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The TOR Signal Transduction Cascade Controls Cellular Differentiation in Response to Nutrients

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Rapamycin binds and inhibits the Tor protein kinases, which function in a nutrient-sensing signal transduction pathway that has been conserved from the yeast *Saccharomyces cerevisiae* to humans. In yeast cells, the Tor pathway has been implicated in regulating cellular responses to nutrients, including proliferation, translation, transcription, autophagy, and ribosome biogenesis. We report here that rapamycin inhibits pseudohyphal filamentous differentiation of *S. cerevisiae* in response to nitrogen limitation. Overexpression of Tap42, a protein phosphatase regulatory subunit, restored pseudohyphal growth in cells exposed to rapamycin. The *tap42-11* mutation compromised pseudohyphal differentiation and rendered it resistant to rapamycin. Cells lacking the Tap42-regulated protein phosphatase Sit4 exhibited a pseudohyphal growth defect and were markedly hypersensitive to rapamycin. Mutations in other Tap42-regulated phosphatases had no effect on pseudohyphal differentiation. Our findings support a model in which pseudohyphal differentiation is controlled by a nutrient-sensing pathway involving the Tor protein kinases and the Tap42–Sit4 protein phosphatase. Activation of the MAP kinase or cAMP pathways, or mutation of the Sok2 repressor, restored filamentation in rapamycin treated cells, supporting models in which the Tor pathway acts in parallel with these known pathways. Filamentous differentiation of diverse fungi was also blocked by rapamycin, demonstrating that the Tor signaling cascade plays a conserved role in regulating filamentous differentiation in response to nutrients.

INTRODUCTION

Diploid cells of the yeast *Saccharomyces cerevisiae* undergo pseudohyphal differentiation in response to nutrient limitation (Gimeno *et al.*, 1992). During pseudohyphal differentiation, cells elongate, adopt a unipolar budding pattern, remain physically attached, and invade the growth substrate. This dimorphic transition is a response to environmental conditions, most notably nitrogen limitation and may enable cells to forage for nutrients under adverse conditions. Several signal transduction pathways, including the MAP kinase and cAMP cascades, control this complex transition (Liu *et al.*, 1993; Cook *et al.*, 1997; Kübler *et al.*, 1997; Lorenz and Heitman, 1997; Robertson and Fink, 1998; Pan and Heitman, 1999; Lorenz *et al.*, 2000a, 2000b).

The Tor protein kinases regulate cell growth in response to nutrient availability (reviewed in Thomas and Hall, 1997; Cutler *et al.*, 1999; Rohde *et al.*, 2001; Schmelzle and Hall, 2000). The Tor proteins are members of the phosphatidyl inositol 3-kinase superfamily that have been conserved throughout evolution from yeast to humans. *S. cerevisiae* expresses two related Tor protein kinases, Tor1 and Tor2 (Heitman *et al.*, 1991a; Cafferkey *et al.*, 1993; Kunz *et al.*, 1993; Helliwell *et al.*, 1994). The Tor1 and Tor2 proteins together play an essential function regulating translation and cell-cycle progression (Kunz *et al.*, 1993; Barbet *et al.*, 1996), whereas Tor2 has an additional unique and essential function involving actin cytoskeleton polarization (Schmidt *et al.*, 1996; Schmidt *et al.*, 1997).

The functions of the Tor protein kinases are specifically inhibited by the natural product rapamycin in complex with the prolyl isomerase FKBP12. Treatment with rapamycin destabilizes the translation initiation factor eIF4G (Berset *et al.*, 1998) and inhibits ribosome biogenesis (Powers and Walter, 1999) and translation (Barbet *et al.*, 1996). In addition, yeast cells treated with rapamycin accumulate in the G(0) phase of the cell cycle, similar to cells starved for nutrients (Barbet *et al.*, 1996). Accordingly, rapamycin treatment results in expression of genes required for utilization of poor nitrogen and carbon sources (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Shamji *et al.* 2000; Kuruvilla *et al.* 2001). Finally, rapamycin induces autophagy, a process of bulk protein degradation induced by starvation (Noda and Ohsumi, 1998; Abeliovich *et al.*, 2000; Kamada *et al.*, 2000; Chan *et al.*, 2001).

