Regulation of Leucine Uptake by tor1+ in Schizosaccharomyces pombe Is Sensitive to Rapamycin

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ABSTRACT

TOR protein kinases are key regulators of cell growth in eukaryotes. TOR is also known as the target protein for the immunosuppressive and potentially anticancer drug rapamycin. The fission yeast Schizosaccharomyces pombe has two TOR homologs, tor1+ is required under starvation and a variety of stresses, while tor2+ is an essential gene. Surprisingly, to date no rapamycin-sensitive TOR-dependent function has been identified in S. pombe. Herein, we show that S. pombe auxotrophs, in particular leucine auxotrophs, are sensitive to rapamycin. This sensitivity is suppressed by deletion of the S. pombe FKBP12 or by introducing a rapamycin-binding defective tor1 allele, suggesting that rapamycin inhibits a tor1-dependent function. Sensitivity of leucine auxotrophs to rapamycin is observed when ammonia is used as the nitrogen source and can be suppressed by its replacement with proline. Consistently, using radioactive labeled leucine, we show that cells treated with rapamycin or disrupted for tor1+ are defective in leucine uptake when the nitrogen source is ammonia but not proline. Recently, it has been reported that tsc1+ and tsc2+, the S. pombe homologs for the mammalian TSC1 and TSC2, are also defective in leucine uptake. TSC1 and TSC2 may antagonize TOR signaling in mammalian cells and Drosophila. We show that reduction of leucine uptake in tor1 mutants is correlated with decreased expression of three putative amino acid permeases that are also downregulated in tsc1 or tsc2. These findings suggest a possible mechanism for regulation of leucine uptake by tor1p and indicate that tor1p, as well as tsc1p and tsc2p, positively regulates leucine uptake in S. pombe.

THE TOR (target of rapamycin) kinases form an evolutionary conserved family of large proteins with a kinase domain that resembles phosphatidylinositol (PI) PI3 and PI4 kinases and are therefore referred to as PI3K-related kinases. Accumulation of data in yeast, Drosophila, and mammalian cells suggests that TOR is a central regulator of cellular growth. TOR controls growth in response to changes in the environment, particularly nutrient availability and cellular energetic status. Cell growth is regulated by TOR on many levels, including translation, ribosome biogenesis, transcription, nutrient permease expression, protein degradation, and autophagy (reviewed in Jacinto and Hall 2003; Fingar and BLENIS 2004; Tokunaga et al. 2004).

Rapamycin, a natural product of the soil bacterium Streptomyces hygroscopicus, inhibits cellular growth and proliferation of certain cell types and hence is considered a potent immunosuppressive and anticancer drug (Bjornsti and Houghton 2004). Rapamycin and its analogs are already in medical use to prevent graft rejection in transplant patients and to inhibit the restenosis that occurs after angioplasty. In addition, rapamycin is in clinical trials as an anticancer treatment against a variety of human tumors (Bjornsti and Houghton 2004; Fingar and BLENIS 2004). Rapamycin binds and inhibits TOR kinases when in complex with FKBP12, a ubiquitous 12-kD prolyl-isomerase. The inhibition of TOR by FKBP12-rapamycin complexes accounts for the inhibition of many growth-related functions that are TOR dependent. Nevertheless, rapamycin also affects cellular processes through direct inhibition of the cellular function of FKBP12. For example, rapamycin inhibits the function of FKBP12 in regulating the ryanodine receptor Ca2+ release channel in mammalian cells (Brillantes et al. 1994; Lehnart et al. 2003). Rapamycin also inhibits several FKBP12-dependent functions in Saccharomyces cerevisiae such as regulation of aspartokinase activity (Alarcon and Heitman 1997). In Schizosaccharomyces pombe, rapamycin inhibits the function of FKBP12 in sexual development, leading to a decrease in mating efficiency (Weisman et al. 2001).

The rapamycin-FKBP12 complex binds the FRB (FKBP12-rapamycin binding) domain in TOR, which lies adjacent to the kinase domain. Although FKBP12-rapamycin inhibits the kinase activity of TOR in vitro, it does not completely inhibit TOR kinase activity in vivo (Peterson et al. 2000). Consistently, rapamycin does not inhibit the S. cerevisiae Tor2p kinase-dependent function in cytoskeleton organization (Schmidt et al. 1996, 1997). The differential effects of rapamycin on

