The Fission Yeast TOR Homolog, *tor1<sup>+</sup>*, Is Required for the Response to Starvation and Other Stresses via a Conserved Serine* 

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Targets of rapamycin (TORs) are conserved phosphatidylinositol kinase-related kinases that are involved in the coordination between nutritional or mitogenic signals and cell growth. Here we report the initial characterization of two *Schizosaccharomyces pombe* TOR homologs, *tor1<sup>+</sup>* and *tor2<sup>+</sup>*. *tor1<sup>+</sup>* is an essential gene, whereas *tor2<sup>+</sup>* is required only under starvation and other stress conditions. Specifically, Δtor1 cells fail to enter stationary phase or undergo sexual development and are sensitive to cold, osmotic stress, and oxidative stress. In complex with the prolyl isomerase FKBP12, the drug rapamycin binds a conserved domain in TORs, FRB, thus inhibiting some of the functions of TORs. Mutations at a conserved serine within the FRB domain of Tor1p is important for the binding of the rapamycin-FKBP12 complex. Studies in mammals (14–16), *S. cerevisiae* (3, 5, 17–19), and *Cryptococcus neoformans* (20) have shown that a mutation at the conserved serine residue confers dominant rapamycin resistance by abolishing the binding to the FKBP12-rapamycin complex. The importance of the conserved serine for this binding is reinforced by the atomic structure of the ternary complex FRB-rapamycin-FKBP12 (21). Despite the high conservation of this serine, TOR proteins that are expressed at normal levels and carry a mutated serine appear to retain wild type activities other than FKBP12-rapamycin binding (5, 22–24).

*Schizosaccharomyces pombe* is genetically tractable yeast that is highly divergent from *S. cerevisiae*. These two yeasts often have distinct differences in carrying out the same cellular functions, which makes their comparative study especially revealing (25). Upon starvation, *S. pombe* cells enter either the stationary phase or the sexual development pathway (reviewed in Ref. 26). We previously reported that rapamycin has a different effect on *S. pombe* compared with its effect on *S. cerevisiae*. Rapamycin does not inhibit the growth of *S. pombe* but specifically inhibits sexual development in response to starvation (27). As a first step toward understanding the response to rapamycin in *S. pombe*, we cloned and initiated a functional analysis of the *S. pombe* TOR homologs, named *tor1<sup>+</sup>* and *tor2<sup>+</sup>*. We show here that at least some of the functions of each of these TORs are distinct. Thus, *tor2<sup>+</sup>* is essential for growth, whereas *tor1<sup>+</sup>* is required only under starvation and other stress conditions. We also demonstrate that the conserved serine residue within the FRB domain of Tor1 is important for the protein function and does not play a detectable role in the response of starved *S. pombe* to rapamycin.

**Experimental Procedures**

**Yeast Strains, Media, and General Techniques**—Yeast strains used in this paper are described in Table I. All media used in this study are based on those described previously (28). EMM-N contains no nitrogen; EMM lowG contains 0.1% glucose. Rapamycin was used as described previously (27). Transformation of *S. pombe* cells was performed by electroporation (29). Assays for mating or sporulation efficiency were carried out as described in (27). Fluorescence-activated Cell Sorter Analysis—Cells were stained with the DNA fluorochrome propidium iodide and analyzed by a Becton Dickinson FACSort as described by (30). Data were analyzed by Cell Quest software for Macintosh.

Disruption of *S. pombe* *tor1<sup>+</sup>*—A fragment containing 5.84 kbp of the C-terminal region of *tor1<sup>+</sup>* gene was amplified by PCR using the Expand Long Template PCR System (Roche Molecular Biochemicals) with a genomic *S. pombe* DNA preparation as a template and the primers

FKBP12-rapamycin binding; kbp, kilobase pair(s); bp, base pairs; PCR, polymerase chain reaction; HA, hemagglutinin.
TOR-mediated Response to Stresses in S. pombe

Table I
Strains used in this study

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| TA01   | ura4-D18  | P. Fantes           |
| TA02   | ura4-D18  | P. Fantes           |
| TA03   | ura4-D18  | P. Fantes           |
| TA04   | ura4-D18  | P. Fantes           |
| TA07   | ura4-D18  | P. Fantes           |
| TA16   | leu1-32   | A. Cohen            |
| TA82   | ura4-D18  | This study          |
| TA99   | leu1-32   | This study          |
| TA100  | leu1-32   | This study          |
| TA120  | leu1-32   | This study          |
| TA137  | leu1-32   | This study          |
| TA157  | his1-102  | This study          |
| TA163  | ade6-M216 | This study          |

* Strains TA99, TA100, and TA120 are haploid segregants of the diploid TA82. Strains TA99 and TA120 are the parents of TA132. TA157 is a haploid segregant from a cross between TA99 and TA16. TA163 is derived from TA99 and is the result of transformation of TA99 with the marker swap construct, *ura4Δ·his1*, a kind gift of P. Fantes (Edinburgh, UK).