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The yeast Tap42 protein and the homologous mammalian protein α4 have been implicated as targets of the Tor signaling cascade. Tap42 is an essential protein that binds the catalytic subunits of protein phosphatase 2A (PP2A) and the related phosphatase Sit4 (Di Como and Arndt, 1996). The Tap42–Sit4 association is dependent on phosphorylation of Tap42 by the TOR pathway (Jiang and Broach, 1999). During starvation, Tap42 disassociates from the phosphatase subunits. This dissociation is also induced by inhibition of Tor function with rapamycin (Di Como and Arndt, 1996). *TAP42* mutations have been identified that confer partial rapamycin resistance, indicating that the essential function of the Tor kinases could be mediated via Tap42. Both plant and mammalian homologues of Tap42 have been identified, and association of the Tap42 homolog α4 with PP2A-type phosphatases is also rapamycin sensitive (Murata *et al.*, 1997; Chen *et al.*, 1998; Inui *et al.*, 1998; Harris *et al.*, 1999). Thus, the Tor proteins may function via conserved Tap42 homologues that regulate phosphatase activities.

Here we describe a novel role for the Tor signaling pathway in regulating the transition to yeast pseudohyphal growth. Rapamycin inhibits pseudohyphal differentiation of yeast cells in response to nitrogen limitation. Rapamycin inhibition of filamentous growth is mediated via FKBP12 and Tor and occurs at concentrations of rapamycin that do not affect vegetative growth. Overexpression or mutation of Tap42 restores pseudohyphal growth in the presence of rapamycin. Moreover, the Tap42-regulated phosphatase Sit4 is essential for pseudohyphal growth. Activation of the MAP kinase or cAMP signaling cascades or relief from repression by the Sok2 pathway restores pseudohyphal growth in the presence of rapamycin. Finally, we show that rapamycin prevents filamentous differentiation of diverse pathogenic fungi. We propose a model whereby a Tor-Tap42–Sit4 nutrient signaling pathway acts in parallel with the MAP kinase and cAMP signal transduction pathways to regulate filamentation.

MATERIALS AND METHODS

Media and Strains

Yeast medium was prepared as previously described: YPD (Sherman, 1991), SLAD (Gimeno *et al.*, 1992), V8 medium (Alspaugh *et al.*, 1997), and Spider medium (Liu *et al.*, 1994). Rapamycin was added to the medium from a concentrated stock solution in 90% ethanol/10% Tween 20. Yeast transformations were performed with the use of the lithium acetate method (Schiestl *et al.*, 1993). Unless otherwise noted, mutant yeast strains were constructed by PCR-mediated gene disruptions, with the use of the G418 resistance gene cassette derived from template plasmid pFA6-kanMX2 as described (Wach *et al.*, 1994; Lorenz *et al.*, 1995). Strains MLY88α and MLY90–1α were generated as previously described (Cardenas *et al.*, 1999). The corresponding diploid strains (MLY88a/α and MLY90a/α) were created by mating with the wild-type MLY41a. In the case of MLY88a/α, this strain was then sporulated, and *MATa* and *MATα* rapamycin-resistant isolates were then mated, and diploids were selected. Unless otherwise indicated, cells grown in synthetic medium were transformed with the *URA3*-bearing pRS426 plasmid to confer uracil prototrophy.

Photomicroscopy

Colony photographs were taken directly on agar plates with a Zeiss microscope (Thornwood, NY) fitted with a 35-mm Nikon camera (Garden City, NY) at a 25× magnification.

