Molecular Biology of the Cell
Vol. 5, 105–118, January 1994

TOR1 and TOR2 Are Structurally and Functionally Similar but not Identical Phosphatidylinositol Kinase Homologues in Yeast

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Submitted September 23; Accepted November 30, 1993
Monitoring Editor: Michael Wigler

The *Saccharomyces cerevisiae* genes *TOR1* and *TOR2* were originally identified by mutations that confer resistance to the immunosuppressant rapamycin. *TOR2* was previously shown to encode an essential 282-kDa phosphatidylinositol kinase (PI kinase) homologue. The *TOR1* gene product is also a large (281 kDa) PI kinase homologue, with 67% identity to TOR2. *TOR1* is not essential, but a *TOR1 TOR2* double disruption uniquely confers a cell cycle (G1) arrest as does exposure to rapamycin; disruption of *TOR2* alone is lethal but does not cause a cell cycle arrest. TOR1-TOR2 and TOR2-TOR1 hybrids indicate that carboxy-terminal domains of TOR1 and TOR2 containing a lipid kinase sequence motif are interchangeable and therefore functionally equivalent; the other portions of TOR1 and TOR2 are not interchangeable. The *TOR1-1* and *TOR2-1* mutations, which confer rapamycin resistance, alter the same potential protein kinase C site in the respective protein's lipid kinase domain. Thus, TOR1 and TOR2 are likely similar but not identical, rapamycin-sensitive PI kinases possibly regulated by phosphorylation. TOR1 and TOR2 may be components of a novel signal transduction pathway controlling progression through G1.

INTRODUCTION

The immunosuppressive drug rapamycin causes yeast cells and activated T cells to arrest in the G1 phase of the cell cycle (Heitman *et al.*, 1992; Sigal and Dumont, 1992; Schreiber and Crabtree, 1992). In the yeast *Saccharomyces cerevisiae*, the three genes *FPR1*, *TOR1*, and *TOR2* are involved in the action of rapamycin. Mutations in any one of these genes renders yeast cells resistant to the toxic effects of the drug (Heitman *et al.*, 1991a). FPR1 is a nonessential cytosolic FK506-binding protein (FKBP) that also binds the FK506 analogue rapamycin (Heitman *et al.*, 1991b; Koltin *et al.*, 1991; Wiederrecht *et al.*, 1991). FKBPs are highly conserved, ubiquitous proteins that have proline isomerase activity in vitro and may fold proteins in vivo. TOR2 is an essential phosphatidylinositol kinase (PI kinase) homolog (Kunz *et al.*, 1993). Cells lacking TOR2 are nonviable but arrest randomly in the cell cycle. As described below, TOR1 is a nonessential PI kinase homologue, however, cells lacking both TOR1 and TOR2 arrest in G1 (Kunz *et al.*, 1993). Thus, the *TOR* gene products appear to be components of a novel signal transduction pathway that controls progression through the G1 phase of the cell cycle. The current model for drug action is that a FKBP-rapamycin complex gains a toxic function that blocks cell proliferation by inhibiting TOR1 and TOR2 (Kunz and Hall, 1993).

Phosphatidylinositol (PI) kinases phosphorylate the inositol ring of PI (for review see Carpenter and Cantley, 1990). PI 4-kinases (type II and III PI kinase) phosphorylate the 4 position of the inositol ring, to produce PI-4-P. PI-4-P is then phosphorylated on the 5 position to produce PI-4,5-P_2, which is subsequently cleaved by phospholipase C to form the standard second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) (Berridge, 1987; Berridge and Irvine, 1989). PI 4-kinase has been detected in various tissues (Hou

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et al., 1988; Walker *et al.*, 1988; Porter *et al.*, 1989), and three PI 4-kinases have been purified and characterized from yeast (Buxeda *et al.*, 1991; Nickels *et al.*, 1992; Flanagan and Thorner, 1992). PI 3-kinase (type I PI-kinase) adds phosphate to the 3 position of the inositol ring of PI, PI-4-P, and PI-4,5-P_2. In mammalian cells, it appears that at least one of the products of PI 3-kinase, PI-3-P, PI-3,4-P_2, and PI-3,4,5-P_3, acts directly as a second messenger signaling cell proliferation. First, 3-phosphorylated phosphoinositides are not substrates for phospholipase C (Lips *et al.*, 1989; Serunian *et al.*, 1989). Second, active PI 3-kinase is associated with activated tyrosine kinases, and in the case of the platelet-derived growth factor receptor, PI 3-kinase is an independent downstream mediator of the PDGF mitogenic signal (Cantley *et al.*, 1991; Valius and Kazlauskas, 1993). Third, the kinetics of appearance of 3-phosphorylated phosphoinositides in PDGF-stimulated cells is consistent with a role as a second messenger (Auger *et al.*, 1989b). Fourth, the ζ isozyme of bovine protein kinase C (PKC) can be activated by PI-3,4-P_2 and PI-3,4,5-P_3 (Nakanishi *et al.*, 1993).

In yeast, the ratio of endogenous PI-3-P to PI-4-P is approximately 60:40, in stark contrast to mammalian cells where the ratio is approximately 10:90 (Whitman *et al.*, 1988; Auger *et al.*, 1989a; Stephens *et al.*, 1989; Hawkins *et al.*, 1993). The yeast PI 3-kinase VPS34 is involved in the sorting of soluble vacuolar proteins. A *VPS34* disruption strain is temperature sensitive for growth and exhibits severe defects in the sorting of vacuolar carboxypeptidase Y, and, in addition, has no detectable PI-3-P (Herman and Emr, 1990; Schu *et al.*, 1993). Thus, in yeast, 3-phosphorylated phosphoinositides have a role in membrane traffic.

The *TOR2* gene encodes a 282-kDa protein with homology to the p110 catalytic subunit of bovine phosphatidylinositol 3-kinase and VPS34 (Herman and Emr, 1990; Hiles *et al.*, 1992; Kunz *et al.*, 1993). The region of highest homology between the three proteins constitutes a lipid kinase consensus sequence (Kunz *et al.*, 1993). In the case of VPS34, the lipid kinase motif is required for PI 3-kinase activity (Schu *et al.*, 1993).

Here we describe the isolation and characterisation of the *TOR1* gene. The gene is 68 and 67% identical to *TOR2* at the nucleotide and predicted amino acid level, respectively, and also contains a so-called lipid kinase domain. Because disruption of *TOR1* and *TOR2* individually and disruption of *TOR1* and *TOR2* in combination all confer different phenotypes, TOR1 and TOR2 have overlapping and distinct functions. TOR2-TOR1 and TOR1-TOR2 hybrid proteins suggest that a shared cell cycle function is mediated by each intact protein's highly conserved (80% identity) carboxy-terminal lipid kinase domain. Furthermore, the lipid kinase domain of TOR1 and TOR2 may be a target of PKC, as suggested by mutations that confer rapamycin resistance.

MATERIALS AND METHODS

Strains, Plasmids and Media

The *S. cerevisiae* strains used in this work are listed in Table 1. All strains except the *tif2*, *ste18*, and *pkc1* strains were isogenic JK9-3d derivatives. SH8 was constructed by crossing NB3-3b with NB6-4c and selecting against the pJK3-3 plasmid. YCplac111 is *CEN4 LEU2 amp*^{r} (Gietz and Sugino, 1988). pSEY18 is 2μ *URA3 amp*^{r} (from S. Emr, University of California, San Diego). Plasmid pPW2 is pSEY18 carrying a 16-kilobase kb genomic DNA insert containing the *TOR1-1* allele (see *Construction of Genomic DNA Library*). Plasmid pPW2-13 is pPW2 lacking the 9-kb *Bgl* II fragment of the genomic DNA insert. Plasmid pPW20 is a gap-repaired derivative of pPW2-13 containing the wild-type *TOR1* allele (see Cloning of *TOR1*). Plasmid pJK3-3 is pSEY18 containing the wild-type *TOR2* allele (Kunz *et al.*, 1993). Plasmid pJK4 is YCplac111 containing *TOR2* (Kunz *et al.*, 1993). Plasmid pJK5 is *CEN4 URA3 GAL1p-TOR2 amp*^{r} (Kunz *et al.*, 1993). Plasmid pTOR1-TOR2 is YCplac111 carrying the *TOR1-TOR2* hybrid gene. Plasmid pTOR2-TOR1 is YCplac111 containing the *TOR2-TOR1* hybrid gene. The pTOR1-TOR2 and pTOR2-TOR1 plasmids were made as described below (see *Construction of TOR1-TOR2 and TOR2-TOR1 Hybrid Genes*). Plasmid pBM743[*PKC1*] (D. Levin, The Johns Hopkins University, Baltimore) is *CEN URA3 GAL1p-PKC1amp*^{r}. The composition of rich medium (YPD), synthetic minimal medium (SD, SGalGly) complemented with the appropriate nutrients for plasmid maintenance, and sporulation medium were as described (Sherman, 1991). YPlactate, YPpyruvate, and YPglycerol were prepared as described (Davis *et al.*, 1992), with the exception that YPglycerol contained 3% glycerol and 3% ethanol. Medium containing 1 M sorbitol was YPD. Rapamycin (kindly provided by Sandoz Pharma Ltd., Basel) was added to the medium to a final concentration of 0.2 μg/ml. Before addition to media, the drug was dissolved (1 mg/ml) in 10% Tween-20 in ethanol.

