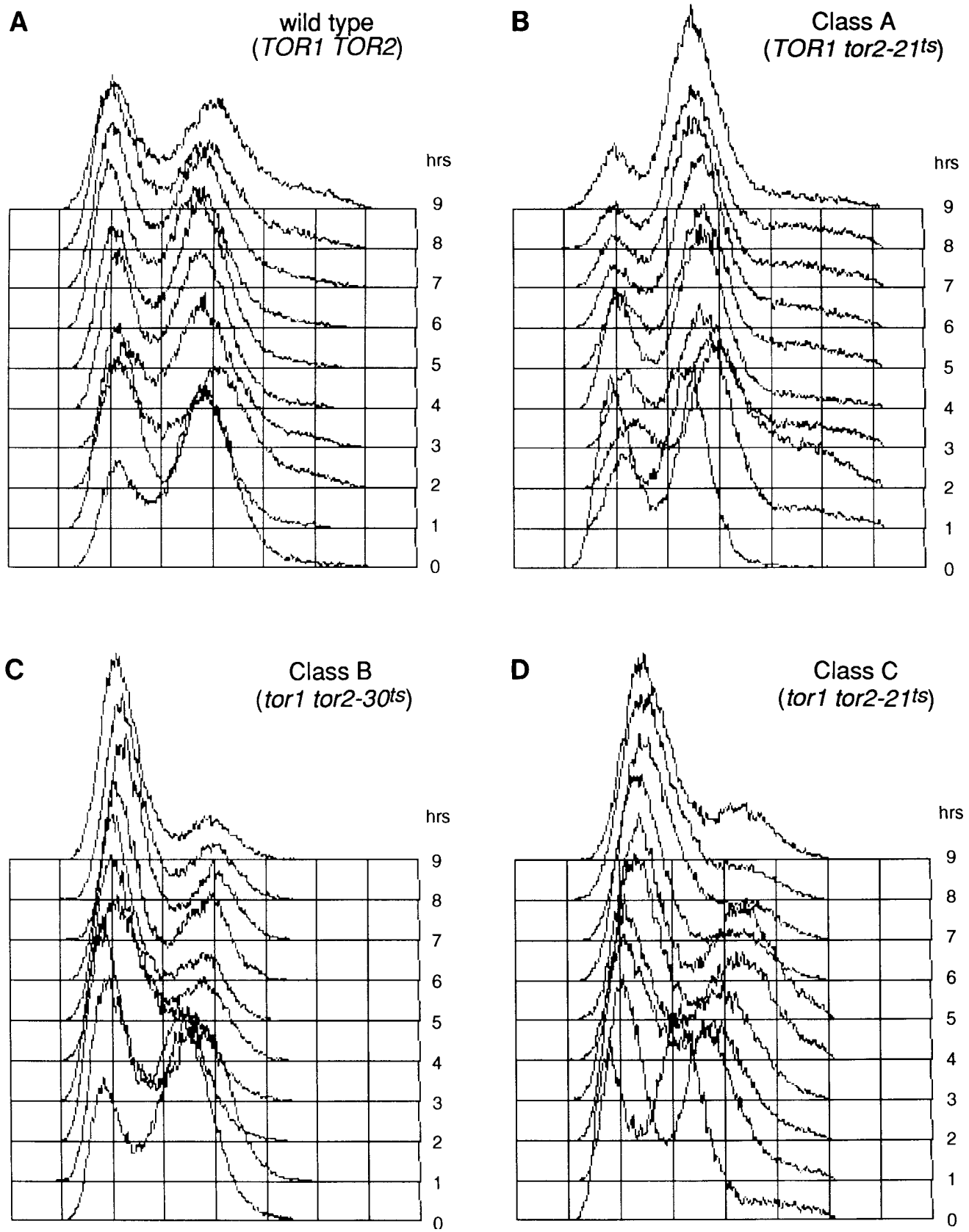

Figure 2.—FACS analysis of wild-type and *tor*^{ts} strains at nonpermissive temperature. Strains are as follows: control (*TOR1 TOR2*) is SH100; class A (*TOR1 tor2-21*^{ts}) is SH121; class B (*tor1 tor2-30*^{ts}) is SH230; class C (*tor1 tor2-21*^{ts}) is SH221. The DNA content in cell populations of each strain was analyzed at hourly intervals after a shift to the nonpermissive temperature; 50,000 events were analyzed. Class B and C strains arrest with 1n DNA content; class A strains arrest with 2n DNA content.