RESULTS

Rapamycin Inhibits Pseudohyphal Differentiation

At low, sublethal connections (10 ng/ml), rapamycin blocked pseudohyphal growth of the Σ1278b diploid yeast strain on low-ammonium medium (SLAD; Figure 1). At these concentrations, rapamycin had no effect on the vegetative growth of wild-type cells, as measured by both colony size and cell growth rates (our unpublished results). Colonies that formed in the presence of rapamycin were similar in size to those in the absence of rapamycin but failed to form any pseudohyphal filaments projecting from the colony borders (Figure 1). Moreover, when the plates were gently washed with a stream of water, most of the cells in colonies exposed to rapamycin were removed, indicating rapamycin also causes a defect in agar invasion and agar adherence. The effects of rapamycin were not nitrogen-source specific, and rapamycin also inhibited pseudohyphal differentiation on medium limiting for glutamine or proline as the sole nitrogen source (our unpublished results). Mutations in the *FPR1* gene encoding the rapamycin-binding protein FKBP12, or dominant mutations in the *TOR1* or *TOR2* genes, restored pseudohyphal growth in the presence of rapamycin (Figure 1 and our unpublished results). These findings indicate that partial inhibition of the Tor1 and Tor2 protein kinases by the FKBP12-rapamycin complex inhibits cellular differentiation without impairing cell growth.

Rapamycin prevented filament formation and agar invasion but did not inhibit all features of pseudohyphal growth. Cells grown on low-ammonium medium in the presence of rapamycin failed to filament but still formed elongated cells characteristic of pseudohyphal differentiation (not shown). Rapamycin also did not inhibit the switch that occurs from bipolar to unipolar budding during pseudohyphal differentiation. Furthermore, a *FLO11-lacZ* reporter gene was expressed normally in cells exposed to rapamycin (our unpublished results). Finally, rapamycin did not inhibit invasive growth on nutrient-rich medium in haploid cells of the Σ1278b strain (our unpublished results; Roberts and Fink, 1994).

The Tor Proteins Regulate Pseudohyphal Growth via Tap42 and Sit4

Tor activity has been shown to regulate the association of Tap42 with protein phosphatase 2A (PP2A) and the related phosphatase Sit4 (Di Como and Arndt, 1996; Jiang and Broach, 1999). When phosphorylated by Tor, Tap42 binds to PP2A catalytic subunits and competes with binding of canonical regulatory phosphatase subunits, including Cdc55 and Tpd3 (Jiang and Broach, 1999). Moreover, cells expressing the *tap42-11* mutant allele are rapamycin resistant (Di Como and Arndt, 1996). We found that overexpression of Tap42 in wild-type cells restored pseudohyphal differentiation on medium containing rapamycin (Figure 2). Overexpression of Tap42 restored both pseudohyphal filament formation (Figure 2) and invasion into the agar medium (our unpublished results). Cells expressing only the Tap42-11 mutant protein formed smaller colonies and exhibited a partial filamentation defect on SLAD medium when compared with cells expressing the wild-type Tap42 protein. Importantly, the filamentous differentiation that did occur in