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TOR-dependent functions is consistent with the presence of two TOR complexes in *S. cerevisiae*, which differ in their ability to bind rapamycin (Loewitt et al. 2002). The TORC1 complex, containing either Tor1p or Tor2p, binds rapamycin and controls cellular growth in a rapamycin-sensitive manner. In contrast, the TORC2 complex, containing only Tor2p, does not bind rapamycin and regulates actin cytoskeleton organization in a rapamycin-independent manner. The mammalian TOR (mTOR) also exerts rapamycin-sensitive, as well as rapamycin-resistant, functions. Accordingly, rapamycin treatment does not equally inhibit the multiple sites of mTOR-dependent phosphorylation of the translational regulators eIF4E-BP1 (also known as PHAS-1) and p70 S6K (McMahon et al. 2002). A recent study comparing the effects of rapamycin treatment to that of a dominant-negative kinase-inactive mTOR allele further demonstrates that rapamycin does not inhibit all TOR kinase-dependent functions (Edinger et al. 2003).

In *S. pombe*, the two TOR homologs, tor1+ and tor2+, exert different and noncomplementary functions. tor1+ is required for entrance into stationary phase and for sexual development, the two principal responses to starvation. tor1+ is also required for growth at extreme temperatures or under osmotic or oxidative stress conditions. The activity of tor1p under stress conditions is mediated via the regulation of gad8p, the mammalian homolog of the mammalian p70 S6K (Matsuo et al. 2003). tor2+ is an essential gene of as-yet-uncharacterized functions. Rapamycin does not affect the growth of wild-type *S. pombe* cells, but specifically inhibits entrance into sexual development (Weisman et al. 1997). Since disruption of the *S. pombe* FKBP12 homolog, *fkh1*+, results in a phenotype highly similar to wild-type cells treated with rapamycin, we previously suggested that rapamycin directly inhibits fkh1p activity in sexual development (Weisman et al. 2001). This suggestion is supported by our ability to isolate *fkh1* mutants that carry mutations at the predicted rapamycin-binding domain and confer dominant resistance to the effect of rapamycin on the sexual development pathway (Weisman et al. 2001). The mechanism(s) that renders *S. pombe* resistant to rapamycin during the growth phase remains unclear. Moreover, to date, no TOR-related function in *S. pombe* has been shown to be sensitive to inhibition by rapamycin. Here, we show that the rapamycin-FKBP12 complex binds tor1p and identify amino acid uptake as a novel tor1p-modulated function that is sensitive to rapamycin-FKBP12 inhibition. Additionally, we show that three putative amino acid permeases are downregulated in *FKBP12* inhibition. Additionally, we show that three ried out as described in previously (Edinger et al. 2003). Rapamycin does not affect the amino acid uptake as a novel TA03, TA04, TA05, TA115, and TA16. All but TA16 are kind gifts of P. Fantes, Edinburgh University, Edinburgh. TA16 is the kind gift of A. Cohen, Hebrew University, Jerusalem.

### MATERIALS AND METHODS

**Yeast strains, media, and general techniques:** Yeast strains used in this article are described in Table 1. Growth media was prepared as described in Moreno et al. (1991). YES was yeast extract supplemented with 75 μg/ml adenine and uracil. EMM (Edinburgh minimal medium) was slightly modified, as described in Stettlter et al. (1996). Minimal medium was supplemented as required. Leucine, histidine, adenine, and uracil were supplemented at the concentrations of 75 μg/ml, and 10 mm proline (Young and Fantes 1987), EMM-N medium contains no nitrogen. Rapamycin was used as described previously (Weisman et al. 1997). Normally, we use rapamycin at a final concentration of 100 ng/ml. Using higher concentrations (up to 250 ng/ml) did not result in more severe effects. Transformation of *S. pombe* cells was performed by electroporation (Prentice 1992). Assays for mating efficiency were carried out as described in Weisman et al. (1997).

#### Full-length tor1 plasmid constructs and site-directed mutagenesis: tor1+ or S184R, S1834E, or S1834A tor1 mutations were expressed under the regulation of the tor1+ promoter from the LEU2-based plasmid pIRT2 (Booher and Beach 1986) or as NH2-terminal HA-tagged proteins from the nmt1 promoter in ura4+ based pSLF275 (Forsburg and Sherman 1997). Mutations at the conserved serine in the tor1p, S1834E or S1834A, were created by site-directed mutagenesis using PCR overlap extension, as described previously (Weisman and Choder 2001). Briefly, pIRT2-tor1+ served as a template in a

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<tr>
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* All laboratory stocks are derived from TA00, TA01, TA02, TA03, TA04, TA05, TA115, and TA16. All but TA16 are kind gifts of P. Fantes, Edinburgh University, Edinburgh. TA16 is the kind gift of A. Cohen, Hebrew University, Jerusalem.
PCR reaction using one primer that contained the desired mutation and another primer 220 bp downstream from the site of the mutation. The resulting PCR product was gel purified and used as a primer for a second round of PCR with primer complementary to sequences 1300 bp upstream of the site of the mutation. The resulting 1580-bp PCR product was cleaved with BglII and BstXI restriction sites that reside near the fragment ends. This fragment was used to replace the corresponding fragment in pIRT2-tol1. Mutations were verified by DNA sequence analysis.