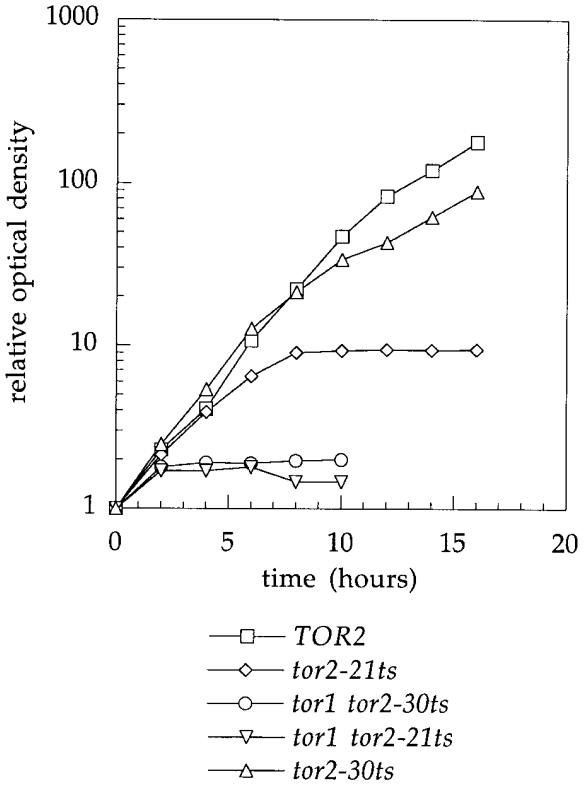

Figure 3.—Growth rates of *TOR2* (wild type, SH100), *tor2-21^{ts}* (class A, SH121), *tor1 tor2-30^{ts}* (class B, SH230), *tor1 tor2-21^{ts}* (class C, SH221), and *tor2-30^{ts}* (class D, SH130) strains. At time zero, exponentially growing cells were diluted to an OD_{600} of approximately 0.05 in prewarmed media and shaken at 37°. All values for a particular strain were normalized to its zero timepoint.

with >80% of the cells in G1 (1n DNA content). Results are shown for the class B mutant SH230 (*tor1 tor2-30^{ts}*) and the class C mutant SH221 (*tor1 tor2-21^{ts}*) (Figure 2; Figure 3). In addition, the average cell size of the strains increased after shift to 37°, for unknown reasons. The G1 arrest is in agreement with previous observations (Kunz *et al.* 1993; Helliwell *et al.* 1994; Barbet *et al.* 1996) and furthermore demonstrates that all the isolated *tor2^{ts}* alleles are temperature sensitive for the shared TOR function, regardless of the state of the other function of TOR2. Thus, no *tor2^{ts}* allele was isolated that was defective for only the unique function.

The class D mutants SH130 (*tor2-30^{ts}*) and SH1119 (*tor2-119^{ts}*) formed normal-sized colonies on solid medium. In liquid medium, strain SH130 exhibited a slight reduction in growth rate, as compared to the control strain, but this pseudomutant eventually reached a stationary phase cell density similar to that of control strain SH100.

The unique function of TOR2 is involved in the organization of the actin cytoskeleton: We have shown recently that the unique function of TOR2 is required for the organization of the actin cytoskeleton. Class A strain SH121 is defective in the cell-cycle-dependent, polarized distribution of the actin cytoskeleton, and overexpression of the actin- and tubulin-specific chaperone TCP20 suppresses both the growth and actin defects of SH121 (*tor2-21^{ts}*) (Schmidt *et al.* 1996). To confirm and extend the finding that the unique function of TOR2 is involved in actin organization, we examined the budding index and actin distribution of the class A mutant SH121 (*tor2-21^{ts}*), the class D mutant SH130 (*tor2-30^{ts}*), and the control strain SH100 (Table 5; Figure 4). Class B and C mutants were not examined because these mutants arrest as unbudded G1 cells in which the actin cytoskeleton is normally not polarized. In agreement with the FACS analysis, which indicated that SH121 cells accumulated in G2/M, SH121 had more cells in the budding stages of the cell cycle. In particular, SH121 preferentially accumulated small-budded cells, indicating that this mutant had a defect in the early stages of bud growth. Interestingly, class D mutant SH130 (*tor2-30^{ts}*) also showed an altered distribution, which, although not as severe as for the class A mutant, also favored budded cells.