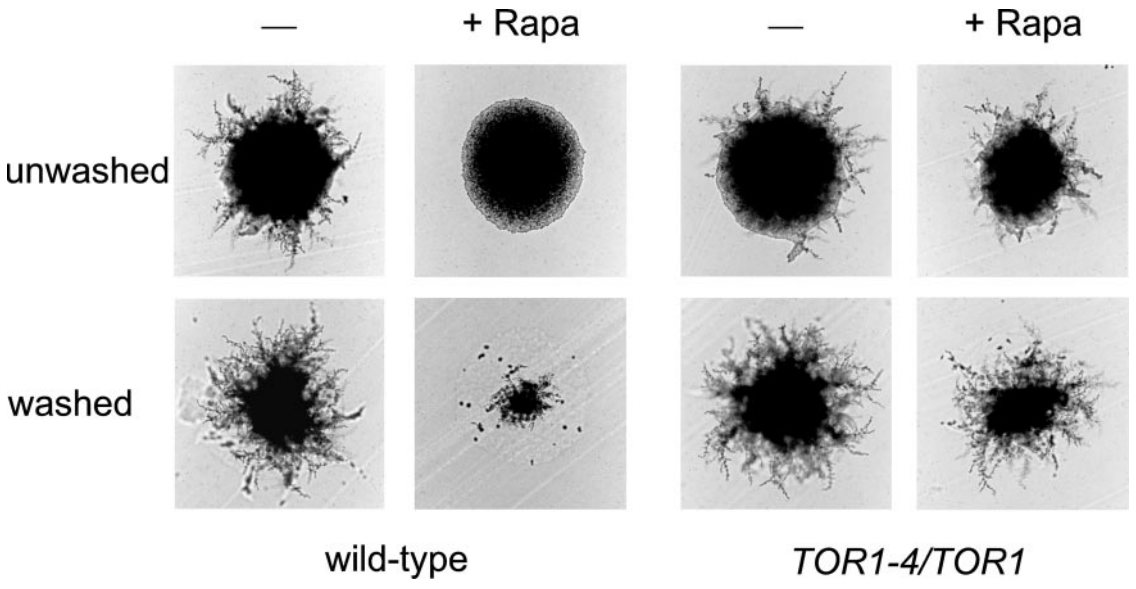

Figure 1. Rapamycin inhibits pseudohyphal filamentous growth and agar invasion. Wild-type (MLY61a/α) and *TOR1-4/TOR1* mutant strains were grown for 3 d at 30°C on SLAD medium without (−) or with 10 ng/ml rapamycin (+ Rapa). Colonies were photographed at 25× magnification before (unwashed) and following (washed) washing with a gentle stream of water to remove noninvasive and nonadherent cells.

cells expressing only the *tap42-11* mutant allele was not inhibited by rapamycin (Figure 2). These findings indicate that the Tap42 phosphatase regulator acts in conjunction with the Tor proteins during pseudohyphal growth.

Tap42 has been shown to bind the products of the *PPH21, PPH22,* and *SIT4* genes (Di Como and Arndt, 1996; Jiang and Broach, 1999). Overexpression of the *SIT4* gene is known to confer partial rapamycin resistance (Di Como and Arndt, 1996). Importantly, we found that cells lacking the Sit4 phosphatase were completely defective in pseudohyphal differentiation, whereas mutations in the PP2A-encoding genes *PPH21* and *PPH22* or the homologous *PPH3* gene had no effect (Figure 3A). In addition, *sit4/sit4* mutant cells exhibited a growth defect on SLAD medium similar to cells compromised for Tap42 function (*tap42-11* mutant cells). Cells lacking the Srk1 protein, which has been implicated in regulating the functions of the *SIT4, TOR1,* and *PP2A* genes (Fernandez-Sarabia *et al.*, 1992; Alarcon *et al.*, 1996; Evans and Stark, 1997), were also unable to undergo pseudohyphal growth. Notably, *srk1/srk1* mutant diploid cells exhibited an unusual morphology and produced ruffled colonies composed of swollen round cells with enlarged vacuoles. These findings support the conclusion that Tor-dependent regulation of Sit4 via Tap42 regulates pseudohyphal differentiation.

We next examined the effects of these PP2A phosphatase mutations on vegetative growth in the presence of rapamycin. The *sit4*Δ mutant was found to be markedly hypersensitive to rapamycin in rich medium by a serial dilution assay, similar to a mutant lacking the Tor1 protein (Figure 3B). Cells lacking the Srk1 regulatory protein were also markedly hypersensitive to rapamycin. Mutations affecting the protein phosphatase 2A catalytic subunits Pph21 and Pph22, the homologous Pph3 protein, or Pph21, Pph22, and Srk1 all conferred partial rapamycin resistance (Figure 3B