Table 1. Yeast strains used in this study

DL164	**a** *trp1 leu2 ura3 his4 can1 pkc1::LEU2*
JH3-3b	JK9-3dα *fpr1::URA3-3*
JH11-1c	JK9-3d**a** *TOR1-1*
JK9-3d**a**/α	**a**/α *leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1/trp1 his4/his4 rme1/rme1 HML**a**/HML**a***
JK9-3d**a**	**a** *leu2-3,112 ura3-52 trp1 his4 rme1 HML**a***
JK9-3dα	α *leu2-3, 112 ura3-52 trp1 his4 rme1 HML**a***
JK350-2a	JK9-3dα *ade2Δ tor1::LEU2-4 tor2::ADE2-3*
JK350-3a	JK9-3dα *ade2Δ tor2::ADE2-3*
MH269-4d	JK9-3d**a** *ade2Δ fpr1::URA3-3*
MH272-1c	MH272-3f**a** *fpr1::URA3-3*
MH272-1d**a**	JK9-3d**a** *HIS4 his3Δ*
MH272-3f**a**/α	JK9-3d**a**/α *HIS4/HIS4 his3Δ/his3Δ ade2Δ/ade2Δ*
MH272-3f**a**	JK9-3d**a** *HIS4 his3Δ ade2Δ*
MH272-3fα	JK9-3dα *HIS4 his3Δ ade2Δ*
MH349-3b	JK9-3dα *tor1::LEU2-4*
MH349-3d	JK9-3d**a** *tor1::LEU2-4*
MH350-1a	JK9-3d**a** *tor1::LEU2-5*
MH350-1d	JK9-3dα *tor1::LEU2-5*
MH353-2b	MH272-3f**a** *tor1::ADE2-6*
MH353-2c	MH272-3fα *tor1::ADE2-6*
NB3-3b	MH272-3f**a** *tor2::ADE2-3*/pJK3-3
NB6-4a	MH272-3f**a** *tor1::HIS3-3*
NB6-4c	MH272-3fα *tor1::HIS3-3*
R1	JK9-3dα *TOR1-1*
R17	JK9-3dα *TOR2-1*
SH8	MH272-3f**a**/α *tor1::HIS3-3/TOR1 tor2::ADE2-3/TOR2*
SS4	**a** *leu2 ura3 trp1 his3 ade2 Δtif2::URA3*
358/pSTE18	**a** *leu2 ura3 trp1 his ste18::LEU2*/pSEY18::*STE18*

General Genetic Manipulations

Standard genetic techniques of crossing, sporulation, and tetrad analysis were as described (Guthrie and Fink, 1991). *S. cerevisiae* transformation was performed by the lithium acetate procedure (Ito *et al.*, 1983). *Escherichia coli* strains MH1 or DH5α were used for propagation and isolation of plasmids as described (Sambrook *et al.*, 1989).

Construction of Genomic DNA Library

Total genomic DNA from the *S. cerevisiae* strain R1 was isolated as described (Philippsen *et al.*, 1991) and subjected to partial *Sau3A* digestion. Fragments were fractionated on a 0.8% low-melting agarose gel and ligated into the *Bam*HI site of pSEY18. The ligation mixture was transformed into *E. coli* strain MH1. Independent transformants (20 000) were pooled and plasmid DNA was isolated. The mean insert size was 12 kb and insert frequency was >80%. The six positive clones isolated from this library were named pPW2, pPW6, pPW11, pPW13, pPW14, and pPW15. Subsequently, pPW2 and pPW14 were found to be identical, as were pPW6 and pPW11.

Cloning of TOR1

The *TOR1-1* gene was cloned by using the semidominant rapamycin-resistance phenotype conferred by this mutant allele at 30 and 37°C. JK9-3da/α diploid wild-type cells were transformed with the yeast genomic library constructed from the *TOR1-1* mutant strain R1 in the vector pSEY18. Several genome equivalents of Ura$^+$ transformants selected at 37°C on minimal plates lacking uracil (SD-Ura) were replica plated at 37°C to prewarmed SD-Ura plates containing rapamycin (0.2 μg/ml). Rapamycin-resistant replica colonies were picked, and the plasmid DNA was isolated (Hoffman and Winston, 1987). After amplification in *E. coli*, the plasmid DNA was reintroduced into the original yeast strain JK9-3da/α and plasmid-dependent resistance to rapamycin at 37°C was confirmed on selective plates containing rapamycin (0.2 μg/ml). To clone the wild-type *TOR1* gene by gap repair (Rothstein, 1991), plasmid pPW2-13 (Figure 1A) was linearized with *Bgl* II and transformed into the wild-type strain JK9-3da. Plasmids from several independent Ura$^+$ transformants were reisolated and mapped by restriction analysis to confirm the gap repair. For integrative mapping, plasmid pPW2 was linearized at the *Bgl* II site within the genomic DNA insert and transformed into strain JK9-3dα. Ura$^+$ transformants were selected on minimal plates and tested for sensitivity to 5-fluoro-orotic acid, indicative of stable integration of the plasmid. Integrants were subsequently crossed to strain JH11-1c. The resultant diploids were sporulated and meiotic segregants were tested for segregation of the *URA3* and *TOR1-1* genes. Rapamycin resistance was used as a marker for the *TOR1-1* allele, and uracil prototrophy was used as a marker for the integrated plasmid. Twenty four tetrads were analyzed.

Mapping the Chromosomal Site of TOR1

The 6.8-kb internal *Kpn* I fragment of plasmid pPW20 was used as a probe for hybridization to a set of overlapping λ-based genomic clones (gift of L. Riles and M. Olson, Washington University, St. Louis). Hybridization was done according to the recommendations of L. Riles. For meiotic mapping, strain R1 (*TOR1-1*) was crossed to strains SS4 (*tif2::URA3*) and 358/pSTE18 (*ste18::LEU2*); resultant diploids were sporulated and dissected, and the segregation pattern of *URA3* (*tif2*), *LEU2* (*ste18*), and rapamycin resistance (*TOR1-1*) was followed.

Plasmids for Deletion Analysis

Plasmids for deletion analysis were obtained by cutting plasmid pPW2 (*TOR1-1*) at sites that were present in both the polylinker and in the insert (or multiple times within the insert) and religating. Alternatively, restriction fragments were subcloned into the multi-copy vector YEplac181, 2μ *LEU2 amp*^{r} (Gietz and Sugino, 1988). Plasmids listed in Figure 1A were all derived from plasmid pPW2 (except for pPW15) and represent the following deletions and subclones (there are *Sal* I, *Sph* I, *Xho* I, *Hin*dIII and *Bam*HI sites in the polylinker of pSEY18 at the left end of the insert, and *Sac* I and *Kpn* I sites in the polylinker at the right end of the insert of pPW2): pPW2-2, Δ*Sal* I deletion; pPW2-6, Δ*Sph* I deletion; pPW2-7, Δ*Hin*dIII deletion; pPW2-8, Δ*Bam*HI deletion; pPW2-9, Δ*Xho* I deletion of pPW2-2; pPW2-10, Δ*Sac* I deletion; pPW2-11, Δ*Xho* I deletion; pPW2-12, Δ*Kpn* I deletion; pPW2-13, Δ*Bgl*II deletion; pPW2-3, the 2.6 kb *Kpn* I fragment was cloned into pSEY18, which was recut with *Bam*HI (in the polylinker) and *Bgl* II (within the *Kpn* I fragment) and ligated with the 9-kb *Bgl* II fragment of pPW2; pPW2-4, constructed as described for pPW2-3 but starting with the 1-kb *Hin*dIII fragment; pPW2-5, the 9-kb *Bgl* II fragment was inserted into the *Bam*HI site of pSEY18. Plasmids were tested for their ability to confer rapamycin resistance on an otherwise sensitive strain JK9-3da.

TOR1 Disruption

The *TOR1* disruption vector pMD19 was constructed by cutting pPW20 with *Sal* I and religating (making pPW20-1), followed by replacement of 4 kb and 140 basepair (bp) *Bam*HI fragments with a 2.3-kb *Bgl* II fragment containing the *LEU2* gene. Plasmid pNB6 was constructed in a similar manner, except that a 1.8-kb *Bgl* II fragment containing the *HIS3* gene was used in place of the *LEU2* insert. Plasmid pMD18 was constructed by cloning the 4-kb *Hin*dIII fragment from pPW20 into pSEY18 and inserting a 2.2-kb *Xho* I-*Sal* I fragment containing the *LEU2* gene in the unique *Xho* I site in the insert. Plasmid pMD16 was constructed by cloning the 5.7-kb *Bam*HI fragment from pPW20 into pGEM7Zf(+) (Promega, Madison, Wisconsin) and inserting a 2.2-kb *Bgl* II *ADE2* gene cassette into the unique *Bgl* II site of the insert. The disruption cassettes were excised from pMD19 (*tor1::LEU2-4*) and pMD18 (*tor1::LEU2-5*) by cutting with *Bgl* II and *Hin*dIII, respectively, and were transformed into JK9-3da/α, selecting for Leu$^+$. The pMD16 disruption cassette (*tor1::ADE2-6*) was excised with *Bam*HI and transformed into MH272-3fa/α, selecting for Ade$^+$. The pNB6 disruption cassette (*tor1::HIS3-3*) was excised with *Bgl* II and transformed into MH272-3fa/α, selecting for His$^+$. The successful disruption of the chromosomal *TOR1* locus was verified in transformants by Southern analysis. Diploid strains that showed the expected hybridization pattern were designated MH349 (*tor1::LEU2-4/TOR1*), MH350 (*tor1::LEU2-5/TOR1*), MH353 (*tor1::ADE2-6/TOR1*), and NB6 (*tor1::HIS3-3/TOR1*). These strains were sporulated, and 16 tetrads each from strains MH349, MH350, MH353, and NB6 were dissected.

TOR Depletion and Analysis of DNA Content by Flow Cytometry

Haploid strains JK9-3da (*TOR1 TOR2*), MH349-3d (*tor1 TOR2*), JK350-3a (*TOR1 tor2*), and JK350-2a (*tor1 tor2*), all containing pJK5 (a *CEN4 URA3* plasmid containing *TOR2* under control of the *GAL1* promoter) (Kunz *et al.*, 1993), were grown in liquid SGalGly-Ura medium. Cells were then used to inoculate SD-Ura (glucose) medium at an OD_{600} of 0.02 and incubated for 16 h at 30°C. In glucose medium, expression of the plasmid-borne *TOR2* is repressed and, because *TOR2* is essential, *TOR1 tor2* and *tor1 tor2* strains arrested growth in early log phase. The *TOR2* cells continued to grow, but were still in log phase when harvested. Three hundred microliters of cells were chilled on ice, sonicated for 2 min in a water bath sonicator, and diluted in 700 μl cold absolute ethanol. After shaking overnight at 4°C, the cells were centrifuged briefly, washed once in 50 mM sodium citrate pH 7.4, and resuspended in 500 μl 50 mM sodium citrate pH 7.4 containing RNase A at 0.25 mg/ml. After incubating 1 h at 37°C, 500 μl of 50 mM sodium citrate, 16 μg/ml propidium iodide was added. A Becton-Dickinson FACScan machine (Lincoln Park, NJ) was used to measure fluorescence, and 10 000 events were analyzed for each strain.

Construction of TOR1-TOR2 and TOR2-TOR1 Hybrid Genes

The pTOR1-TOR2 plasmid was constructed by cutting the vector pJK4 with *Sac* I and *Bam*HI, and replacing the excised region with a 7.8-

kb *Sac* I-*Bam*HI fragment of pPW20 containing the promoter and codons 1-1680 of *TOR1*. To obtain the 7.8-kb *Sac* I-*Bam*HI fragment, pPW20 (pSEY18::*TOR1*) was first cut with *Sal* I and religated (to remove other *Sac* I and *Bam*HI sites 3′ of the desired region) and then subjected to a *Sac* I digest, followed by a *Bam*HI partial digest. The partial digest was loaded onto low melting agarose and, after electrophoresis, the relevant band was excised and ligated into the prepared vector as described (Sambrook *et al.*, 1989). The pTOR2-TOR1 plasmid was made by excising the 4.4-kb *Bam*HI-*Sal* I fragment from plasmid pJK4 and replacing it with the 4.8-kb *Bam*HI-*Sal* I fragment from pPW20 containing codons 1681–2470 and the 3′-end of *TOR1*.