Two-hybrid plasmid constructions and assays: The two-hybrid plasmids used are pGAD424 and pGBT9, generously provided by J. Heitman, Duke University Medical Center, Durham, North Carolina. The region corresponding to the FRB domain of tor1p (amino acids 1883–2078) was PCR amplified with primers 221 (5′-CGGAATTCGAAATACACACTTCAAGTTCTGAGCACTGAGAAAGCACTGAGAGTGTAATATGAGGTGTCGAAAAGCAAGTTATTTC) and 224 (5′-CGGGATCCAGGCACAGTTTCTTTCTTTCAATCACGCTGCCGTTCTCACGCTTGAAAGTGAGATGCAAGGATGTTTTC) using pIRT2-tol1 as a template. The PCR product was cloned at the EcoRI-BamHI sites of pGBT9 to create the fusion protein GAL4(BD)-tor1p(FRB). Similarly, pIRT2 plasmids carrying tol1 mutations S1834R, S1834E, or S1834A were used as templates for PCR amplification and cloning into pGAD424. GAL4(AD)-fkhl1 fusion proteins were created by PCR amplification of the ORF of fkh1 using primers 220 (5′-AAAACCTGAGTTAAGCCTTCTTATCATTGATAGCAG) and 225 (5′-TCCCCCAGGAGTGGTTGTCGAAAGCCAGTTATTTCTGAGGACATGTTTTC) and subsequent cloning into the pGAD424 plasmid.

β-Galactosidase assays and Western blot analysis: β-Galactosidase assays were performed as described (Pañé et al. 1999). Overnight cultures of two-hybrid strains coexpressing the fusion proteins were grown in SD-Leu-Trp medium at 30°C. Where indicated, 0.1 μg/ml of rapamycin was added. For Western blot analysis, equivalent amounts of proteins, corresponding to 60 μg of protein, were fractionated by SDSpolyacrylamide gel electrophoresis and analyzed with α-GAL4(BD) or α-GAL4(AD) (UBI), using the ECL detection system (Amersham, Buckinghamshire, UK). Western blot gels were quantified using the NIHimage (version 1.62) computer program.

Measurement of leucine uptake: The method described in Karagiannis et al. (1999) was followed with slight modifications adopted from Cohen et al. (2003). Wild-type (TA00) or Δtor1 cells (TA390) were grown to log phase in minimal medium. Wild-type cells were grown either in the absence or in the presence of 0.1 μg/ml rapamycin. A total of 0.5 ml of logarithmic cells (total of ~4 × 10⁶ cells) grown in minimal media were harvested and resuspended in 0.5 ml minimal media containing 0.01 or 2 mM leucine together with [3H]-labeled leucine (1–5 μCi of 1,4-3H(N)-leucine 50 Ci/mmol; NEN Life Science). Cells were incubated at 30°C and samples were taken at 2, 4, and 6 min and mixed with a chilled solution (minimal medium containing 10 mM leucine). Cells were washed three times before being resuspended in water containing 0.5% SDS. Leucine uptake was expressed in specific activity units (picomoles per 10⁶ cells per hour).

Northern blot analysis: Yeast RNA was extracted from logarithmic growing cells. Ten micrograms of total RNA was separated on 1% agarose formaldehyde gels and transferred to nylon membrane. Probes for 7G5.06, isp5, c869.10, and adh1 were PCR amplified using S. pombe DNA extracts as template. The PCR products were separated and extracted from 1% agarose gels and labeled with [α-³²P]dCTP, using the random oligonucleotide labeling procedure.

RESULTS

The growth of strains auxotrophic for leucine is sensitive to rapamycin in an FKB12-dependent manner: To study the mechanism(s) that render S. pombe resistant to rapamycin during growth, we executed a genetic screen designed to isolate rapamycin-sensitive mutants. During this screen it became apparent that the growth of the auxotrophic strain TA2 (leu1-32 ade6-M210 ura4-D18 h+), which was partially sensitive to rapamycin, while the isogenic prototrophic strain was completely resistant to rapamycin. The sensitivity of TA2 to rapamycin is apparent on normally supplemented EMM but not on rich medium (YES; Figure 1A).