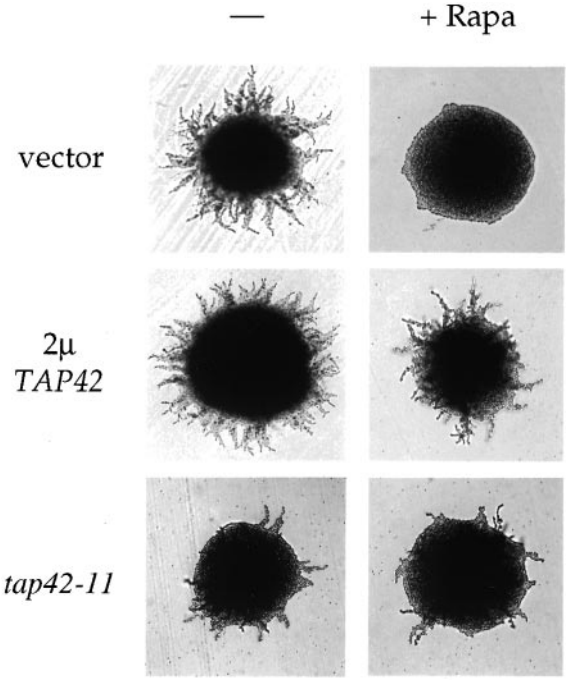

Figure 2. Tap42 controls pseudohyphal growth. Wild-type cells (MLY61a/α) transformed with the control 2-μm plasmid (pRS426, vector) or a 2-μm plasmid bearing the *TAP42* gene (CB2516, 2 μ *TAP42*), or diploid *tap42*Δ/*tap42*Δ mutant cells expressing the *tap42-11* mutant allele from a centromeric plasmid (CY5755, 2 μ *tap42-11*) were grown on SLAD medium without (−) or with (+ Rapa) 10 ng/ml rapamycin (RAPA) at 30°C for 3 d. Colonies were photographed at 25× magnification.