DNA Manipulations

Recombinant DNA manipulations were performed according to standard methods (Sambrook *et al.*, 1989). The DNA probes for Southern analysis were labelled using the random-primed labelling kit (Amersham International, Bucks, UK) following the manufacturer's recommendation. DNA probes for the physical mapping of the *TOR1* gene were made by random-primed ^{32}P-labelling using random hexanucleotides as primers, [α-^{32}P]dATP, and the Klenow fragment of DNA polymerase I. Total yeast genomic DNA for Southern analyses was extracted as described (Guthrie and Fink, 1991), fractionated by gel electrophoresis on an agarose gel in Tris borate EDTA (TBE) and transferred to nylon membranes. Hybridization was according to the manufacturer's recommendation (Amersham International). For sequencing, restriction fragments were cloned into the vector pGEM7Zf(+) (Promega) and sets of nested deletions were obtained by the exonuclease III method using the Erase-a-Base system (Promega). Custom-made oligonucleotides were used as primers for sequencing regions not accessible with the deletions. DNA sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the T7 sequencing system from Pharmacia (Piscataway, NJ). To determine the sequence of the *TOR1-1* mutation, a 4.0-kb *Hin*dIII fragment containing the region of interest (Figure 1A) was cloned into pSEY18 for use as a template. To determine the sequence of the *TOR2-1* mutation, a 1.2-kb *Sal* I-*Hin*dIII fragment of the relevant region was cloned for use as a template.

RESULTS

Isolation of TOR1

The *TOR1-1* mutant allele confers resistance to rapamycin and is semidominant at 30 or 37°C and recessive at 24°C. The *TOR1* gene was isolated using a strategy identical to that used to isolate *TOR2* (Kunz *et al.*, 1993) in which the mutant allele was cloned first by taking advantage of the dominant rapamycin resistant phenotype at high temperature. A library was constructed from *TOR1-1* mutant strain R1 in the multicopy vector pSEY18 (2μ *URA3*) and transformed into JK9-3da/α. Ura$^+$ transformants were selected at 37°C and screened on solid minimal media lacking uracil and containing rapamycin at a concentration of 0.2 μg/ml. Plasmids that conferred growth at 37°C on medium containing rapamycin were isolated. Four different plasmids, pPW2, pPW6, pPW13, and pPW15, were obtained from among six that contained overlapping genomic inserts able to confer resistance to rapamycin. Plasmids pPW2 and pPW15 containing the smaller inserts, 16 and 15 kb respectively, were chosen for further characterization. The restriction map of the pPW2 insert and the overlapping region of the pPW15 insert are shown in Figure 1A. pPW15 is denoted in Figure 1A as giving partial resistance (+/−) because transformants with this plasmid did not grow in the presence of rapamycin as well as transformants with the other three plasmids. The insert of pPW2 was shown to correspond to the *TOR1* locus by integrative mapping (see MATERIALS AND METHODS).

The wild-type *TOR1* gene was isolated by gap repair of the cloned mutant allele. Plasmid pPW2-13, pPW2 lacking the internal 9-kb *Bgl* II fragment (Figure 1A), was linearized with *Bgl* II and transformed into wild-type strain JK9-3d. Plasmids from Ura$^+$ transformants were isolated and amplified in *E. coli*. A typical recovered plasmid, pPW20, was identical by restriction analysis to pPW2 (Figure 1A) but, unlike pPW2, was unable to confer rapamycin resistance. Thus, gap-repaired plasmid pPW20 contained the wild-type *TOR1* allele. This was further confirmed by the subsequent demonstration (see below) that the *TOR1-1* lesion is a single base pair change (Figure 2) within the *Bgl* II fragment deleted for the gap repair.

Physical and Genetic Mapping of TOR1

To determine the map position of *TOR1*, the internal 6.8-kb *Kpn* I fragment (Figure 1A) derived from pPW20 was radiolabeled and used to probe an ordered λ-based *S. cerevisiae* library. Two clones, one corresponding to a position ~100 kb centromere-proximal to *TIF2* on the left arm of chromosome X and the other corresponding to a position ~25 kb centromere-proximal to *STE18* on the right arm of the same chromosome, were detected. To further define the map position of *TOR1*, strain R1 (*TOR1-1*) was crossed to strain SS4 (*tif2::URA3*) and strain 358/pSTE18 (*ste18::LEU2*). Sporulated diploids were dissected, and segregation of the relevant markers was checked. On the basis of a parental ditype:nonparental ditype:tetratype ratio of 28:0:11, *TOR1* was found to be 14.1 cM centromere-proximal to *STE18*. No linkage was detected between *TOR1* and *TIF2*.

TOR1 Is Homologous to TOR2 and PI 3-kinases

The position of the *TOR1* gene in the 16-kb insert of pPW2 was located by testing the ability of different deletion constructs to confer rapamycin resistance when transformed into a wild-type strain. As illustrated in Figure 1A, an ~11-kb region extending from the left most *Bgl* II site to beyond the right most *Kpn* I site was required to provide full rapamycin resistance. The nucleotide sequence of this region from plasmid pPW20 containing the wild-type *TOR1* allele was determined. The sequence revealed a 7410 nucleotide open reading frame (ORF) (Figure 2) whose ends are in good agreement with the boundaries defined by the deletion analysis. Furthermore, Northern analysis using the internal 4.2-kb *Hin*dIII fragment as a probe detected a message of ~8 kb that disappeared in a *TOR1* disruption strain.

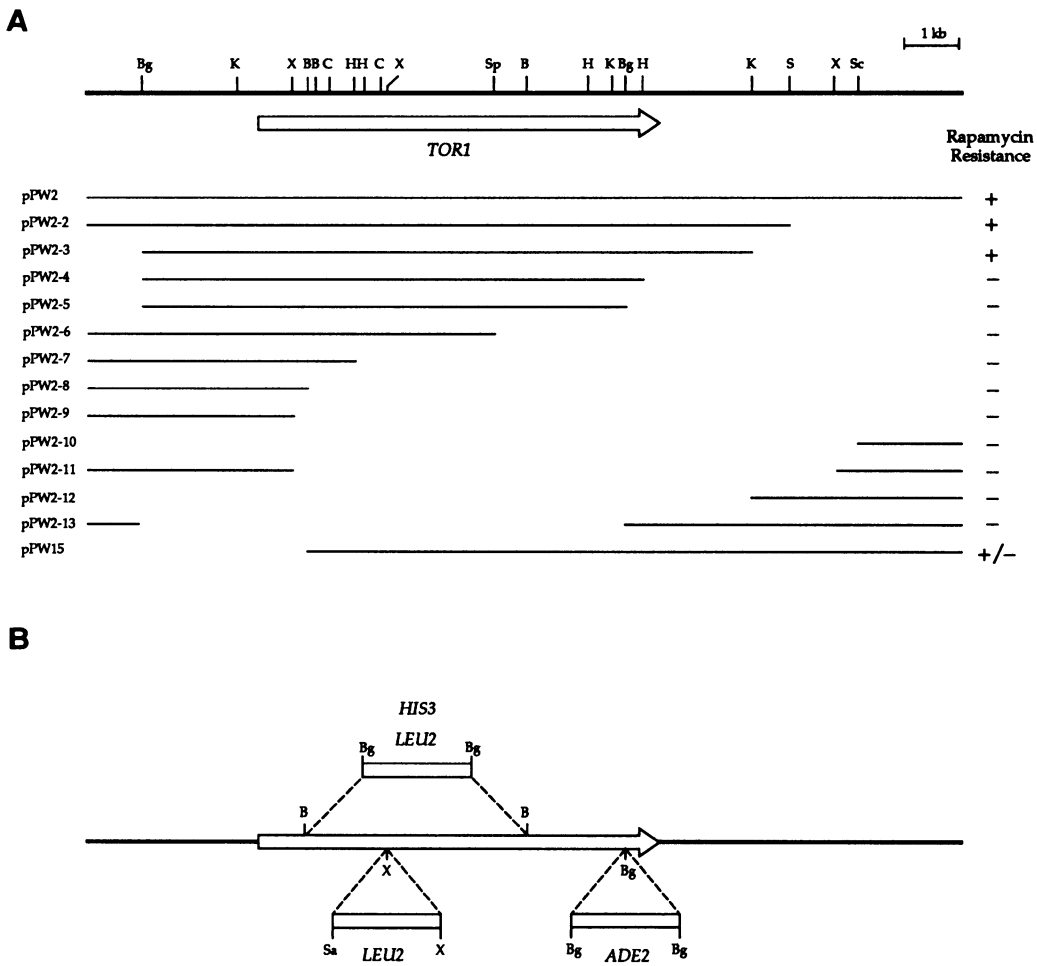

Figure 1. Restriction map of *TOR1*, functional assay of deletion constructs, and *TOR1* disruptions. (A) Plasmid pPW2, pPW15 and derivatives containing the DNA indicated by a line were tested for ability to confer rapamycin resistance when transformed into wild-type strain JK9-3da/α. (+) and (−) indicate ability and inability, respectively, to confer rapamycin resistance. ⇒ indicates the position of the *TOR1* ORF as determined by DNA sequence analysis. Restriction enzyme sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl* II; K, *Kpn* I; X, *Xho* I; C, *Cla* I; H, *Hin*dIII; Sp, *Sph* I; S, *Sal* I; Sc, *Sac* I. *Bam*HI and *Sal* I sites to the right of the right-most indicated *Sal* I site have been omitted. (B) *TOR1* disruptions. *tor1::HIS3*-3 and *tor1::LEU2-4*, a *LEU2* or *HIS3* cassette was inserted as a *Bgl* II fragment into a *Bam*HI deletion of the coding region. *tor1::LEU2-5*, an *Xho* I-*Sal* I *LEU2* fragment was inserted into the a *Xho* I site in the coding region. *tor1::ADE2*, a *Bgl* II *ADE2* cassette was inserted into the *Bgl* II site in the lipid kinase sequence motif.

Thus, TOR1 is a protein of 2470 amino acids with a calculated molecular weight of 281 kDa and a predicted pI of 7.33.