Examining the growth of strains auxotrophic for a single nutrient on normally supplemented minimal medium revealed that the growth of leucine auxotrophs is particularly sensitive to rapamycin. Growth of uracil auxotrophs and, to a lesser extent, growth of histidine auxotrophs is less sensitive to rapamycin. Growth of adenine auxotrophs is not inhibited by rapamycin under these conditions (Figure 1B). Thus, rapamycin differentially affects growth of auxotrophic strains, leucine auxotrophy resulting in the strongest effect.

Two well-established targets for the action of rapamycin are the prolyl-isomerase FKBP12 and TOR kinases. Inhibition of TOR by rapamycin is dependent on the formation of FKB12-rapamycin complexes (see Introduction). To examine whether growth inhibition of leucine auxotrophs is the result of the action of rapamycin alone or rapamycin-FKB12 complexes, we examined the effect of rapamycin on Δfkh1 leu1-32 double mutants. Deletion of fkh1+ completely abolished rapamycin sensitivity in leu1-32 strains (Figure 1C), indicating that FKB12-rapamycin complexes are responsible for the growth inhibition.

Rapamycin sensitivity of leucine auxotrophs is rescued by a tor1 allele defective in rapamycin binding: If rapamycin sensitivity in auxotrophs is the result of TOR inhibition by FKB12-rapamycin, it is expected that mutations in tor1p that fail to bind FKB12-rapamycin would confer rapamycin resistance. The domain responsible for FKB12-rapamycin binding, the FRB domain, is highly conserved in eukaryotes. In several model systems, particularly in S. cerevisiae and mammalian cells, it has been demonstrated that mutating the conserved serine within the FRB domain into a bulky amino acid, such as arginine (R) or glutamic acid (E) disrupts binding to rapamycin-FKB12. In contrast, mutating this serine into the relatively small amino acid alanine does not interfere with rapamycin binding (Helliwell et al. 1994; Stan et al. 1994; Chen et al. 1995; Lorenz and Heitman 1995).

To test the ability of the FRB domain of wild type or mutated tor1p to bind FKB12-rapamycin, we used the two-hybrid assay. To this end, the GAL4-DNA binding domain (GAL4-BD) was fused to the FRB domain of tor1p and the GAL4-DNA activating domain to the S. pombe FKB12, fkh1p. The fusion proteins were coexpressed in a rapamycin-resistant FKB12-deficient two-hybrid host strain (Lorenz and Heitman 1995). We found that the FRB domain of tor1p is capable of inter-
acting with fkh1p and that this interaction occurs only in the presence of rapamycin (Figure 2A). Mutations into arginine (S1834R) or glutamic acid (S1834E) dramatically reduced FKBP12-rapamycin binding, while substitution of the conserved serine in tor1p into alanine (S1834A) exhibited an even higher binding activity compared to wild type (Figure 2B). Binding activities are normalized to the protein levels of the GAL4(BD)-tor1p(FRB) fusion proteins (Figure 2C). Thus, as has been demonstrated for other eukaryotes, the FRB domain of tor1p binds FKBP12-rapamycin, and mutating the conserved serine within the FRB domain into a bulky amino acid disrupts rapamycin-FKBP12 binding.

Previously, we reported that the S1834R mutation is deleterious for tor1p function, although it did not affect the level of protein expression (Weisman and Choder 2001). Therefore, we tested the functionality of tor1 alleles carrying the S1834E or S1834A mutations. We transformed the mutated alleles of tor1 into Δtor1 cells and examined their ability to complement the sterility and growth defects associated with deletion of tor1+. We found that S1834E could not fully complement tor1p function in sexual development; however, the defect observed in S1834E mutants is moderate compared to that in S1834R mutants (Figure 2D). In addition, while S1834R could not fully complement the growth defects of Δtor1 cells under osmotic stress conditions (Weisman and Choder 2001), S1834E was indistinguishable from wild type in this respect (data not shown). Interestingly, the S1834A mutation did not impair tor1p function, as it fully complemented the sexual development defect in Δtor1 mutants (Figure 2D) or the growth defect under stress conditions (data not shown). Interestingly, our data are reminiscent of data found for mTOR rapamycin-binding defective alleles. Mutating S2035 in mTOR into bulky amino acids caused defects in kinase activity; the extent of the defect depended on the specific substitution, while mutating S2035 into alanine resulted in an allele indistinguishable from wild-type mTOR (McMahon et al. 2002).