104 (5′-TTGAAAGATCTCGAGCAAAATATTTCG) and 105 (5′-AA-
GATTTTGAGCATTGGGAC). The resulting PCR fragment was subcloned into a pGEM-T vector (Promega) to give pGEMtor1. A 3.63-kbp HindIII fragment of pGEMtor1 containing the kinase and FRB-like domains was replaced with an *His*III fragment containing the entire *ura4* gene, resulting in the plasmid ptor1::ura4. NotI and SacI were used to release the 4-kbp *tor1*::ura4 disruption fragment, and this was gel purified and transformed into the diploid TA07. Stable *ura4* diploids were selected, and their DNA was extracted and subjected to PCR analysis with primers 105 and 137 (5′-TTGAATATAGGACTACAGCC- CAC), which lies 100 bp upstream of the *tor1* ORF. This fragment was used to replace the corresponding fragment in pIRT1::ura4.

The wild type and mutant *tor1* genes were amplified from the plasmids pIRT2-*tor1* and pIRT2-*tor1S1834R* using the primers 141 (containing a *BamHI* site, see above) and 199 (ATAAGAGATCCGAGCATTGGGAC) (see Table I, lanes B-G). The resulting 1.58-kbp PCR product was cloned into the *his1*-resistant vector pSLF273 (34), downstream and in frame with the *his1* coding sequence. The resulting construct was used to transform the S. pombe *his1* mutant strain TP159, resulting in the plasmid carrying *tor1S1834R* with the *his1* ORF. This plasmid was used to transform the *his1* mutant strain TP159, resulting in the plasmid carrying *tor1S1834R* with the *his1* ORF.

To express *tor1* and *tor2* genes, we used the Expand Long Template PCR System (Roche Molecular Biochemicals) with a genomic *S. pombe* DNA preparation as the template. For amplifying *tor2* we used primers 152 (5′-ATAAGAGATCCGAGCATTGGGAC) and 155 (5′-CGGGATCCGTTTCTGGTAGGTGACAGTCCC). The resulting 1.58-kbp PCR fragment was digested with *BamHI* and *SacI* sites and used as an internal loading control.

**RESULTS**

**Identification of TOR Homologs in the S. pombe Genome**

The DNA sequence of most of the *S. pombe* genome has been determined through the coordination of the Sanger Center. Based on sequence comparisons, we identified two TOR homologs in chromosome II. We named one of these, on cosmid SPBC30D10, *tor1* and the second, on two overlapping cosmids, SPBC216 and SPBC646, *tor2*. The open reading
isolated Δtor1 haploid clones. Under optimal growth conditions, the growth rate and cell morphology of Δtor1 cells were indistinguishable from that of wild type cells (Fig. 3A). However, Δtor1 cells exited the logarithmic phase at a lower cell density, were abnormally long, and lost viability rapidly (Fig. 3B). The loss of viability of Δtor1 depends on the growth medium. Whereas Δtor1 cells died when they reached saturation in rich medium, they maintained viability comparable with wild type cells when grown to saturation in minimal medium (Fig. 3B). This suggests that Δtor1 cells are defective in the response to particular sets of conditions rather than in the actual cellular processes that allow cells to acquire the stationary phase physiology.

We also noted that Δtor1 cells failed to cross with wild type strains. Microscopic examination suggested a defect at an early stage of sexual development, before conjugation had occurred. Media limiting for either nitrogen or carbon sources are conventionally used for a quantitative analysis of conjugation (mating). Under these conditions, haploid cells of the opposite mating types can conjugate to form a diploid zygote, which rapidly undergoes meiosis and sporulation and produces an ascus (reviewed in Ref. 26). Under either nitrogen or carbon starvation, ~60% of wild type cells underwent mating compared with less than 1% of Δtor1 cells. The sterile phenotype of Δtor1 was efficiently suppressed when we reintroduced tor1+ (Fig. 4A).