TOR1 is remarkably similar to TOR2 (2474 amino acids). A comparison of the sequences of the *TOR1* and *TOR2* genes revealed 68% identity at the nucleotide level and 67% identity for the predicted amino acid sequences (Figures 3 and 5). TOR1, like TOR2, is also related to the p110 catalytic subunit of bovine PI 3-kinase and the yeast PI 3-kinase VPS34 (Herman and Emr, 1990; Hiles *et al.*, 1992). Amino acids 1240–2441 of TOR1 are 22.3% identical and 47.8% similar to VPS34 (875 amino acids). Amino acids 901–2435 of TOR1 are 20.3% identical and 45.6% similar to p110 (1068 amino acids). Furthermore, the carboxy-terminal halves of all four proteins contain a lipid kinase motif (Figures 2 and 3) (Kunz *et al.*, 1993). The lipid kinase motif is within amino acids 2123–2296 of TOR1 and amino acids 2127–2300 of TOR2 (Figure 3). Thus, TOR1 is a PI 3-kinase homologue as is TOR2.

TOR1 contains a Ca^{2+}-dependent lipid-binding (CaLB) domain. The CaLB domain is a sequence motif originally identified as a conserved region in the Ca^{2+}-dependent isozymes of mammalian PKC (α, β and γ) and in phosphoinositidase C, and more recently implicated in the Ca^{2+}-dependent binding of cytosolic phospholipase A_2 to membranes (Baker, 1989; Clark *et al.*, 1991). A CaLB domain is also found in TOR2, p110 and several other cytosolic proteins (but not VPS34) that may be activated by Ca^{2+} to act on or utilize mem-

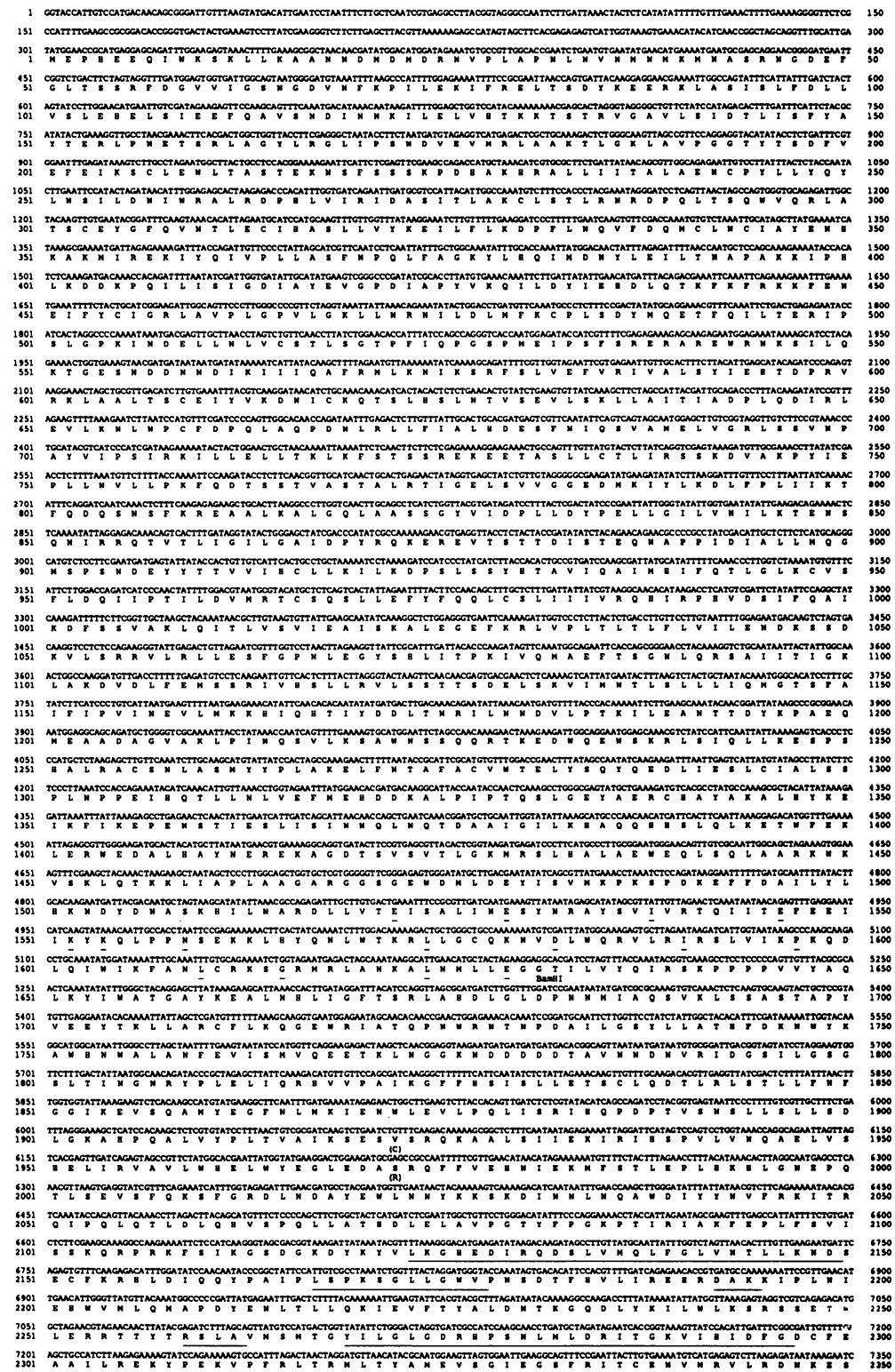

Figure 2. Nucleotide sequence of *TOR1* and deduced amino acid sequence. Complete nucleotide sequence of the *TOR1* ORF and adjacent 5′ and 3′ sequence. The deduced amino acid sequence is denoted using the one letter code. The position of the lipid kinase motif is indicated with continuous lines underneath the amino acid sequence (within amino acids 2123–2296). Individually underlined residues correspond to the CaLB domain (within amino acids 1522–1629). The position of the *Bam*HI restriction enzyme recognition site (GGATCC), used in the construction of hybrids (Figure 5), is indicated. The nucleotide (C) and amino acid (R) in parentheses at positions 6215 and 1972, respectively, indicate the changes introduced by the *TOR1-1* mutation.

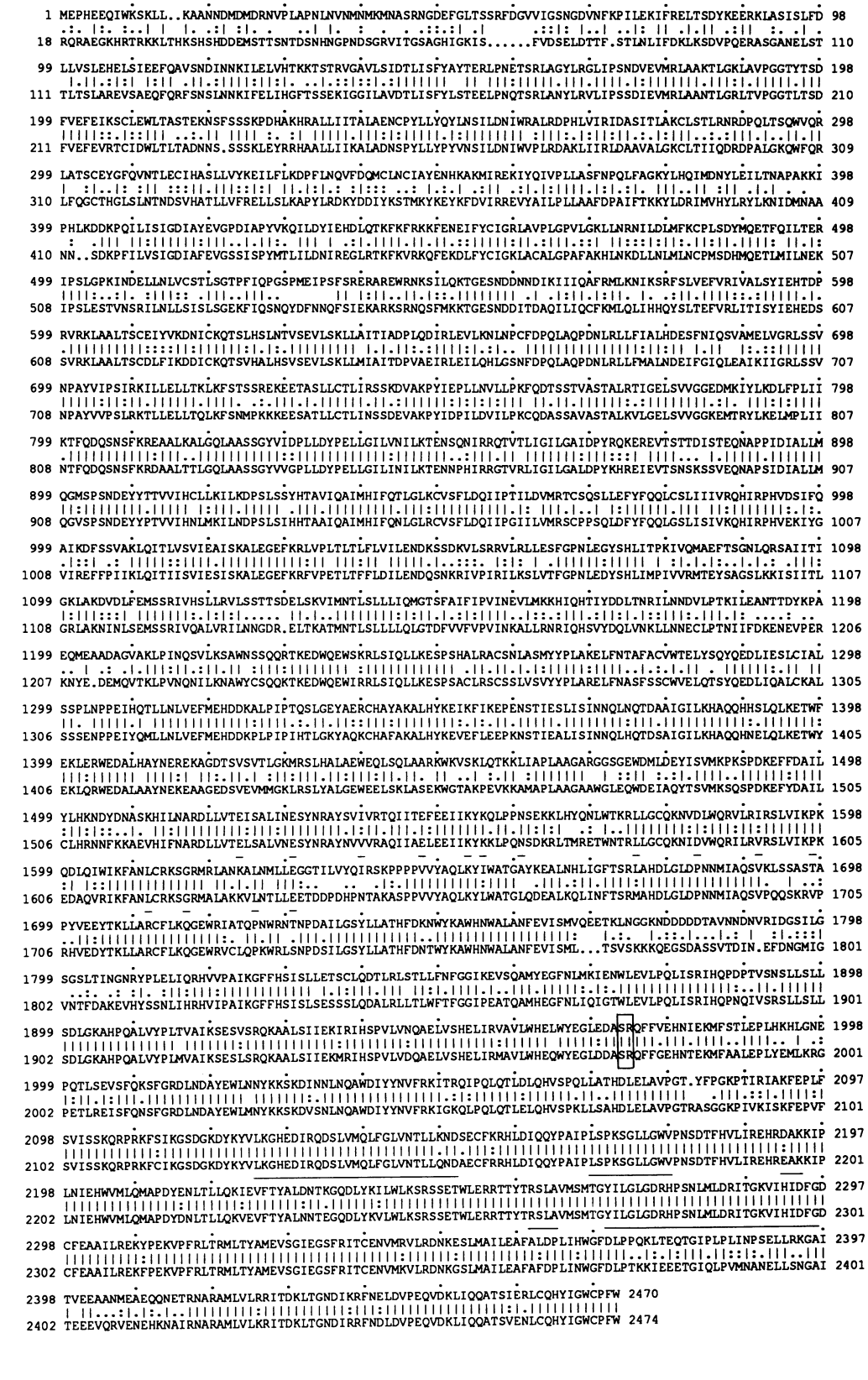

Figure 3. TOR1 is highly homologous to TOR2. The predicted amino acid sequences of TOR1 (top) and TOR2 (bottom) were aligned using the Bestfit program (Genetics Computer Group package, Devereux *et al.*, 1984). A vertical line between two amino acids indicates identity; two dots represent a conservative difference. The lipid kinase motif is indicated by continuous lines underneath the sequences. Conserved amino acids of the CaLB domain are underlined individually. The potential PKC site (SR) altered in TOR1-1 and TOR2-1 is boxed.

brane phospholipids (Clark *et al.*, 1991; Stephens *et al.*, 1993). The CaLB domain is within amino acids 1522–1629 of TOR1 and in the corresponding position in TOR2 and p110 (Figure 3) (Stephens *et al.*, 1993). Interestingly, both intracellular Ca^{2+} and the TORs are required for cell cycle progression in yeast (see below) (Iida *et al.*, 1990).