The budded cells in this analysis were assessed for actin distribution (Table 5; Figure 4). Actin was considered to be abnormally distributed (apolar) if more than five actin patches could still be seen in the mother cell and if these patches were not adjacent to the bud neck. Class A mutant SH121 (*tor2-21^{ts}*) exhibited poor polarization of actin patches, with 80% of the cells analyzed having improperly distributed actin (Figure 4). Additionally, very few or no actin cables could be seen in the mutant cells, implying defects in cable assembly. The other class A mutant SH1156 *(tor2-156^{ts}*) exhibited a similar defect in actin distribution (data not shown). Interestingly, 47% of the class D mutant (SH130) cells had an altered actin distribution as compared with 16% of the control cells (SH100). This intermediate effect was apparently not sufficient to severely affect growth, since SH130 (*tor2-30^{ts}*) formed normal colonies. Yeast

TABLE 5

Cell cycle and actin distribution of class A and D *tor^{ts}* mutants

Mutant	Allele	Cell cycle distribution (%)				Actin (%)	
		⊙	⊙*	⊙*	⊙	⊙	⊙
Wild type	*TOR2*	43	25	21	12	84	16
Class A	*tor2-21*	24	56	14	6	20	80
Class D	*tor2-30*	30	42	16	12	53	47

Wild-type *TOR2* (SH100), class A *tor2-21^{ts}* (SH121), and class D *tor2-30^{ts}* (SH130) strains were analyzed for cell cycle distribution and actin localization 8 hr after a shift to nonpermissive temperature. Only cells marked with an asterisk in the cell cycle distribution were analyzed for their actin localization.

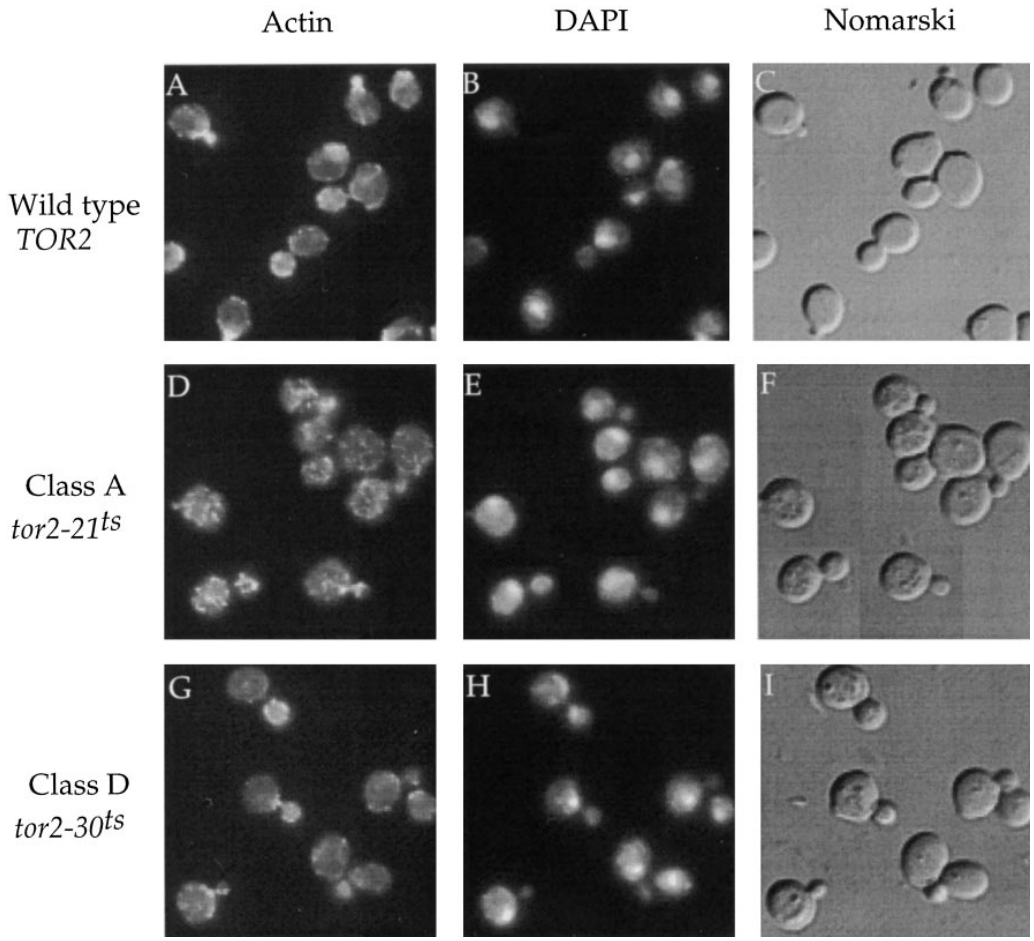

Figure 4.—Loss of only the unique function of TOR2 causes a severe actin defect. Analysis of the actin distribution (Actin), nuclear distribution (DAPI), and cell morphology (Nomarski) of wild-type *TOR2* (SH100), class A *tor2-21*^{ts} (SH121), and class D *tor2-30*^{ts} (SH130). Cells were fixed for actin and DNA staining 8 hr after shift to the nonpermissive temperature in rich medium.

cells can tolerate a certain amount of cytoskeletal perturbation and still grow relatively normally, as observed previously with mutants defective in actin itself or in actin-binding proteins (Novick and Botstein 1985). Thus, it appears that in a class A mutant there is a severe defect in actin polarization that slows down or prevents bud growth without affecting DNA replication. This defect leads to a cell population dominated by small-budded cells with a replicated genome. The unique function of TOR2 is thus involved in the correct localization of actin and in bud growth. The defect in bud growth is presumably a secondary consequence of the actin defect.