Nitrogen or carbon starvation is also a signal for diploid cells to enter meiosis (reviewed in Ref. 26). Under these conditions most of the wild type diploid cells, >60%, underwent sporulation, whereas <1% of homozygous Δtor1 diploids sporulated (results not shown). Thus, in addition to its role in mating, tor1+ is also required for meiosis/sporulation.

Analysis of the DNA content of growing and starved Δtor1 cells also indicates that these cells are defective in their response to starvation. In the absence of a mating partner, starved S. pombe cells become arrested in either G1 or G2, depending on the growth medium; nitrogen starvation arrests cells mainly at the G1 phase, and carbon starvation arrests cells mainly at the G2 phase (35). The DNA profile of Δtor1 cells under optimal growth conditions shows a major 2n DNA peak, characteristic of growing wild type cells (see Refs. 36 and 37 and Fig. 4B). However, under nitrogen starvation, Δtor1 cells show an abnormal DNA profile as cells failed to arrest in G1 (Fig. 4B). Because G1 arrest is a prerequisite for mating, the failure of Δtor1 cells to arrest their growth in G1 may be associated with their inability to undergo sexual development. tor1+ Is Required for Growth under Osmotic or Oxidative Stress Conditions—We noted that the phenotype of Δtor1 cells was particularly similar to that of cells disrupted for atf1+, a gene that encodes a bZIP (basic leucine zipper) transcription factor (37). Under starvation conditions, both Δtor1 and Δatf1 cannot arrest in G1, exhibit an abnormal elongated morphology, lose viability in rich but not minimal medium, and are sterile. atf1+ has also been implicated in regulating the cellular response to a variety of stress conditions, such as cold, osmotic stress, and oxidative stress (37–39). We found that tor1+ is also required under these stress conditions; unlike wild type cells,
morality of starved wild type and Δtor1 cells was determined by plating efficiency on a complete medium. Cells were grown to stationary phase in rich or minimal (EMM) medium. After the cells entered the stationary phase, at the indicated time points, cell viability was determined by plating efficiency on a complete medium. C, the morphology of starved wild type and Δtor1 cells. Photographs were taken 48 h after cells exited the logarithmic phase in rich medium.

Fig. 3. tor1Δ is required for entrance into stationary phase. A, growth curves of wild type (WT, TA100) and Δtor1 (TA99) cells in a rich (YE) medium. B, viability in stationary phase. Cells were grown to stationary phase in rich or minimal (EMM) medium. After the cells entered the stationary phase, at the indicated time points, cell viability was determined by plating efficiency on a complete medium. C, the morphology of starved wild type and Δtor1 cells. Photographs were taken 48 h after cells exited the logarithmic phase in rich medium.

Fig. 4. Phenotypes of Δtor1 cells under starvation, osmotic, and oxidative stress. A, heterothallic Δtor1 strain (TA99) was transformed with the S. pombe vector pIRT2 or pIRT2-tor1Δ. The transformants were mixed with wild type (WT) cells of the opposite mating type (TA02) and induced to undergo sexual development in EMM-N medium. Wild type is TA16 induced to undergo sexual development in EMM-N. B, exponentially growing wild type (TA100) and Δtor1 (TA99) cells in minimal medium (log) were collected, washed and resuspended in nitrogen free (EMM-N), low glucose (EMM(loose)), or EMM (Stationary) medium, and incubated at 25 °C for 3 days. Aliquots were removed from growing and starved cells, and the DNA content of individual cells was measured by fluorescence-activated cell sorter. C, Δtor1 cells transformed with vector only or plasmid carrying tor1Δ were streaked onto YE agar plates supplemented with 1 M KCl or 0.5 M NaCl and incubated for 5 days at 32 °C. D, Δtor1 cells transformed with vector only or plasmid carrying tor1Δ were streaked onto YE agar plates supplemented with 0 or 5 mM H2O2 and incubated for 5 days at 32 °C.

Δtor1 could not form colonies on medium containing 0.5 M NaCl or 1 M KCl (Fig. 4C) or on medium containing 5 mM H2O2 (Fig. 4D) or below 20 °C (results not shown). Taken together, our findings reveal a striking similarity between the phenotypes of Δtor1 and Δatf1 cells. It remains to be determined whether tor1Δ and atf1Δ are involved in the same signaling pathway.