TOR1 is leucine rich, and the spacing of the leucines in amino acids 312–333 is characteristic of a leucine zipper (Busch and Sassone-Corsi, 1990). However, leucine zippers form alpha helices, and a secondary structure prediction based on the algorithms of Kyte and Doolittle (1982) indicates that the potential leucine zipper in TOR1 does not have the tendency to form an alpha helix because of a proline at position 331. TOR2 is also leucine rich but does not have any sequences resembling a leucine zipper.

TOR1 Is not Essential but Cells Lacking TOR1 and TOR2 Arrest in the G1 Phase of the Cell Cycle

To determine whether the *TOR1* gene is essential for cell viability, several disruptions within the *TOR1* ORF were constructed (Figure 1B). Diploids heterozygous for each of the disruptions indicated in Figure 1B were sporulated and dissected. Spores were incubated at 30°C on rich (YPD) medium. Four viable progeny were obtained in each case, indicating that *TOR1*, in contrast to *TOR2*, is not essential for vegetative growth. The *tor1* segregants grew slightly slower but otherwise behaved like an isogenic wild-type strain. The generation time of *tor1* strains grown in YPD liquid cultures at 30°C was 10–15% longer than that of a wild-type strain. At high and low temperatures this slow growth phenotype was slightly accentuated; at 24°C and 37°C, the generation time of the *tor1* strains was increased 15–25% compared to a wild-type strain (Figure 6, A and B). This slow growth is not a petite phenotype (respiratory deficiency) because growth on nonfermentable carbon sources (YPglycerol, YPpyruvate, YPlactate) was not altered any more than growth on YPD. The *tor1* mutants also mated normally as assayed by a patch mating assay, and homozygous *tor1* diploids sporulated as well as an isogenic wild-type strain. Furthermore, disruption of *TOR1* did not confer resistance to rapomycin, as expected. No significant differences in behavior among strains containing the different disruption alleles shown in Figure 1B were detected.

Although disruption of *TOR1* alone confers only a subtle growth defect, it confers a very pronounced phenotype when combined with a disruption of *TOR2*. The *TOR1 TOR2* double disruption causes cells to arrest growth in the G1 phase of the cell cycle as previously determined by a terminal phenotype of large unbudded cells (Kunz *et al.*, 1993). To characterize further the arrest caused by TOR depletion, we performed flow cytometry (FACS analysis) on a wild-type strain (TOR1 TOR2) and strains lacking TOR1 (*tor1 TOR2*), TOR2 (*TOR1 tor2*), or both (*tor1 tor2*) (see MATERIALS AND METHODS). Populations of *TOR1 TOR2*, *tor1 TOR2*, and *TOR1 tor2* cells exhibited a normal distribution of DNA content, with approximately equal numbers of cells in the G1 (1n) and G2/M (2n) phase of the cell cycle (Figure 4). The *tor1 tor2* strain showed a DNA distribution typical of cells arrested in the G1 phase of the cell cycle (Figure 4). Thus, depletion of both TOR1 and TOR2 causes cells to arrest in the G1 phase of the cell cycle, as indicated by a terminal phenotype of large unbudded cells (Kunz *et al.*, 1993) and by a 1n DNA content. Further analysis is required to define precisely where in G1 the cells are arrested. The FACS analysis also confirms that strains lacking TOR1 and TOR2 individually do not have an obvious cell cycle defect.

The above results taken together indicate that TOR1 and TOR2 are structurally and functionally related. However, the finding that strains lacking either one of the two TORs have a detectable growth defect (Kunz *et al.*, 1993) suggests that the functions of TOR1 and TOR2 are not completely redundant. Alternatively, growth is stringently controlled by the level of active TOR protein in the cell.

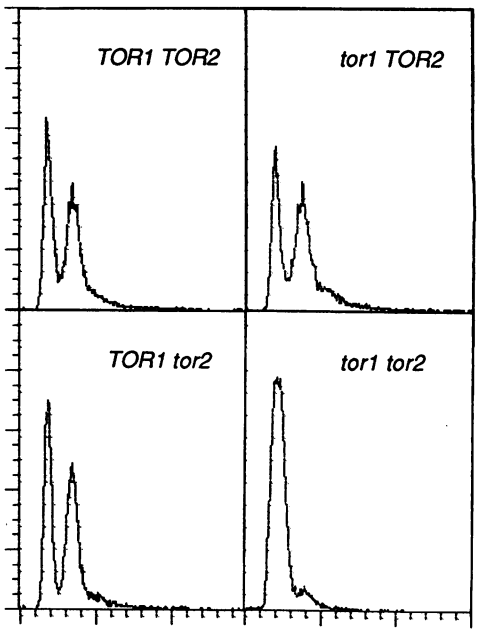

Figure 4. TOR-depleted cells arrest growth with G1 DNA content. DNA content of haploid strains JK9-3da (*TOR1 TOR2*), MH349-3d (*tor1 TOR2*), JK350-3a (*TOR1 tor2*), and JK350-2a (*tor1 tor2*) was determined by flow cytometry (see MATERIALS AND METHODS). Results are plotted as the relative number of events on the vertical axis against fluorescence (DNA content) on the horizontal axis. Populations of *TOR1 TOR2*, *tor1 TOR2*, and *TOR1 tor2* cells contain both 1n (G1, left-hand peak) and 2n (G2/M, right-hand peak) DNA. Only *tor1 tor2* cells arrest growth in the G1 phase of the cell cycle, as indicated by an almost exclusively 1n DNA content.

Because rapamycin resistance can be conferred by mutations in *FPR1*, *TOR1*, or *TOR2*, we examined whether there is any interplay between disruptions in these three genes. Strain JH3-3b (*fpr1::URA3-3*) was crossed with strains MH349-3d (*tor1::LEU2-4*) and MH350-1a (*tor1::LEU2-5*). Four viable spores were obtained from the resultant diploids, including segregants with the two disruption markers, indicating that a *tor1 fpr1* combination is not lethal. Strains MH269-4d and MH272-1c (*fpr1::URA3-3*) were crossed with strain JK350-3a (*tor2::ADE2-3*) containing a plasmid-borne copy of *TOR2* (pJK3-3). Dissection of diploids from which pJK3-3 had been cleared yielded only two viable spores per tetrad. Approximately one-half of the recovered segregants were Ura$^+$ (*fpr1::URA3-3*), but all were Ade$^-$ (*TOR2*), indicating that *fpr1* cannot suppress the lethality of a *TOR2* disruption. Thus, as expected, there appears to be no genetic interaction between *FPR1*, *TOR1*, and *TOR2* disruptions.

The Lipid Kinase Domains of TOR1 and TOR2 Are Interchangeable

Our results so far suggest that TOR1 and TOR2 have common and unique functions. To examine the possible differences and similarities in the functions of TOR1 and TOR2 and how these functional differences and similarities might correlate with the structures of the two proteins, we constructed TOR1-TOR2 and TOR2-TOR1 hybrids by gene fusion.

A comparison of overlapping fragments of TOR1 against TOR2 revealed three major peaks of identity corresponding approximately to amino acids 300–1100, 1100–1700, and 1700–2470 (Figure 5). Each peak may represent a functional and structural domain. Because the third domain contains the lipid kinase sequence motif, we constructed the hybrids such that this so-called lipid kinase domain was reciprocally exchanged (Figure 5). Taking advantage of a conserved *Bam*HI site (Figure 2) to construct the hybrid genes, the promoter region and codons 1–1680 of *TOR1* were fused to codons 1688–2474 and the 3′-end of *TOR2* (*TOR1-TOR2*), and the promoter region and codons 1–1687 of *TOR2* were fused to codons 1681–2470 and the 3′-end of *TOR1* (*TOR2-TOR1*) in low copy number (*LEU2 CEN4*) plasmids. The plasmid-encoded hybrid proteins were then assayed for TOR1 and TOR2 function by complementation analyses.

To assay for TOR1 and TOR2 function, the hybrid genes were introduced into a *tor1::HIS3-3/TOR1 tor2::ADE2-3/TOR2* heterozygous diploid (SH8), and the transformed diploids were then sporulated and dissected. TOR2 function was assayed as follows. Because the *tor2::ADE2-3* disruption is lethal, viable Ade$^+$ segregants would be obtained only if a plasmid-borne hybrid gene were present (Leu$^+$) and able to provide the missing TOR2 function. Eighteen tetrads of strain SH8

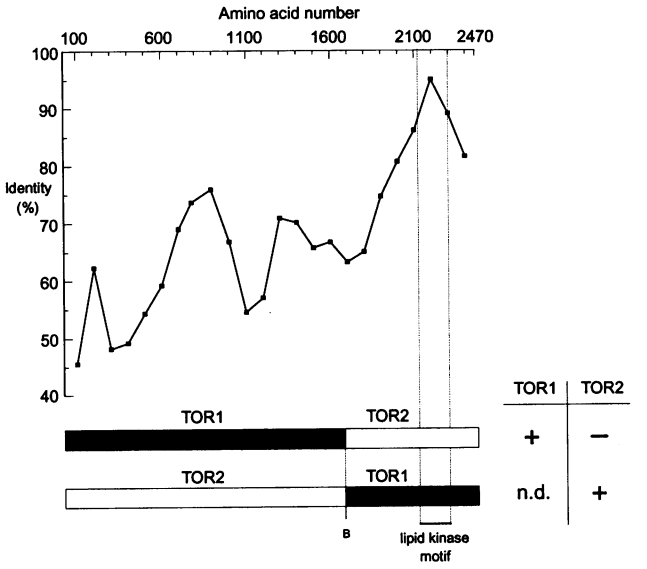

Figure 5. Distribution of TOR1 to TOR2 identity and structure of hybrids. Sequential 50% overlapping 200 amino acid fragments of TOR1 were compared to TOR2 using the Bestfit program (Genetics Computer Group package, Devereux *et al.*, 1984), and the resultant percentage values were plotted at the midpoint of each fragment. The last fragment used was amino acids 2301 to 2470 and was plotted at position 2400. The region of TOR1 and TOR2 corresponding to the identity peak containing the lipid kinase motif is referred to as the lipid kinase domain (amino acid ~1700 to the carboxy terminus of each protein). Structures of TOR1-TOR2 and TOR2-TOR1 hybrids in which lipid kinase domains were reciprocally exchanged are indicated below the graph. ▩ represents TOR1 sequence; □ represents TOR2 sequence. B indicates the *Bam*HI site used to construct the gene fusions. The position of the lipid kinase motif is indicated. + and − refer to function. n.d., not determined.

containing the TOR2-TOR1 hybrid (SH8/pTOR2-TOR1) were dissected; 15 Ade$^+$ segregants were obtained, all of which were Leu$^+$, indicating that the TOR2-TOR1 hybrid had TOR2 function. Thirty one tetrads of SH8/pTOR1-TOR2 were dissected; no Ade$^+$ segregants were obtained even though in several cases the plasmid could be inferred to have segregated with Ade$^+$, indicating that the TOR1-TOR2 hybrid did not have TOR2 function (Figure 5).