The shared function of TOR2 is involved in protein synthesis: We have recently demonstrated that depletion of TOR1 and TOR2 or rapamycin treatment of wild-type cells causes an immediate and severe block in protein synthesis. Furthermore, we have shown that this block occurs at the level of translation initiation and is the cause of the early G1 arrest seen with the loss of TOR or rapamycin treatment (Barbet *et al.* 1996). We wished to confirm these observations for class B and C mutants (*tor1 tor2*^{ts}). Figure 5 shows the results of continuous labeling experiments that measure the amount of labeled methionine incorporated into protein in these mutants at times after a shift to nonpermissive temperature, thereby giving an indication of the rate of protein synthesis. As expected, class B (SH230 and SH2119) and C (SH221 and SH2156) mutants exhibited a rapid and severe defect in the amount of label incorporated into protein (Figure 5 and data not shown), in agreement with previous data and congruent with the fact that these mutants arrested within one cell cycle and in G1 (Figure 2, Figure 3, and data not shown). As both types of mutants exhibited a similarly severe translation defect, the unique function of TOR2 did not appear to have a role in translation. To assess further whether the unique function of TOR2 could be involved in the regulation of translation, we examined the rates of label incorporation in the two class A mutants. The class A mutants, SH121 (*tor2-21*^{ts}) and SH1156 (*tor2-156*^{ts}), showed a relatively mild reduction in incorporation rates, suggesting that the unique function of TOR2 is not directly involved in the regulation of translation. This mild loss of translation seen with SH121 and SH1156 is presumably an indirect consequence of the reduction in growth of these strains after shift to nonpermissive temperature.

Overexpression of *MSS4, PKC1, PLC1, RHO2, ROM2,* or *SUR1* rescues a class A mutant: The above analysis

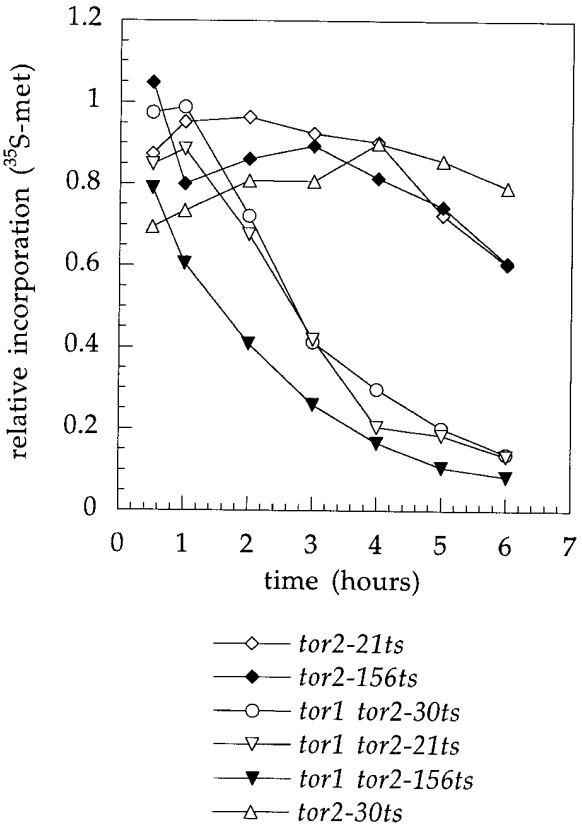

Figure 5.—Protein synthesis of *tor*^{ts} strains after shift to nonpermissive temperature. ^{35}S-methionine incorporation rates of *tor2-21*^{ts} (class A, SH121), *tor2-156*^{ts} (class A, SH1156), *tor1 tor2-30*^{ts} (class B, SH230), *tor1 tor2-21*^{ts} (class C, SH221), *tor1 tor2-156*^{ts} (class C, SH2156), and *tor2-30*^{ts} (class D, SH130) mutants normalized to a *TOR2* (wild type, SH100) strain. The experiment was performed as described in materials and methods. The data from a representative experiment are shown. The label was added, and the cells were shifted to 37° at time zero.

of class A, class B, and class C mutants supports the existence of at least two functions for TOR2 (Table 3). Class A mutants arrest within a few generations as small-budded cells in G2/M and with an actin defect, whereas class B and C mutants arrest within one generation in G1 because of a translation initiation defect. To identify the two pathways by which TOR2 may control actin organization and translation initiation, we isolated multicopy suppressors of class A mutant SH121 and of class B mutant SH229 (see materials and methods).