Ser1834 in the FRB Domain of Tor1 Is Required for Tor1 Activity—We previously reported that rapamycin specifically inhibits sexual development in S. pombe (27). As indicated above, Δtor1 cells are unable to undergo sexual development. Because rapamycin is known to inhibit the TOR proteins in mammals, S. cerevisiae, and C. neoformans, we considered the possibility that rapamycin exerts its effect by inhibiting the function of the S. pombe Tor1 during sexual development. A conserved serine residue in TORs has been identified as the site for missense mutations (serine substituted with arginine, isoleucine, or glutamic acid) conferring dominant rapamycin resistance (see the Introduction). We mutated the equivalent serine residue in S. pombe Tor1, Ser1834, into arginine (“Experimental Procedures”).

The tor1Δ and tor1S1834R genes, cloned into the S. pombe expression vector pIRT2 (“Experimental Procedures”) were transformed into Δtor1 strains TA99 or TA157. TA99 and TA157 are isogenic except that TA157 is a homothallic strain (cells can switch their mating types between h+ and h− every other generation), whereas TA99 is a heterothallic strain composed of h+ cells only. Surprisingly, we found that the mutation at Ser1834 diminished the activity of Tor1; the mating efficiency was extremely low when TA157 cells carrying tor1S1834R were induced to undergo mating (0.9%, Fig. 5A). In crosses between wild type and Δtor1 cells (TA99) carrying tor1S1834R, we observed that the wild type cells partially suppressed the sterility of Δtor1 cells carrying tor1S1834R (Fig. 5A). The mutation S1834R also diminished the activity of Tor1 under osmotic stress conditions (Fig. 5C) or in acquisition of stationary phase physiology (data not shown). Because mating is very inefficient in tor1S1834R mutants, it was no surprise that this mutated allele did not confer dominant resistance to rapamycin in wild type TA16 transformants (Fig. 5B).

Our finding that Ser1834 is critical for the function of Tor1 is surprising given that equivalent mutations did not affect TOR function in S. cerevisiae. To ascertain that the only defect of the tor1S1834R resided in S1834, the plasmid carrying tor1S1834R was used as the template in a PCR reaction performed to replace Arg1834 back with serine (see “Experimental Proce-
We examined the functions of the cells. Fig. 7 shows that promoter, were introduced into wild type ADH1 stably expressed.

homology with the S. cerevisiae tor1 against the S. pombe with the S. pombe S1834 is required for Tor1 function in S. cerevisiae required for the rapamycin-sensitive TOR function of S. TOR proteins (3, 5, 19), except that to the effect of equivalent point mutations in the S. cerevisiae domiant rapamycin resistance in S. cerevisiae required for the rapamycin resistance at a high concentration of rapamycin. It also implies that the conserved serine in S. pombe tor1 S1834 does not confer rapamycin resistance in S. cerevisiae. This is similar to the effect of equivalent point mutations in the S. cerevisiae TOR proteins (3, 5, 19), except that tor1S1834R did not confer rapamycin resistance at a high concentration of rapamycin (100 ng/ml, results not shown). The dominant resistance exhibited by the S. pombe tor1S1834R indicates that the S. pombe TOR homolog can complement the function of the S. cerevisiae TORs. It also implies that the conserved serine in tor1+ is critical for the binding of FKBP12-rapamycin when expressed in S. cerevisiae cells. In conclusion, it appears that although S1834 is required for Tor1 function in S. pombe, it is not required for the rapamycin-sensitive TOR function of S. cerevisiae.

**DISCUSSION**

We report here the identification and initial characterization of the S. pombe homologs of the TOR genes. We found the S. pombe tor2 gene is essential for growth, whereas tor1 is required only under starvation, osmotic stress, and oxidative stress.

The inability of Δtor1 cells to respond appropriately to starvation conditions may suggest that the S. pombe tor1+, like its S. cerevisiae TOR homologs (2, 40), participates in signal transduction pathways that are involved in nutrient sensing. However, there are significant differences between the functions of the S. cerevisiae and S. pombe TORs. First, the S. pombe tor1+ has a positive role in the sexual development pathway and entry into stationary phase, whereas the activity of the S. cerevisiae TORs is required to repress meiosis (41) and entry into stationary phase (6–10, 42). Second, the S. pombe tor1+ is required for the appropriate response to a variety of stress conditions, whereas there is no evidence that the S. cerevisiae TOR homologs are involved in the response to stresses other than starvation. Third, each of the two S. pombe TOR homologs carries out a distinct function that is not shared by the other homolog. In contrast, the S. cerevisiae TOR1 is a nonessential gene, and its function in regulating growth in response to nutrient availability is shared with TOR2 (3–5).