Having shown that S1834 is critical for rapamycin binding in S. pombe tor1p, and bearing in mind the effects of mutating this residue on the protein function, we examined the ability of tor1 mutations to rescue rapamycin-mediated growth inhibition. Significantly, S1834E, but not S1834A, rescued leucine auxotrophs from the inhib-
Figure 2.—tor1 mutants defective in rapamycin binding rescue the growth defect of leucine auxotrophs in the presence of rapamycin. (A) Fusion GAL4(AD)-fkh1p and GAL4(BD)-tor1p(FRB) proteins were constructed and expressed in the two-hybrid rapamycin-resistant S. cerevisiae strain (SYM4; Lorenz and Heitman 1995). Cells coexpressing both fusion proteins or only the GAL4(BD)-tor1p(FRB) fusion protein were spotted onto plates containing X-gal in the absence or the presence of 0.1 μg/ml rapamycin. (B) Fusion GAL4 (BD)-tor1(FRB) proteins carrying specific mutations at S1834 were coexpressed with GAL4(AD)-fkh1p in SYM4 in medium containing 0.1 μg/ml rapamycin (WT, wild type; SE, S1834E; SR, S1834R; SA, S1834A). β-Galactosidase activity measured by the liquid test is normalized to the amount of the fusion protein as assessed by Western blot analysis in B. (C) GAL4(BD)-tor1p(FRB) proteins expressed in SYM4 were detected by Western blot analysis using α-GAL4 antibodies. (D) Mating efficiency of the Δtor1 h⁰ strain (TA157) transformed with plasmid carrying no insert, tor1⁺, or tor1 mutations in the FRB domain. Mating efficiency (%) was determined following 3 days incubation at 30°C in either EMM-N or EMM. Mating efficiency typical for wild-type cells is in boldface type. (E) Δtor1 leu1-32 (TA353) transformed with pSLF273 (ura4⁺-based plasmids) carrying tor1⁺, S1834E, or S1834A mutants was streaked onto minimal plates with no rapamycin (−R) or with 0.1 μg/ml rapamycin (+R). Only cells transformed with the S1834E allele could grow in the presence of rapamycin.

Rapamycin growth inhibition of leucine auxotrophs is suppressed by excess of leucine or by replacing the nitrogen source in the medium: The inability of leucine auxotrophs to grow on minimal medium in the presence of rapamycin suggests a defect in leucine uptake. To test this possibility, we examined the growth of leucine auxotrophs on rapamycin-containing plates in the presence of excess leucine. An increase of the concentration of leucine from 75 to 500 μg/ml partially suppressed rapamycin sensitivity, supporting our hypothesis that rapamycin inhibits leucine uptake from the environment (Figure 3A).

Amino acid permeases in S. cerevisiae are divided into two classes, according to their regulation and function (Sophianopoulou and Diallinas 1995). One class is derepressed under poor nitrogen conditions and is believed to transport amino acids for nitrogen source. The other class is expressed under good nitrogen conditions and regulates the uptake of specific amino acids for the use of protein synthesis. Less is known about the function and regulation of amino acid permeases in S. pombe. However, amino acid import as the function of the nitrogen source has been reported during the study.
of high pH sensitivity (Karagiannis et al. 1999). The growth of leucine auxotrophs is sensitive to high pH in the presence of ammonia, whereas deletion of the E3 ubiquitin ligase pub1\(^+\), the S. pombe homolog of the nitrogen permease inactivator NPI1/RSP5, abolishes pH sensitivity (Karagiannis et al. 1999). Similarly, we have found that the inhibitory effect of rapamycin on leucine auxotrophs is dependent on the presence of ammonia. Thus, when proline is used as the nitrogen source or when the ammonia concentration is decreased, rapamycin does not inhibit growth of leucine auxotrophs (Figure 3B). These data suggest that rapamycin inhibits the growth of leucine auxotrophs due to insufficient leucine uptake in a mechanism that involves amino acid permeases regulated by ammonia.