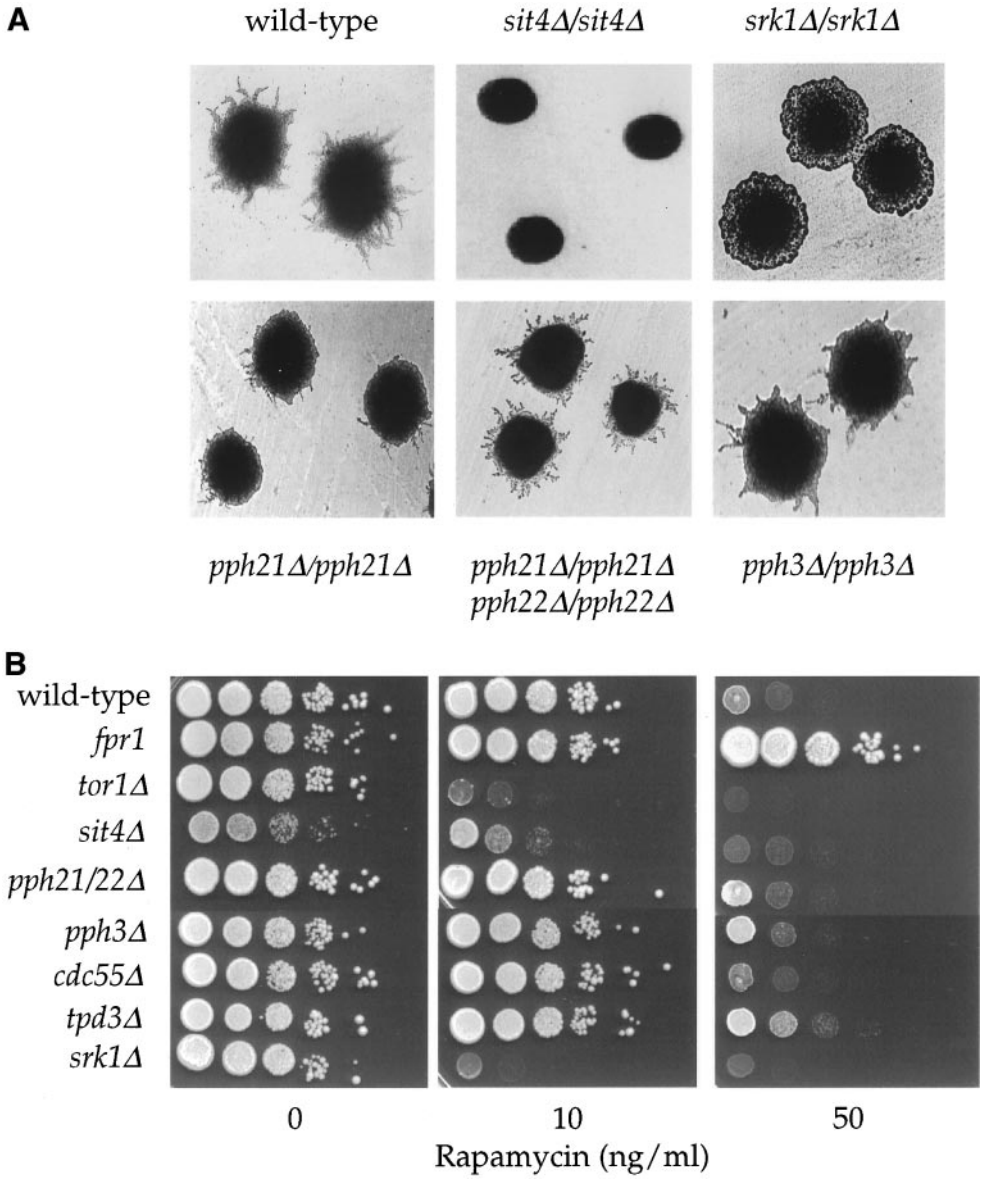

Figure 3. Cells lacking the Sit4 phosphatase are defective in pseudohyphal growth and exhibit rapamycin hypersensitive growth. (A) Wild-type (MLY61a/α), *sit4/sit4* (SCY94a/α), *srk1/srk1* (SCY95a/α), *pph21/pph21* (SCY98a/α), *pph21/pph21 pph22/pph22* (SCY105a/α), and *pph3/pph3* (SCY100a/α) mutant strains were grown on SLAD medium for 5 d at 24°C, and colonies were photographed at 25× magnification. (B) Wild-type (MLY41a), *fpr1* (MLY88a), *tor1*Δ (MLY109a), *sit4*Δ (SCY94a), *pph21 pph22*Δ (SCY105a), *pph3*Δ (SCY100a), *cdc55*Δ (SCY91a), *tpd3*Δ (SCY92a), and *srk1*Δ (SCY95a) mutant strains were serially diluted onto YPD medium without (0) or with 10 or 50 ng/ml rapamycin and grown for 3 d at 24°C.

and our unpublished results). Furthermore, a mutation removing the protein phosphatase regulatory subunits Cdc55 or Tpd3 also conferred partial rapamycin resistance, in accord with an earlier report (Jiang and Broach, 1999). Mutations affecting the Sap4, Sap155, Sap185, or Sap190 proteins, which are known to associate with the Sit4 phosphatase (Di Como and Arndt, 1996), had no effect on pseudohyphal differentiation or rapamycin sensitivity (our unpublished results).

These findings provide further support for a model in which the Tap42–Sit4 complex mediates Tor signaling. Depletion or elimination of this complex increases cell sensitivity to rapamycin, whereas increasing this complex confers partial rapamycin resistance. In accord with this model, mutations that remove phosphatase regulatory subunits that compete with Tap42 for Sit4 increase the amount of the Tap42–Sit4 complex and confer relative rapamycin resistance. Likewise, deletion of the catalytic subunits, Pph21, Pph22, and Pph3 that compete with Sit4 for Tap42 also confers partial rapamycin resistance.