TOR1 function was assayed as follows. Because *tor2* (*tor2::ADE2-3*) segregants undergo three to four rounds of division before arresting growth randomly in the cell cycle and *tor1 tor2* (tor1::HIS3-3 tor2::ADE2-3) segregants germinate but remain arrested in G1 (Kunz *et al.*, 1993), we could assay the TOR1-TOR2 hybrid for TOR1 function by monitoring microscopically the segregants of SH8/pTOR1-TOR2 for number of cell divisions and the cell cycle arrest point. Ten segregants of SH8/pTOR1-TOR2 inferred to be disrupted for both *TOR1* (*tor1::HIS3-3*) and *TOR2* (*tor2::ADE2-3*) and to contain TOR1-TOR2 expressed from the *LEU2 CEN4* plasmid (inferred His$^+$ Ade$^+$ Leu$^+$), underwent three to five rounds of division and arrested randomly in the cell

cycle. Segregants inferred to be *tor1 tor2* and to lack the hybrid (His^+ Ade^+ Leu^-) arrested as single unbudded cells, as expected. Thus, the TOR1-TOR2 hybrid had TOR1 function (Figure 5). We could not assay the TOR2-TOR1 hybrid for TOR1 function in this manner because this hybrid has TOR2 function and the relevant segregants therefore lack only TOR1 that does not prevent growth.

To examine further the hybrids for TOR1 function, we determined whether the hybrids could suppress the slow growth phenotype of a *tor1* mutant. The low copy number (*LEU2 CEN4*) plasmids bearing the hybrid genes or an intact *TOR2* gene were transformed into *tor1* strain MH353-2b (*tor1::ADE2-6*). As shown in Figure 6, the TOR1-TOR2, TOR2-TOR1, and *TOR2* genes could all complement the *tor1* mutation and suppress the slow growth (small colony) phenotype. This suggested that the TOR1-TOR2 hybrid had TOR1 function, as seen previously, and that increased dosage of TOR2, either as a TOR2-TOR1 hybrid or as intact TOR2, could also provide TOR1 function. However, because intact TOR2 can provide TOR1 function in this assay and in a similar assay with a *tor1 tor2* strain, we are hesitant to conclude that TOR2-TOR1 can indeed provide TOR1 function. For reasons that we cannot yet explain, suppression of the slow growth phenotype of a *tor1* mutation with plasmid-borne *TOR2* or *TOR2-TOR1* is not a sufficiently sensitive assay to distinguish TOR1 function from TOR2 function. Thus, we were unable to determine with this assay or the one described above whether the TOR2-TOR1 hybrid has TOR1 function.

The above results (Figure 5) indicate that the carboxy-terminal lipid kinase domains of TOR1 and TOR2 are interchangeable and therefore functionally equivalent. The lipid kinase domains of TOR1 and TOR2 have a function, as strongly suggested by the consensus sequence, because lesions in these regions abolish TOR activity (Figure 1) (Kunz *et al.*, 1993). Because the amino-terminal segment and/or upstream regions (promoter and untranslated regions) of each hybrid determines whether it behaves as TOR1 or TOR2, one or both of these elements is not interchangeable and therefore not functionally equivalent.

The TOR1-TOR2 and TOR2-TOR1 Hybrids Are Targets of Rapamycin

Because *TOR1* and *TOR2* were originally identified by mutations that confer resistance to rapamycin, a shared function of the two proteins is presumably their ability to interact directly with and to be inhibited by FKBP-rapamycin. To probe further the structure and function of the hybrids, we examined whether their activity was rapamycin sensitive. *tor1 tor2*, *TOR1 tor2*, and *tor1 TOR2* segregants of SH8 containing the plasmid-borne *TOR2-TOR1* gene and *tor1 TOR2* segregants of SH8 expressing the TOR1-TOR2 hybrid were sensitive to rapamycin

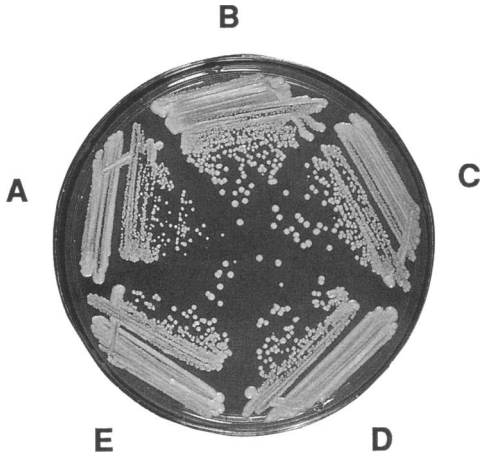

Figure 6. Suppression of the *tor1* slow growth phenotype by hybrid *TOR* genes. Strains were incubated at 24°C for 60 h on YPD. (A) MH353-2b/YCplac111 (*tor1 TOR2*). (B) MH272-1da/YCplac111 (*TOR1 TOR2*). (C) MH353-2b/pTOR1-TOR2 (*tor1 TOR2/TOR1-TOR2*). (D) MH353-2b/pTOR2-TOR1 (*tor1 TOR2/TOR2-TOR1*). (E) MH353-2b/pJK4 (*tor1 TOR2/TOR2*).

(0.2 μg/ml). This sensitivity was indistinguishable from that of a wild-type *TOR1 TOR2* strain. Thus, the hybrids were structurally and functionally intact as targets of FKBP-rapamycin.

The TOR1-1 and TOR2-1 Mutations Alter the Same Potential PKC Site in Each Protein's Lipid Kinase Domain

To identify the position of the *TOR1-1* mutation, a *TOR2-TOR1* hybrid similar to the one described above was constructed with *TOR1-1* DNA. The *TOR2-TOR1-1* hybrid conferred rapamycin resistance, unlike the wild-type *TOR2-TOR1* hybrid, indicating that the *TOR1-1* lesion was promoter-distal to the *Bam*HI site (Figure 2) used for construction of the hybrids. To map further the *TOR1-1* mutation, deletion variants and subclones of *TOR1-1* (Figure 1A) were assayed for their ability to transfer the *TOR1-1* lesion to a wild-type chromosomal *TOR1* allele by in vivo recombination. Haploid strain JK9-3d transformed with plasmid-borne fragments of *TOR1-1* were incubated on YPD medium containing rapamycin and examined for papillation of rapamycin-resistant colonies. The appearance of rapamycin-resistant colonies at high frequency indicated that the plasmid-borne *TOR1-1* fragment being tested contained the lesion and was able to regenerate an intact *TOR1-1* allele. This papillation assay placed the *TOR1-1* mutation within ~1 kb of the *Bam*HI site. Nucleotide sequence of the 1-kb region promoter-distal to the *Bam*HI site revealed a C to A transversion changing $serine_{1972}$ in the TOR1 lipid kinase domain to arginine (Figures 2 and 7). $Serine_{1972}$ is followed by an arginine and is therefore possibly phosphorylated by PKC. Mammalian

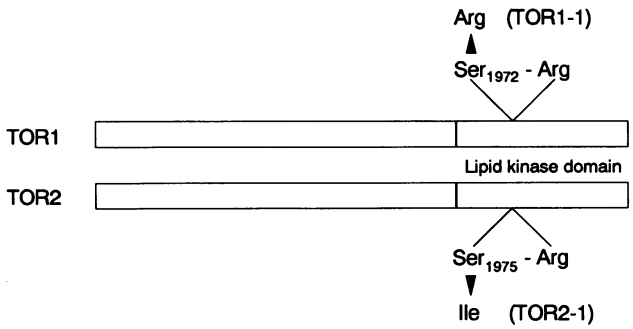

Figure 7. Alteration of the same potential PKC site in the lipid kinase domain of TOR1 (TOR1-1) or TOR2 (TOR2-1) confers rapamycin resistance. PKC reacts preferentially with serine followed by lysine or arginine in the +1 or +2 position (Kishimoto *et al.*, 1985; Woodgett *et al.*, 1986).

PKC reacts preferentially with serine followed by lysine or arginine in the +1 or +2 position (Kishimoto *et al.*, 1985; Woodgett *et al.*, 1986), and mammalian-like PKC isozymes have been described in yeast (Levin *et al.*, 1990; Simon *et al.*, 1991; Riedel *et al.*, 1993).

Because the potential PKC site altered in TOR1-1 is also present at the corresponding position in the TOR2 lipid kinase domain ($serine_{1975}$-arginine) (Figures 3 and 7), we examined whether this site is also altered in the rapamycin resistance-conferring *TOR2-1* allele (Heitman *et al.*, 1991a). The sequence of the relevant region of the previously cloned *TOR2-1* allele (Kunz *et al.*, 1993) was determined, and the PKC site was indeed found to be mutated. The *TOR2-1* allele contains a G to T transversion in the middle position of codon 1975, changing $serine_{1975}$ to isoleucine. Thus, the *TOR1-1* and *TOR2-1* mutations eliminate the same serine in the respective protein's lipid kinase domain; both serines may be phosphorylated by PKC. Interestingly, the TORs and PKCs (mammalian α, β and γ isozymes) both have a CaLB domain, suggesting that both are translocated to a membrane in a Ca^{2+}-dependent manner.

We examined whether the TORs are in the same pathway as PKC1, a yeast homologue of the Ca^{2+}-dependent isozymes of mammalian PKC (Levin *et al.*, 1990). Because loss of PKC1 results in a cell lysis defect that is suppressed by osmotic stabilizing agents (Levin *et al.*, 1992), we first examined whether 1 M sorbitol has any effect on growth of a wild-type strain and *TOR* mutant strains in the presence and absence of rapamycin. One molar sorbitol had no effect on the sensitivity of a wild-type strain (JK9-3d) or on the resistance of *TOR1-1* (R1), *TOR2-1* (R17), and *fpr1* (JH3-3b) strains to rapamycin (0.2 μg/ml). The lethality of a *TOR2* disruption was likewise not affected by 1 M sorbitol; *TOR1 tor2* (JK350-3a) and *tor1 tor2* (JK350-2a) strains containing plasmid-borne *TOR2* under control of the *GAL1* promoter (pJK5) (Kunz *et al.*, 1993) failed to grow on glucose media with or without 1 M sorbitol. Growth of a *TOR1* disruption strain (MH349-3d) was also unaltered by 1 M sorbitol. We next examined whether *PKC1* disruption or overexpression confers rapamycin resistance. *PKC1* disruption strain DL164 (Levin *et al.*, 1990) grown in the presence of 1 M sorbitol was sensitive to rapamycin. Strain DL164 containing plasmid-borne *PKC1* under control of the GAL1 promoter (pBM743[*PKC1*]) was also sensitive to rapamycin in the presence of galactose. Thus, PKC1 does not appear to act on TOR1 or TOR2 nor to have a role in a rapamycin sensitive pathway. The TORs may be targets of other more recently described but not yet cloned mammalian-like, Ca^{2+}-dependent PKCs (Simon *et al.*, 1991; Riedel *et al.*, 1993).