To identify the pathway mediating the TOR2-unique function, we isolated high-copy suppressors that rescue class A mutant SH121 (*tor2-21*^{ts}). Plasmids isolated as multicopy suppressors were sequenced and subcloned to identify the ORF with suppressor activity. *TOR2* itself was isolated as a suppressor four times. Other multicopy suppressors were *MSS4*, *PKC1*, *PLC1*, *RHO2*, and *SUR1* (Figure 6; Table 6).

MSS4, a strong suppressor obtained once (clone pSH121.10), encodes an essential PI-4-P 5-kinase homolog (Yoshida *et al.* 1994b). *FAB1* encodes the only other PI-4-P 5-kinase homolog in yeast (Yamamoto *et al.* 1995), and we thus examined whether overexpression of *FAB1* could also rescue SH121. *FAB1* on a high-copy-number vector (pEMY105) was unable to suppress the growth defect of SH121 at nonpermissive temperature (data not shown). We also examined whether overexpression of either *STT4* (YEp352-STT4) or *PIK1* (pGB1) could rescue SH121; these two genes encode the two PI 4-kinases in yeast, and *MSS4* was first isolated as a multicopy suppressor of an *STT4* mutation (Yoshida *et al.* 1994a,b). Neither *STT4* or *PIK1* on high copy number vectors suppressed the growth arrest of SH121. Finally, we examined if overexpression of *MSS4* could rescue a strain disrupted for *TOR2*. Strain MH346 (*tor2*::*ADE2*/*TOR2*) containing the *MSS4* multicopy suppressor was sporulated and dissected. For each tetrad, only the two *TOR2* segregants of the four progeny were viable, and of these, most had retained the high-copy-number *MSS4* plasmid. Thus, overexpression of *MSS4* could not suppress a *tor2*-null allele, unlike the *tor2*^{ts} allele, suggesting that suppression by *MSS4* requires residual TOR2 activity.

PKC1, a strong suppressor obtained twice (clones pSH121.8 and pSH121.25), encodes the yeast homolog of protein kinase C (Levin *et al.* 1990). Multiple copies of *PKC1* could not suppress a *tor2*-null allele (*tor2*::*ADE2*), as assayed as described above for *MSS4*.

PLC1, a moderate suppressor obtained once (clone pSH121.56), encodes the yeast homolog of phospholipase C (Flick and Thorner 1993; Payne and Fitzgerald-Hayes 1993; Yoko-o *et al.* 1993). A subclone of pSH121.56 (pSH25) containing only *PLC1* was also only a moderate suppressor of *tor2-21*^{ts}. The *PLC1* plasmid pSH25 did not suppress a *tor2*-null allele (*tor2*::*ADE2*).

RHO2, a strong suppressor obtained once (clone pSH121.33), encodes a small Rho-like GTPase. We also found *RHO2* as a multicopy suppressor of a *tor2* mutation by an independent and concurrent approach (Schmidt *et al.* 1997). In this other study, we also found that overexpression of *ROM2*, encoding the GDP/GTP exchange factor (GEF) for RHO2, rescues strain SH121, although we never obtained *ROM2* in the multicopy suppressor isolation performed here.

SUR1, a weak suppressor obtained three times (clones pSH121.28, pSH121.30, and pSH121.59), encodes a protein with no homology to known proteins. The function of SUR1 is unknown, but it is possibly involved in the maintenance of phospholipid levels (see discussion).

Overexpression of *PLC1* or *MSS4* rescues a class B mutant: To identify the pathway mediating the *TOR*-shared function, we isolated multicopy suppressors that rescue class B mutant SH229 (*tor1 tor2-29*^{ts}). *TOR2* itself