**Rapamycin sensitivity of leucine auxotrophs is suppressed by mutations in the stress-activated MAPK cascade:** Studies of the sensitivity of leucine auxotrophs to high pH in S. pombe suggested that stress-activated spc1p MAPK cascade is involved in amino acid uptake (Karagiannis et al. 1999). Accordingly, Δwis1 or Δspc1 mutants suppressed the growth inhibition of leucine auxotrophs when placed in the genetic background of a partially active pub1 mutant (Karagiannis et al. 1999). If rapamycin inhibits leucine uptake, then it might be expected that mutations in the spc1p MAPK cascade may also suppress sensitivity to rapamycin. Indeed, loss of function of either wis1\(^+\) or spc1\(^+\) suppressed sensitivity of leucine auxotrophs to rapamycin (Figure 4), supporting our suggestion that the effect of rapamycin involves a defect in leucine uptake. Interestingly, deletion of atf1\(^+\), which encodes a transcription factor that is activated by spc1p (Shiozaki and Russell 1996; Wilkinson et al. 1996) did not suppress rapamycin sensitivity (Figure 4). This finding suggests that in respect to regulation of leucine uptake, the wis1p-spc1p cascade functions through an atf1p-independent mechanism. The mechanism by which mutations in components of the
MAPK cascade suppress leucine uptake defect is not yet known.

**Leucine uptake is inhibited in Δtor1 or rapamycin-treated cells:** To directly test the hypothesis that rapamycin inhibits leucine uptake by blocking a tor1p-dependent function, we used 3H-labeled leucine to measure leucine uptake of Δtor1 cells and wild-type cells treated with rapamycin. Two leucine-uptake systems were identified in *S. pombe*, a high- and a low-affinity system (Sychrova et al. 1989). We first measured leucine uptake in media that contains ammonia as the nitrogen source (EMM) in the presence of 0.01 mM leucine, conditions under which only the high-affinity system is expected to transport leucine at a significant capacity (Karagiannis et al. 1999; Matsumoto et al. 2002). As shown in Figure 5A, the uptake of rapamycin-treated cells was ~40% of the untreated wild-type cells. Similarly, the rate of uptake by Δtor1 cells was ~40% of the wild-type cells. We also measured leucine uptake in EMM media in the presence of 2 mM leucine, conditions under which both systems are expected to import leucine, although the majority of leucine uptake can be attributed to the function of the low-affinity system (Karagiannis et al. 1999; Matsumoto et al. 2002). In the presence of 2 mM leucine, leucine uptake by rapamycin-treated cells is reduced to ~50% of the untreated wild-type strain, while leucine uptake by Δtor1 cells is even further reduced to ~30% of the wild-type strain (Figure 5B). Importantly, uptake of radioactively labeled leucine was not reduced when cells treated with rapamycin or deleted for tor1 were grown in medium containing proline instead of ammonia (Figure 5C), consistent with our observation that the growth of leucine auxotrophs is not inhibited on proline plates (Figure 3B). Taken together, we conclude that rapamycin-treated or Δtor1 cells grown in ammonia-containing medium are defective in leucine uptake. In contrast, leucine uptake is not significantly reduced in rapamycin-treated or Δtor1 cells grown in proline-containing medium.

**Expression of three putative amino acid permeases is downregulated in Δtor1 or rapamycin-treated cells:** Recently, it has been reported that the expression of three putative amino acid permeases is reduced in the *S. pombe* tsc1 and tsc2 mutants that are defective in amino acid uptake (Van Slegtenhorst et al. 2004). The three amino acid permeases 7G5.06, isp5, and c869.10 show high sequence similarity to the general amino acid permease, GAP1, in *S. cerevisiae* (Van Slegtenhorst et al. 2004). Wild-type cells treated with rapamycin or Δtor1 cells are defective in leucine uptake. Wild-type or Δtor1 cultures were grown to midlog phase in (A and B) EMM or in (C) medium containing proline as a nitrogen source (proline); cells were then collected and uptake of radioactively labeled leucine was measured in the presence of 0.01 or 2 mM leucine (see MATERIALS AND METHODS). Wild-type cells were grown either in the absence (WT) or in the presence (WT + R) of 0.1 µg/ml rapamycin.
Figure 6.—Expression of putative amino acid permeases in wild-type and Δtor1 cells. (A) Changes in expression of the putative amino acid permeases 7G5.06 and isp5 were determined by Northern blot analysis. RNA was extracted from cells grown to midlog in either EMM or EMM-Pro. Lanes 1 and 4, wild-type cells (WT); lanes 2 and 5, wild-type cells grown in the presence of 0.1 μg/ml rapamycin; lanes 3 and 6, Δtor1 cells (Δ). (B) Changes in expression of the putative amino acid permease c869.10 were determined as in A. (C) Northern blots were quantified using the NIHimage (version 1.62) computer program. Changes in expression are presented relative to the level of expression in minimal EMM medium (considered as 100%). R, 0.1 μg/ml rapamycin; Pro, EMM-Pro.