DISCUSSION

The *TOR1* and *TOR2* genes of *S. cerevisiae* were originally identified by mutations that render cells resistant to the immunosuppressant rapamycin. Here we describe the cloning and characterization of *TOR1*. The predicted TOR1 protein is 281 kDa (2470 amino acids) and 67% identical to TOR2 (2474 amino acids). TOR1 and TOR2, in turn, are homologous to the p110 catalytic subunit of bovine PI 3-kinase and the yeast VPS34 PI 3-kinase (Herman and Emr, 1990; Hiles *et al.*, 1992; Kunz *et al.*, 1993). All four proteins contain a carboxy-terminal lipid kinase motif. TOR1, TOR2, and p110 contain a CaLB domain (Clark *et al.*, 1991; Stephens *et al.*, 1993). Disruptions of *TOR1* and *TOR2* indicate that either TOR1 or TOR2 is required for progression through the G1 phase of the cell cycle (Kunz *et al.*, 1993).

Four observations suggest that TOR1 and TOR2 have a common function. First, *TOR1* and *TOR2* disruptions are synergistic. Second, both proteins are targets of rapamycin. Third, the two proteins are highly homologous, particularly in the carboxy-terminal lipid kinase domain. Fourth, as observed earlier, specific rapamycin resistance-conferring alleles of *TOR1* and *TOR2* do not complement each other (nonallelic noncomplementation) (Heitman *et al.*, 1991a). Other observations suggest that the common function of TOR1 and TOR2 is a similar, cell cycle-related, rapamycin-sensitive PI kinase activity. First, both a *TOR1 TOR2* double disruption and exposure to rapamycin cause cells to arrest in the G1 phase of the cell cycle. Second, TOR1 and TOR2 are homologous to PI kinases, enzymes frequently implicated in control of the cell cycle. Third, the lipid kinase domains of TOR1 and TOR2 are interchangeable and therefore functionally equivalent. Lastly, mutations that make TOR1 and TOR2 resistant to rapamycin alter a site in the lipid kinase domain of each protein. Thus, the TORs appear to be similar PI kinases, possibly PI 3-kinases, that may signal the cell to transit through G1.

It remains to be determined what type of cell cycle-related PI kinase TOR1 and TOR2 may be. Although some of the early studies on the physiological role of

different phosphorylated phosphoinositides in yeast are difficult to interpret because they predate the discovery of PI 3-kinase (Whitman *et al.*, 1988; Auger *et al.*, 1989a), several observations have implicated PI 3-kinase, PI 4-kinase, and PI 5-kinase in the control of the yeast cell cycle. Activation of a sterol auxotroph by addition of ergosterol coincides with an increase in PI-4-P (Dahl *et al.*, 1987). Activation of G1-arrested yeast cells by addition of glucose correlates with an increase in the levels of PI-4-P and PI-3-P (Hawkins *et al.*, 1993). The synthesis of DAG and IP_3 (via PI-4-P) is not stimulated by glucose (Schomerus and Küntzel, 1992), but the breakdown of PI-4,5-P_2 to DAG and IP_3 has been shown to be required for proliferation of yeast cells (Uno *et al.*, 1988). The lack of detectable PI-3-P in the vacuolar sorting mutant *vps34* does not preclude the existence of a cell cycle-related PI 3-kinase (Schu *et al.*, 1993). PI 3-kinases other than VPS34 may occur in much lower abundance than VPS34 or may be activated only in response to a specific signal. Furthermore, a cell cycle-related PI 3-kinase may have a product other than PI-3-P such that the absence of PI-3-P would not be relevant when considering the cell cycle. Hawkins *et al.* (1993) have detected a molecule in yeast similar to PI-3,4-P_2. Three previously identified PI 4-kinases do not necessarily preclude the existence of two more such kinases either (Buxeda *et al.*, 1991; Flanagan and Thorner, 1992; Nickels *et al.*, 1992). Efforts to determine what type of PI kinase the TORs might be have so far been inconclusive.

Although similar, the two TORs are also different because neither TOR can completely compensate for the loss of the other; disruptions of *TOR1* and *TOR2* individually confer different growth defects. The two TORs could be different qualitatively or quantitatively. However, we believe the difference must, at least in part, be qualitative because even increased dosage of TOR1 cannot compensate for loss of TOR2; the *TOR1* gene on a high copy number plasmid cannot rescue a *tor2* mutant. Furthermore, *TOR1* and *TOR2* mRNA levels are similar and constant throughout the cell cycle (Schneider and Hall, unpublished data). Qualitative differences between TOR1 and TOR2 could be, for example, differences in regulation (posttranscriptional) or cellular localization. Alternatively, either or both proteins could have a function unrelated to the proteins' presumed PI kinase activity. Because the regions of *TOR1* and *TOR2* other than that encoding the lipid kinase domain are not interchangeable, we anticipate that these regions are responsible for the functional difference(s) between TOR1 and TOR2.

The finding that the rapamycin resistance-conferring *TOR1-1* and *TOR2-1* mutations alter the same potential PKC site in each protein's lipid kinase domain (Figure 7) has interesting implications with regard to rapamycin action and PI kinase function. Assuming the mutated $serine_{1972}$ and $serine_{1975}$ are indeed phosphorylated and loss of the phosphoserine is responsible for rapamycin resistance, two models can be envisaged for drug action. First, the FKBP-rapamycin complex could inhibit a phosphoserine phosphatase that normally activates TOR by removal of the phosphate from $serine_{1972}$ in TOR1 and from $serine_{1975}$ in TOR2. This is similar to the action of the FKBP-FK506 complex in inhibiting the serine/threonine phosphatase calcineurin in yeast and mammalian cells (Liu *et al.*, 1991; Foor *et al.*, 1992; Schreiber, 1992); FK506 is a structural analogue of rapamycin and is also an immunosuppressant. Inherent in this model is that TOR activity (presumably PI kinase activity) is negatively regulated by serine phosphorylation, as previously observed for mammalian PI 3-kinase (Carpenter *et al.*, 1993). However, because *TOR1-1* or *TOR2-1* does not confer a growth defect, TOR is also regulated in some other manner, or a constitutively active TOR does not have a deleterious effect on the cell cycle. According to the second model, FKBP-rapamycin inhibits TOR by binding directly and specifically to the phosphoserine residues in TOR1 and TOR2. This would be analogous to the specific binding of SH2 domains to phosphotyrosine residues in receptor protein-tyrosine kinases (Pawson and Schlessinger, 1993). FKBP-rapamycin may competitively inhibit TOR by preventing the binding of a yet-to-be identified activator molecule that normally binds the TOR phosphoserines. Alternatively, FKBP-rapamycin does not mimic another molecule but simply inhibits TOR noncompetitively. Although we do not yet know whether TOR1 or TOR2 are indeed phosphorylated, both mammalian PI 3-kinase and the yeast PI 3-kinase VPS34 appear to be regulated by phosphorylation. Mammalian PI 3-kinase is serine/threonine and tyrosine phosphorylated. Serine phosphorylation by a tightly associated serine/threonine kinase negatively regulates mammalian PI 3-kinase activity in vitro, and tyrosine phosphorylation likely mediates regulatory interactions with other proteins (Cantley *et al.*, 1991; Carpenter *et al.*, 1993; Kavanaugh *et al.*, 1993; Reif *et al.*, 1993; Stephens *et al.*, 1993). VPS34 is activated by the associated serine/threonine kinase VPS15 (Stack *et al.*, 1993). Should $serine_{1972}$ of TOR1 and $serine_{1975}$ of TOR2 not be phosphorylated, the simplest model for rapamycin action is that FKBP-rapamycin binds directly to TOR and these serines are important for such an interaction.

Why should yeast have two similar but not identical PI kinases? The yeast cell cycle is activated in response to different and simultaneous environmental cues. Unless nutrients are available and a critical cell size has been attained, a cell remains arrested in G1. Each of these different signals could be relayed through a different PI kinase. Different PI kinases could thus be required to act in concert for optimal production of a second messenger needed to activate the cell cycle. Although we can only speculate as to the answer to the above question, it is worth noting that there are, also

for unknown reasons, two forms of both the p85 regulatory subunit and the p110 catalytic subunit of PI 3-kinase in mammalian cells (Carpenter *et al.*, 1990; Otsu *et al.*, 1991; Hiles *et al.*, 1992; Reif *et al.*, 1993).

ACKNOWLEDGMENTS

We thank Jose Garcia-Bustos for the physical mapping of *TOR1*, Nicholas Barbet for the *tor1::HIS3-3* disruption, Peter Erb for instruction on use of the FACS machine, Reinhard Dölz for help with computing, and members of the laboratory for helpful comments. The research was supported by a grant from the Swiss National Science Foundation awarded to M.N.H. The accession number for the sequence reported in this paper is X74857.

REFERENCES

Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., and Cantley, L.C. (1989b). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell *57*, 167–175.

Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., and Cantley, L.C. (1989b). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell *57*, 167–175.

Baker, M.E. (1989). Similarity between phospholipase C and the regulatory domain of protein kinase. Mol. Cell. Endocrinol. *61*, 129–131.

Berridge, M.J. (1987). Inositol trisphosphate and diacyglycerol: two interacting second messengers. Annu. Rev. Biochem. *54*, 205–235.

Berridge, M.J., and Irvine, R.F. (1989). Inositol phosphates and cell signalling. Nature *341*, 197–205.

Busch, S.J., and Sassone-Corsi, P. (1990). Dimers, leucine zippers, and DNA-binding domains. Trends Genet. *6*, 36–40.

Buxeda, R.J., Nickels, J.T., Jr., Belunis, C.J., and Carman, G.M. (1991). Phosphatidylinositol 4-kinase from *Saccharomyces cerevisiae*. J. Biol. Chem. *266*, 13859–13865.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Solotoff, S. (1991). Oncogenes and signal transduction. Cell *64*, 281–302.

Carpenter, C.L., Auger, K.R., Duckworth, B.C., Hou, W.-M., Schaffhausen, B., and Cantley, L.C. (1993). A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. Mol. Cell. Biol. *13*, 1657–1665.

Carpenter, C.L., and Cantley, L.C. (1990). Phosphoinositide kinases. Biochemistry *29*, 11149–11156.

Carpenter, C.L., Duckworth, B.C., Auger, K.R., Cohen, B., Schaffhausen, B.S., and Cantley, L. (1990). Purification and characterization of phosphoinositide 3-kinase from rat liver. J. Biol. Chem. *265*, 19704–19711.