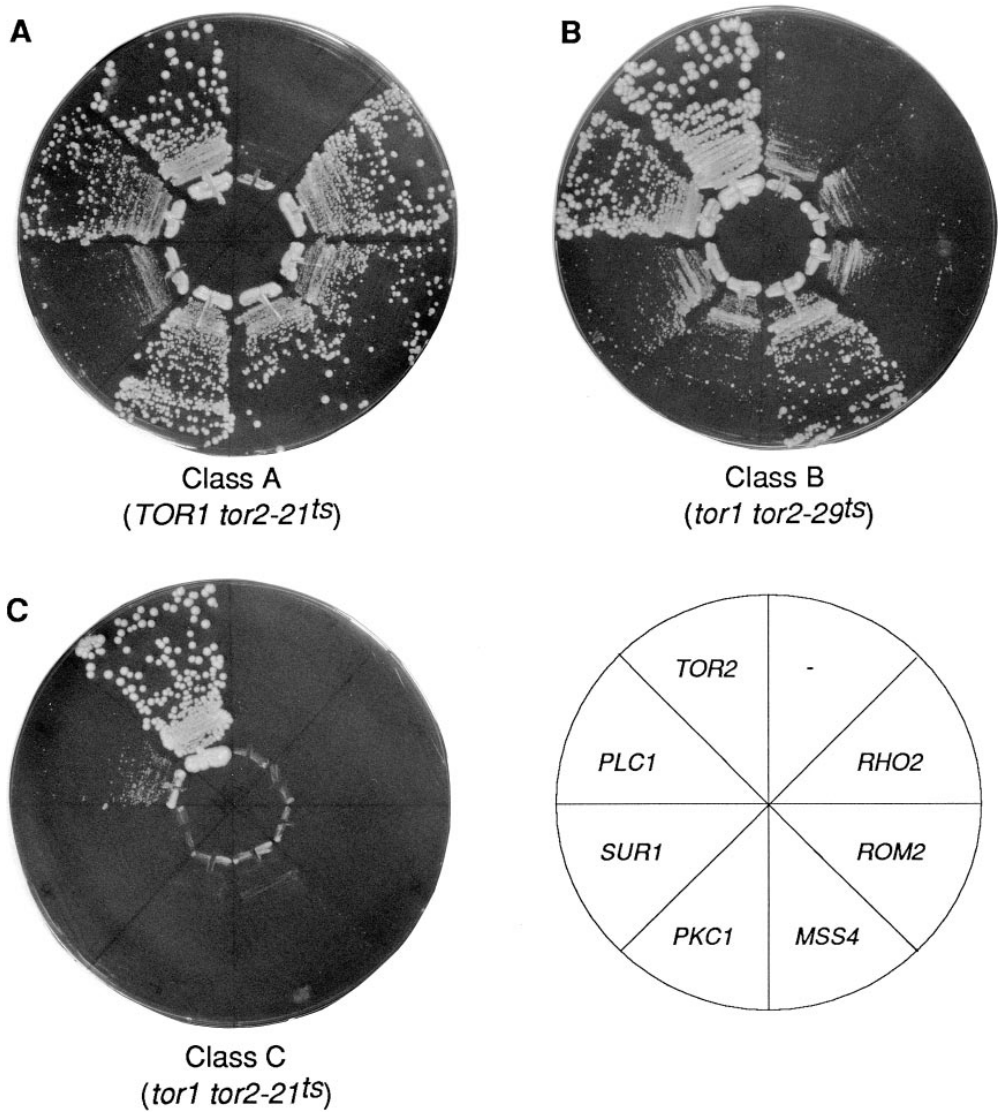

Figure 6.—Suppressor analysis of *tor*^{ts} strains lacking either the unique (class A), the shared (class B), or both (class C) functions of TOR2. Class A (*TOR1 tor2-21*^{ts}) strain is SH121; class B (*tor1 tor2-29*^{ts}) strain is SH229; class C (*tor1 tor2-21*^{ts}) strain is SH221. The strains were transformed with 2μ *URA3* plasmids bearing the genes denoted in the figure (*RHO2*, pC-186; *ROM2*, pAS30; *MSS4*, pSH22; *PKC1*, pSH24; *SUR1*, pSH27; *PLC1*, pSH25; *TOR2*, pJK3-3), streaked on rich medium, and incubated for 2.5 (plates A and C) or 3.5 (plate B) days at 37°. Table 6 summarizes these data.

was isolated as a multicopy suppressor 12 times. Five other suppressor plasmids were obtained and found to have overlapping fragments containing the *PLC1* gene in common. We confirmed that overexpression of *PLC1* alone was sufficient for suppression by transforming the class B mutants SH229 (*tor1 tor2-29*^{ts}) and SH2103 (*tor1 tor2-103*^{ts}) with pSH25 (pSEY18::*PLC1*) and assessing growth on rich medium at the nonpermissive temperature (Figure 6 and data not shown). The suppression by *PLC1* was moderate.

We then examined whether any of the multicopy suppressors, other than *PLC1*, that rescued a class A mutant could also rescue a class B mutant (Figure 6B; Table 6). Neither *PKC1*, *RHO2*, *ROM2*, nor *SUR1* could rescue class B mutant SH229, as judged by growth on solid rich medium at 37°, even after prolonged incubation. *MSS4* was able to rescue strain SH229 at high temperature; however, the suppression by *MSS4* was weaker than that by *PLC1* and evident only after prolonged incubation.