2004). tsc1 and tsc2 are the S. pombe homologs of the mammalian TSC1 and TSC2, which are implicated in regulation of TOR-dependent signaling (reviewed in Pan et al. 2004). Like their mammalian counterparts, the gene products of tsc1 and tsc2 physically interact, indicating evolutionary conservation that extends beyond the primary amino acid sequence (Matsumoto et al. 2002). Intriguingly, similar to deletion of tor1, disruption of either tsc1 or tsc2 results in a defect in leucine uptake (Matsumoto et al. 2002; Van Slgtenhorst et al. 2004).

We examined the expression levels of 7G5.06, isp5, and c869.10 by Northern blot analysis in wild-type cells in the absence or presence of rapamycin and in Δtor1 cells. We found that the expression of all three amino acid permeases in ammonia medium (EMM) is reduced either by rapamycin or by deletion of tor1 (Figure 6, A and B, lanes 1–3). The level of expression of 7G5.06 and isp5 is reduced to undetectable levels and the level of c869.10 expression is reduced fivefold, compared to untreated wild-type cells (changes in gene expression are normalized to standard control as depicted in Figure 6C). Therefore, growth inhibition or reduction in leucine uptake by rapamycin is correlated with reduction in expression of these three amino acid permeases.

As mentioned above, the amino acid sequence of 7G5.06, isp5, and c869.10 shows high similarity with the Gap1 amino acid permease. Since GAP1 transcription
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Δtor1 cells are sensitive to rapamycin in an FKBP12-dependent manner: It has previously been reported that deletion of tor1 renders cells sensitive to rapamycin (Kawai et al. 2001; Weisman 2004). This rapamycin sensitivity may be explained if tor1p and tor2p share a rapamycin-sensitive function (Weisman 2004). Accordingly, if tor2p is the target for rapamycin action in Δtor1 cells, it is expected that FKBP12-rapamycin complexes are responsible for the toxicity. We examined whether rapamycin sensitivity of Δtor1 is dependent on the presence of FKBP12. As seen in Figure 7A, Δfkh1 restored rapamycin resistance in Δtor1 cells, indicating that FKBP12-rapamycin complexes are responsible for the rapamycin-sensitive phenotype.

We also found that, unlike leu1-32 mutants, Δtor1 leu1-32 mutants are sensitive to rapamycin on rich medium as well as on minimal medium (Figure 7A). In addition, Δtor1 cells carrying no additional mutations for auxotrophy are also sensitive to rapamycin (data not shown). A simple model to explain these findings is that tor1p and tor2p share a common rapamycin-sensitive function. This function is important for regulating uptake of amino acids, as well as another, yet-unidentified process (Figure 7B). Alternatively, only tor1p regulates leucine uptake in a rapamycin-sensitive manner, while tor1p and tor2p share an additional rapamycin-sensitive function, which is not dependent on the composition of the medium.

DISCUSSION

Rapamycin sensitivity of leucine auxotrophs results from the inhibition of tor1p and depends on the presence of ammonia in the medium: While the fission yeast tor1p and tor2p proteins play essential roles in growth under stress or normal conditions, respectively, rapamycin does not inhibit growth of wild-type S. pombe cells (Weisman et al. 1997). Here we show that the FRB domain of tor1p binds the FKBP12-rapamycin protein-drug complex. Moreover, rapamycin inhibits tor1p-dependent function in leucine uptake. We show that, unlike wild type, auxotrophic cells, particularly leucine auxotrophs, are sensitive to growth in the presence of rapamycin. Both experiments of growth and direct measurements of radioactively labeled leucine uptake suggest that the growth inhibition of leucine auxotrophs is due to insufficient leucine uptake. Deletion of fkh1, the FKBP12 homolog in fission yeast, completely abolished rapamycin sensitivity of leucine auxotrophs. This finding suggests that FKBP12 is not the target for rapamycin inhibition, but rather that FKBP12-rapamycin complexes are responsible for the growth-inhibition effect. Our studies of rapamycin-binding defective tor1 alleles corroborate this hypothesis, as we demonstrate that mutating the conserved serine in tor1p into glutamic acid (S1834E) abolished the binding of rapamycin-FKBP12.
and conferred rapamycin resistance in leucine auxotrophs.