Mutational analysis of tor1+ revealed that the conserved serine residue within the FRB domain of Tor1 plays a critical role in the protein cellular function. Thus, although the mutation at Ser1834 did not affect the level of protein expression (Fig. 6), tor1S1834R can only partially complement the defects observed in Δtor1 cells (Fig. 5). Although Ser1834 is required for Tor1 function, its equivalent serine residues in the S. cerevisiae TOR proteins (3, 5, 18) and possibly the human TOR protein (22–24) do not appear to play an essential role in the studied functions of the proteins. However, given the conserved nature of this serine residue, its role in other TOR homologs may become evident under yet unidentified conditions.

None of the functions of the S. pombe TORs described in this work appear to be inhibited by rapamycin. Rapamycin specifically inhibits sexual development in S. pombe, at an early stage, before mating (27). Cells disrupted for tor1+ are deficient in their ability to undergo mating. However, the effects of Tor1 and rapamycin on sexual development appear to be unrelated. The inability of Δtor1 cells to enter sexual development seems to be part of a general defect in responding to nutritional deprivation. Thus, Δtor1 cells fail to enter stationary phase, arrest in G1 in response to starvation, or undergo meiosis/sporulation. In contrast, rapamycin specifically inhibits the sexual development pathway and does not interfere with other responses to starvation. Thus, cells treated with rapamycin can enter stationary phase properly, are only slightly defective in meiosis/sporulation (27), and can arrest their growth in G1 under starvation conditions. Our finding that tor1S1834R could not alleviate the inhibitory effect of rapamycin on sexual development (Fig. 5B) is consistent with our suggestion that rapamycin does not exert its effect in S. pombe by forming a toxic FKBP12-rapamycin complex that inhibits the Tor1 function.

If neither Tor1 nor Tor2 is the protein target for rapamycin, than what is the target for the action of rapamycin in S. pombe? Recent findings in our lab show that cells disrupted for the S. pombe FKBP12 homolog exhibit a phenotype highly similar to treatment with rapamycin. Thus, it is most probable that rapamycin inhibits sexual development by inhibiting the cellular

R. Weisman and M. Choder, unpublished results.

![Fig. 6. Wild type and mutant HA-TOR1 fusion proteins are stably expressed. Top panel, HA-Tor1 and HA-Tor1S1834R fusion proteins were expressed in strain TA163 and detected by Western blot with antibodies against the HA epitope. Bottom panel, the same extracts as in the top panel were detected using a FKBP12 antiserum raised against the S. cerevisiae FKBP12 homolog. This antibody cross-reacts with the S. pombe FKBP12 protein and is used here as a control for protein loading.](image)

![Fig. 7. The S. pombe tor1S1834R can confer rapamycin resistance in S. cerevisiae. S. cerevisiae wild type cells, JK9–3d (19) transformed with plasmids containing the S. pombe tor1+ or tor1S1834R genes, were streaked on minimal plates containing 0 or 10 ng/ml rapamycin.](image)
function of FKBP12 in sexual development\(^3\) and not by inhibiting TOR-related function.

Why rapamycin does not inhibit neither the function of Tor1 or Tor2? Our functional analysis of tor1\(^{\text{S1834R}}\) and tor1\(^{\text{S1834R}}\) in S. cerevisiae cells (Fig. 5A) suggests that Tor1 can bind the FKBP12-rapamycin complex, at least in S. cerevisiae cells. It is possible that Tor1 interacts with FKBP12-rapamycin complexes in S. pombe, but this interaction does not inhibit the studied functions of Tor1 or Tor2. By analogy, the function of the S. cerevisiae Tor2p in the control of the actin cytoskeleton organization is not inhibited by rapamycin (5).

The features of S. pombe TOR proteins, together with other studies of TOR functions, indicate that these proteins are involved in many distinct cellular functions. Given that the C-terminal region containing the FRB and the kinase domains of the TORs is highly conserved, the differences between the TORs might reside in the less conserved N-terminal region. This is an intriguing possibility yet to be explored.

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