As *PLC1* was isolated as a multicopy supppressor of a TOR-shared function defect, we also examined whether high-copy *PLC1* could suppress a *tor1* mutation. *tor1* strains have a slow-growth phenotype that is exacer-

TABLE 6

Multicopy suppressors of *tor*^{ts} class A, B, and C mutants

Suppressor gene	Class A *tor2-21*^{ts}	Class B *tor1 tor2-29*^{ts}	Class C *tor1 tor2-21*^{ts}
TOR2	+++	+++	+++
PLC1	++	++	+
MSS4	+++	+/−	−
ROM2	+++	−	−
RHO2	+++	−	−
PKC1	+++	−	−
SUR1	+	−	−

The pluses and minuses refer to suppression of growth defect of the indicated *tor*^{ts} mutant.

bated at low and high temperatures, and at least at 39° *tor1* cells accumulate in the G1 phase of the cell cycle (S. B. Helliwell and M. N. Hall, unpublished results). We transformed plasmids bearing *TOR1*, *PLC1*, and an empty vector into MH349-3d (*tor1*::*LEU2-4*) and examined the growth of the resulting transformants at 24° and 39°. At both temperatures, multicopy *PLC1* was able to partially suppress the growth defect of the *tor1* strain (data not shown), confirming that overexpression of *PLC1* could suppress a defect in the TOR-shared function. As *PLC1* was able to suppress defects in either the shared or the unique function of *TOR2*, we examined whether it could rescue a class C mutant that lacks all TOR function. pSH25 (pSEY18::*PLC1*) was transformed into class C mutant SH221 (*tor1 tor2-21^{ts}*) and assessed for growth at the nonpermissive temperature. After 2.5 days incubation at 37°, transformants formed small colonies, indicating that overexpression of *PLC1* could weakly suppress a complete TOR deficiency (Figure 6C). Overexpression of *MSS4*, *PKC1*, *RHO2*, *ROM2*, or *SUR1* did not rescue strain SH221.

DISCUSSION

TOR1 and *TOR2* encode 67% identical PI kinase homologs, and mutations in either gene confer rapamycin resistance (Heitman *et al.* 1991; Kunz *et al.* 1993; Helliwell *et al.* 1994). *TOR2* has two essential functions, only one of which can be performed by *TOR1*. We have shown previously that the TOR-shared function is involved in the control of translation initiation; loss of this function causes cells to arrest in early G1 within one cell cycle (Barbet *et al.* 1996). Recent evidence indicates that the unique function of *TOR2* is involved in the control of the actin cytoskeleton (Schmidt *et al.* 1996; 1997). In this study, we sought to genetically dissect these two functions at the level of the *TOR2* gene by generating a large number of *tor2^{ts}* mutants. We expected that some of these mutants would be conditional for one function while being unaffected in the other. This would support the model that TOR2 is part of two separate signaling pathways and would facilitate the analysis of each pathway individually.

Twenty-nine *tor2^{ts}* alleles were obtained and characterized. All of the *tor2^{ts}* alleles were defective in the TOR-shared function such that all *tor1 tor2^{ts}* strains arrested growth like strains depleted for TOR. However, the same alleles varied in their ability to provide the TOR2-unique function such that they caused growth defects of varying severity when in a strain background containing a wild-type *TOR1* gene. The alleles varied from being unaffected (*tor2-30^{ts}* and *tor2-119^{ts}*) to being fully defective for the unique function (*tor2-21^{ts}* and *tor2-156^{ts}*). No *TOR2* allele was isolated that was temperature sensitive for only the unique function, for unknown reasons.

The alleles obtained allowed the construction of three classes of *tor* mutants that arrest growth for different reasons. Class A mutants, strains SH121 (*tor2-21^{ts}*) and SH1156 (*tor2-156^{ts}*), are *tor2-unique^{ts}* and arrest within a few generations, primarily as small-budded cells with a 2n DNA content. These mutants are defective in the organization of the actin cytoskeleton. The actin defect is presumably the cause of growth arrest (Schmidt *et al.* 1996), and may account for the terminal phenotype of small-budded cells. Class B mutants, strains SH230 (*tor1 tor2-30^{ts}*) and SH229 (*tor1 tor2-29^{ts}*), and class C mutants, strains SH221 (*tor1 tor2-21^{ts}*) and SH2156 (*tor1 tor2-156^{ts}*), are *tor-shared^{ts}* and *tor-null^{ts}*, respectively. These mutants arrest rapidly, in G1, and are defective in translation. We have previously shown that the loss of translation is responsible for the G1 arrest phenotype. Importantly, class D strains, SH130 (*tor2-30^{ts}*) and SH129 (*tor2-29^{ts}*), are viable pseudomutants, indicating that these *tor2^{ts}* alleles encode a TOR2 protein that can carry out the unique function, but not the shared function. Thus, the mutants support the model that TOR2 performs two different growth-controlling functions: one function is involved in G1 progression via control of translation initiation, and the other function is involved in bud growth via control of the actin cytoskeleton. These two functions, or readouts, of TOR2 are presumably mediated by two separate TOR2 signaling pathways.