In growth medium containing proline as the nitrogen source, rapamycin does not inhibit the growth of leucine auxotrophs or reduce uptake of radioactively labeled leucine (Figure 3B and Figure 5). Thus, we suggest that in the presence of ammonia, when the expression of ammonia-sensitive permeases is poor, further reduction of gene expression of these permeases by rapamycin leads to growth inhibition of auxotrophic strains. Interestingly, this effect of rapamycin on leucine uptake resembles the effect of high pH (Karagiannis et al. 1999). Like rapamycin treatment, high pH conditions particularly affect the growth of leucine auxotrophs and can be suppressed either by replacement of the nitrogen source in the medium from ammonia to proline or by deletion of either wis1+ or spc1+, the stress-activated MAPK kinase and MAPK, respectively. High pH may diminish leucine uptake by lowering the primary electrochemical gradient across the membrane, thus causing a decrease in the uptake of secondary transport processes (Karagiannis et al. 1999). It will be interesting to determine the mechanism by which mutations in the stress-activated MAPK pathway rescue leucine-uptake defects.

**TOR signaling and nutrient uptake mechanisms:** Studies in S. cerevisiae and mammalian cells suggest that TOR proteins regulate cell growth partly by regulating the activity of plasma membrane transporters, including amino acid permeases. However, the mechanism(s) by which TOR proteins regulate these transporters and how they contribute to cell growth regulation are far from being clear. Studies in mammalian cells have implicated rapamycin and mTOR in transcription regulation of amino acid permeases (Peng et al. 2002) and trafficking of the amino acid transporter 4F2hc (Edinger et al. 2003). Our data showing that tor1p regulates amino acid uptake in a rapamycin-sensitive manner thus support a highly evolutionarily conserved role of TOR signaling in regulation of amino acid uptake.

In S. cerevisiae, TOR proteins regulate both specific amino acid permeases, such as the high-affinity tryptophan permease Tat2p (Schmidt et al. 1998; Beck et al. 1999), and general amino acid permeases, such as Gap1p. The regulation of Gap1p activity by TOR can result in apparently opposite effects. Inactivation of TOR by high levels of rapamycin leads to induction of GAP1 transcription (Schmidt et al. 1998; Beck and Hall 1999; Cardenas et al. 1999; Hardwick et al. 1999). In contrast, partial inactivation of TOR results in low Gap1p activity at the plasma membrane (Chen and Kaiser 2003).

Notably, our studies suggest a different type of regulation of amino acid permeases, compared to S. cerevisiae. Thus, while in S. cerevisiae treatment with rapamycin leads to effects that resemble response to poor nitrogen conditions, including upregulation of GAP1 transcription, our data suggest that this is not the case in S. pombe. On the contrary, we show here that the transcripts of the three amino acid permeases, 7G5.06, isp5, or c869.10, are induced by the poor nitrogen source proline, yet rapamycin treatment leads to reduction in the expression of these permeases. Moreover, our data show that rapamycin treatment does not mimic a condition equivalent to either poor or high quality of nitrogen, but rather leads to decrease in expression of 7G5.06, isp5, or c869.10 in either ammonia or proline media. Since in ammonia medium the level of transcription is already low, further reduction in transcription may explain the inefficient uptake of amino acid and the growth sensitivity of leucine auxotrophs.

Our results are particularly intriguing in view of recent findings that link TOR and tuberous sclerosis complex (TSC) signaling pathways. TSC1 and TSC2 are tumor suppressor genes mutated in tuberous sclerosis, a human syndrome characterized by the widespread development of benign tumors and severe neurological problems. In Drosophila and mammals, it has been suggested that TSC1 and TSC2 work as a complex to antagonize mTOR activity (Radimerski et al. 2000; Gao et al. 2002; Inoki et al. 2002; Tee et al. 2002). However, more recently it has also been suggested that the TSC-2 complex is required for PI3K/mTOR signaling, possibly by repressing a negative feedback loop from mTOR/S6K to the insulin receptor-1 (Harrington et al. 2004). As in higher eukaryotes, the gene products of the S. pombe homologs tsc1+ and tsc2+ work as a complex (Matsumoto et al. 2002). Disruption of either tsc1+ or tsc2+ results in amino acid uptake defects and partial sterility (Matsumoto et al. 2002; van Slegtenhorst et al. 2004). We have shown that tor1p is required for sexual development (Weisman and Choder 2001) and is a positive regulator of amino acid transport (this study). Moreover, tor1p or tsc1p or tsc2p mutants are defective in expression of the amino acid permeases 7G5.06, isp5, and c869.10 (van Slegtenhorst et al. 2004 and Figure 6). Thus, at present, both tor1p and tsc1p-tsc2p appear to act as positive regulators of amino acid uptake and sexual development, which does not fit a simple model in which tsc1p-tsc2p are negative regulators of tor1p in S. pombe. Given the conservation in protein structure of TOR and TSC between S. pombe and human cells, it will be interesting to determine the genetic relationship between these two pathways in fission yeast.

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