Clark, J.D., Lin, L.-L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N., and Knopf, J.L. (1991). A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. Cell *65*, 1043–1051.

Dahl, C., Biemann, H.-P., and Dahl, J. (1987). A protein kinase antigenically related to $pp60^{v\text{-}src}$ possibly involved in yeast cell cycle control: positive in vivo regulation by sterol. Proc. Natl. Acad. Sci. USA *84*, 4012–4016.

Davis, E.S., Becker, A., Heitman, J., Hall, M.N., and Brennan, M.B. (1992). A novel yeast cyclophilin gene essential for lactate metabolism at high temperature. Proc. Natl. Acad. Sci. USA *89*, 11169–11173.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. *12*, 387–395.

Flanagan, C.A., and Thorner, J. (1992). Purification and characterization of a soluble phosphatidylinositol 4-kinase from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. *267*, 24117–24125.

Foor, F., Parent, S.A., Morin, N., Dahl, A.M., Ramadan, N., Chrebet, G., Bostian, K.A., and Nielsen, J.B. (1992). Calcineurin mediates inhibition by FK506 and cyclosporin of recovery from α-factor arrest in yeast. Nature *230*, 682–684.

Gietz, R.D., and Sugino, A. (1988). Calcineurin mediates inhibition by FK506 and cyclosporin of recovery from α-factor arrest in yeast. Gene *74*, 527–534.

Guthrie, C., and Fink, G.R., (eds.) (1991). Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology, vol. 194, New York: Academic Press.

Hawkins, P.T., Stephens, L.R., and Piggott, J.R. (1993). Analysis of inositol metabolites produced by *Saccharomyces cerevisiae* in response to glucose stimulation. J. Biol. Chem. *268*, 3374–3383.

Heitman, J., Movva, N.R., and Hall, M.N. (1991a). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science *253*, 905–909.

Heitman, J., Movva, N.R., and Hall, M.N. (1992). Proline isomerases at the crossroads of protein folding, signal transduction, and immunosuppression. New Biol. *4*, 448–460.

Heitman, J., Movva, N.R., Hiestand, P.C., and Hall, M.N. (1991b). FK506-binding protein proline rotamase is a target for the immunosuppressant rapamycin in yeast. Proc. Natl. Acad. Sci. USA *88*, 1948–1952.

Herman, P.K., and Emr, S.D. (1990). Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *10*, 6742–6754.

Hiles, I.D., Otsu, M., Volinia, S., Fry, M.J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N.F., Hsuan, J.J., Courtneidge, S.A., Parker, P.J., and Waterfield, M.D. (1992). Phosphatidylinositol 3-kinase: structure and expression of the 110kd catalytic subunit. Cell *70*, 419–429.

Hoffman, C.S., and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene *57*, 267–272.

Hou, W.-M., Zhang, Z.-L., and Tai, H.-H. (1988). Purification and characterisation of phosphatidylinositol kinase from porcine liver microsomes. Biochim. Biophys. Acta *959*, 67–75.

Iida, H., Sakaguchi, S., Yagawa, Y., and Anraku, Y. (1990). Cell cycle control by Ca^{2+} in *Saccharomyces cerevisiae*. J. Biol. Chem. *265*, 21216–21222.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

Kavanaugh, W.M., Klippel, A., Escobedo, J.A., and Williams, L.T. (1992). Modification of the 85-kilodalton subunit of phosphatidylinositol 3-kinase in platelet derived growth factor stimulated cells. Mol. Cell. Biol. *12*, 3415–3424.

Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y. (1985). Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. *260*, 12492–12499.

Koltin, Y., Faucette, L., Bergsma, D.J., Levy, M.A., Cafferkey, R., Koser, P., Johnson, R., and Livi, G.P. (1991). Rapamycin sensitivity in *Saccharomyces cerevisiae* is mediated by peptidyl-prolyl *cis-trans* isomerase

related to human FK506-binding protein. Mol. Cell. Biol. *11*, 1718–1723.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N.R., and Hall, M.N. (1993). Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homologue required for G1 progression. Cell *73*, 585–596.

Kunz, J., and Hall, M.N. (1993). Cyclosporin A, FK506 and rapamycin: more than just immunosuppression. Trends Biochem. Sci. *18*, 334–338.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. *157*, 105–132.

Levin, D.E., and Bartlett-Heubusch, E. (1992). Mutants in the *S. cerevisiae PKC1* gene display a cell-cycle specific osmotic stability defect. J. Cell Biol. *116*, 1221–1229.

Levin, D.E., Fields, F.O., Kunisawa, R., Bishop, J.M., and Thorner, J. (1990). A candidate protein kinase C gene, *PKC1*, is required for the *S. cerevisiae* cell cycle. Cell *62*, 213–224.

Lips, D.L., Majerus, P.W., Gorga, F.R., Young, A.T., and Benjamin, T.L. (1989). Phosphatidylinositol 3-phosphate is present in normal and transformed fibroblasts and is resistant to hydrolysis by bovine brain phospholipase C II. J. Biol. Chem. *264*, 8759–8763.

Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell *66*, 807–815.

Nakanishi, H., Brewer, K.A., and Exton, J.H. (1993). Activation of the ζ isozyme of protein kinase C by phosphatidylinositol-3,4,5-triphosphate. J. Biol. Chem. *268*, 13–16.

Nickels, J.T. Jr., Buxeda, R.J., and Carman, G.M. (1992). Purification, characterization, and kinetic analysis of a 55-kDa form of phosphatidylinositol 4-kinase from *Saccharomyces cerevisiae*. J. Biol. Chem. *267*, 16297–16304.

Otsu, M., Hiles, I., Gout, I., Fry, M.J., Ruiz-Larrea, F., Panayatou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A.D., Morgan, S.J., Courtneidge, S.A., Parker, P.J., and Waterfield, M.D. (1991). Characterisation of two 85 kD proteins that associate with receptor tyrosine kinases, middle-T/pp60$^{c\text{-}src}$ complexes, and PI 3-kinase. Cell *65*, 91–104.

Pawson, T., and Schlessinger, J. (1993). SH2 and SH3 domains. Curr. Biol. *3*, 434–442.

Philippsen, P., Stotz, A., and Scherf, C. (1991). DNA of *Saccharomyces cerevisiae*. Meth. Enzymol. *194*, 169–182.

Porter, F.D., Li, Y.-S., and Deuel, T.F. (1989). Purification and characterisation of a phosphatidylinositol 4-kinase from bovine uteri. J. Biol. Chem. *263*, 8989–8995.

Reif, K., Gout, I., Waterfield, M.D., and Cantrell, D.A. (1993). Divergent regulation of phosphatidylinositol 3-kinase P85a and P85b isoforms upon T cell activation. J. Biol. Chem. *268*, 10780–10788.

Riedel, H., Parissenti, A.M., Hansen, H., Su, L., and Shieh, H.-L. (1993). Stimulation of calcium uptake in *Saccharomyces cerevisiae* by bovine protein kinase. J. Biol. Chem. *268*, 3456–3462.

Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Meth. Enzymol. *194*, 281–301.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schomerus, C., and Küntzel, H. (1992). *CDC25*-dependent induction of inositol 1,4,5-triphosphate and diacylglycerol in *Saccharomyces cerevisiae* by nitrogen. FEBS Lett. *307*, 249–252.

Schreiber, S.L. (1992). Immunophilin-sensitive protein phosphatase action in cell signaling pathways. Cell *70*, 365–368.

Schreiber, S.L., and Crabtree, G.R. (1992). The mechanism of action of cyclosporin A and FK506. Immunol. Today *13*, 136–142.

Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D., and Emr, S.D. (1993). A yeast gene essential for protein sorting in the secretory pathway encodes phosphatidylinositol 3-kinase. Science *260*, 88–91.

Serunian, L.A., Haber, M., Fukui, T., Kim, J.W., Rhee, S.G., Lowenstein, J.M., and Cantley, L.C. (1989). Polyphosphoinositides produced by phosphatidylinositol 3-kinase are poor substrates for phospholipase C from rat liver and bovine brain. J. Biol. Chem. *264*, 17809–17815.

Sherman, F. (1991). Getting started with yeast. Meth. Enzymol. *194*, 3–21.

Sigal, N.H., and Dumont, F.J. (1992). Cyclosporin A, FK-506, and rapamycin: pharmacologic probes of lymphocyte signal transduction. Annu. Rev. Immunol. *10*, 519–560.

Simon, A.J., Milner, Y., Saville, S.P., Dvir, A., Mochly-Rosen, D., and Orr, E. (1991). The identification and purification of a mammalian-like protein kinase C in the yeast *Saccharomyces cerevisiae*. Proc. R. Soc. Lond. Biol. Sc. *243*, 165–171.

Stack, J.H., Herman, P.K., Schu, P.V., and Emr, S.D. (1993). A membrane associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. EMBO J. *12*, 2195–2204.

Stephens, L.R., Hawkins, P.T., and Downes, C.P. (1989). Metabolic and structural evidence for the existence of a third species of polyphosphoinositide in cells: D-phosphatidyl-*myo*-inositol 3-phosphate. Biochem. J. *259*, 267–276.

Stephens, L.R., Jackson, T.R., and Hawkins, P.T. (1993). Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-triphosphate: a new intracellular signalling system? Biochim. Biophys. Acta *1179*, 27–75.

Uno, I., Fukami, K., Kato, H., Takenawa, T., and Ishikawa, T. (1988). Essential role for phosphatidylinositol 4,5-bisphosphate in yeast cell proliferation. Nature *333*, 188–190.

Valius, M., and Kazlauskas, A. (1993). Phospholipase C-γ1 and phosphatidylinositol 3-kinase are the downstream mediators of the PDGF receptor's mitogenic signal. Cell *73*, 321–334.

Walker, D.H., Dougherty, N., and Pike, L.J. (1988). Purification and characterisation of a phosphatidylinositol kinase from A431 cells. Biochemistry *27*, 6504–6511.

Whitman, M., Downes, C.P., Keeler, M., Keller, T., and Cantley, L. (1988). Type I phosphatidylinositol kinase makes a novel phospholipid, phosphatidylinositol-3-phosphate. Nature *332*, 644–646.

Wiederrecht, G., Brizuela, L., Elliston, K., Sigal, N.H., and Siekierka, J.J. (1991). FKB1 encodes a nonessential FK506-binding protein in *Saccharomyces cerevisiae* and contains regions suggesting homology to cyclophilins. Proc. Natl. Acad. Sci. USA *88*, 1029–1033.

Woodgett, J.R., Gould, K.L., and Hunter, T. (1986). Substrate specificity of protein kinase. Eur. J. Biochem. *161*, 177–184.