Suppressor analyses with the mutants deficient in either the shared function or the unique function provided insight into the nature of the possible signaling events downstream of TOR2. Many genes were isolated that when overexpressed allow growth of a *tor2^{ts}* strain lacking the TOR2-unique function. These genes encode proteins (MSS4, PKC1, PLC1, RHO2, ROM2, and SUR1) directly or indirectly implicated in signaling and, in particular, in protein kinase C signaling. MSS4 is a PI-4-P 5-kinase homolog (Yoshida *et al.* 1994b) and may be upstream of the yeast protein kinase C PKC1, which was also isolated as a multicopy suppressor. PLC1 is the yeast homolog of mammalian PI-phospholipase Cδ, which has been shown to exhibit a calcium dependence with respect to its hydrolytic activity toward PI and PI-4,5-P_2 (PIP_2) (Flick and Thorner 1993). RHO2 and ROM2 are components of a Rho-type GTPase switch that also controls PKC1 (Nonaka *et al.* 1995; Ozaki *et al.* 1996). SUR1 is a protein of unkown function involved in maintenance of phospholipid levels. *SUR1* was originally identified as a mutation that suppresses the growth defect of strains lacking *RVS161* or *RVS167* (Desfarges *et al.* 1993; Takita *et al.* 1995). Importantly, *rvs161* and *rvs167* mutants exhibit an altered actin cytoskeleton and an inability to maintain viability upon carbon or nitrogen starvation (Crouzet *et al.* 1991; Bauer *et al.* 1993). Analysis of a *sur1-1* mutant demonstrated reduced levels and altered ratios of membrane phospholipids (Desfarges *et al.* 1993).

Overexpression of MSS4 or PLC1 were also found to rescue a mutant lacking the TOR-shared function. Additional studies will be required to determine whether MSS4, PKC1, PLC1, and SUR1 are in a signaling pathway with TOR2 or whether they are in a parallel pathway and are suppressing via a bypass mechanism. However, the suppressors strongly suggest that there are signaling events downstream of TOR2 with regard to both the shared and the unique functions of TOR2.

Furthermore, we anticipate that at least some of the suppressors, including PKC1, are in a TOR2 pathway. First, the growth arrest phenotype of the class A mutant SH121 (*tor2-21^{ts}*) is similar to the arrest phenotype of strains expressing a conditional allele of *PKC1*; both *tor2-21^{ts}* and *pkc1^{ts}* mutants arrest with increased numbers of small-budded cells containing replicated DNA, and both mutants are rescued by the addition of 0.1 m $CaCl_2$, 0.5 m NaCl, or the osmotic stabilizer sorbitol to the growth medium (Levin and Bartlett-Heubusch 1992). Second, we have recently shown that TOR2 signals to the actin cytoskeleton by activating a GTPase switch composed of RHO1, RHO2, ROM2, and SAC7 (Schmidt *et al.* 1997), and others have shown recently that PKC1 is regulated by RHO1 (Nonaka *et al.* 1995; Kamada *et al.* 1996). However, *pkc1* mutants have not been shown to have actin defects, although mutants altered in MPK1, a mitogen-activated protein kinase (MAPK) regulated by PKC1, exhibit delocalized actin (Mazzoni *et al.* 1993). PKC1 is responsible for the regulation of genes involved in the remodeling and synthesis of the cell wall at the growing bud tip (Igual *et al.* 1996), suggesting that TOR2 is a global controller of both actin reorganization and cell wall biosynthesis. More careful analysis of the roles played by TOR2, RHO1, PKC1, and the cell integrity pathway will shed light on the importance of TOR2 in both cell wall biosynthesis and actin reorganization in polarized cell growth.

A surprising observation was that two of the multicopy suppressors, *MSS4* and *PLC1*, rescue both class A and class B mutants. Regardless of whether MSS4 and PLC1 are in a TOR2 pathway or in a parallel pathway, this finding suggests that the two signaling functions of TOR2 are more related than previously thought. It remains to be determined how the two different readouts of TOR2—translation initiation and organization of the actin cytoskeleton—are affected by the same signaling proteins. There may be crosstalk between two TOR2 signaling pathways such that overexpressing a protein in one pathway affects the other. Alternatively, two pathways may have common components or the common suppressors may have two functions. A sharing of components between two TOR2 signaling pathways could ensure that temporal (protein synthesis) and spatial (actin cytoskeleton organization) control of cell growth are coordinated (Schmidt *et al.* 1997).

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