Activated MAP Kinase, PKA, or Sok2 Signaling Pathways Render Pseudohyphal Growth Rapamycin Resistant

Three signal transduction cascades have been shown to regulate pseudohyphal growth. A MAP kinase cascade that shares elements of the pheromone signaling pathway is necessary for filamentous growth (Liu *et al.*, 1993; Madhani and Fink, 1997; Rupp *et al.*, 1999). A second pathway is comprised of the G-protein coupled receptor Gpr1, the heterotrimeric G-protein alpha subunit Gpa2, and the Tpk2

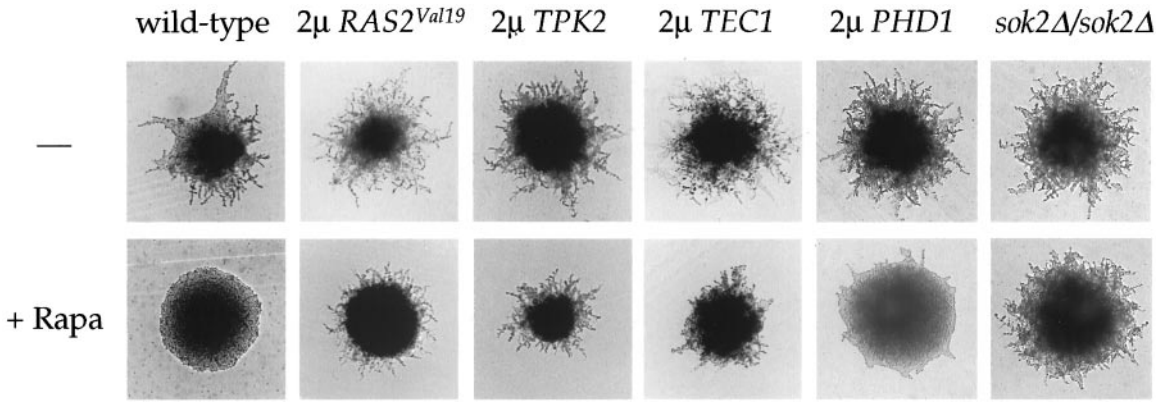

Figure 4. Activation of MAP kinase or cAMP signaling restores pseudohyphal growth in the presence of rapamycin. Wild-type (MLY61a/α) cells transformed with the control plasmid (pRS426), a centromeric plasmid expressing Ras2-^{Val19}, 2-μm plasmids overexpressing Tpk2, Tec1, or Phd1, and isogenic *sok2/sok2* mutant cells were grown on SLAD medium without (−) or with (+ Rapa) 10 ng/ml rapamycin. Cells were grown for 3 d at 30°C and colonies were photographed at 25× magnification.

catalytic subunit of cAMP-dependent protein kinase (Ward *et al.*, 1995; Kübler *et al.*, 1997; Lorenz and Heitman, 1997; Pan and Heitman, 1999; Lorenz *et al.*, 2000a, 2000b). A third pathway is a transcription factor cascade involving Sok2, Phd1, Ash1, and Swi5, which together regulate expression of the cell surface flocculin Flo11 and other enzymes that mediate mother-daughter cell separation (Pan and Heitman, 2000). Mutations in these signaling pathways compromise or enhance pseudohyphal differentiation. Notably, because of partial redundancy between these signaling pathways, stimulation of one pathway can suppress mutations in the other pathways and restore pseudohyphal growth (Lorenz and Heitman, 1997, 1998a, 1998b; Mösch *et al.*, 1999; Pan and Heitman, 1999, 2000; Rupp *et al.*, 1999).

Activation of the MAP kinase or cAMP pathways, or mutation of the Sok2 repressor, was found to restore pseudohyphal growth in the presence of rapamycin (Figure 4). For example, overexpression of the Tec1 transcription factor regulated by the MAP kinase pathway restored filamentation. Similarly, expression of a constitutively activated Ste11-4 mutant kinase restored pseudohyphal growth in the presence of rapamycin (our unpublished results). Activation of the cAMP signaling pathway similarly rendered pseudohyphal growth resistant to rapamycin. Overexpression of the Tpk2 catalytic subunit of PKA restored pseudohyphal growth in the presence of rapamycin (Figure 4), as did the addition of 1 mM exogenous cAMP in cells lacking the cAMP phosphodiesterase Pde2 (our unpublished results). Expression of an activated *RAS2* mutant (val19), which acts at a branch point and activates both cAMP and MAP kinase signaling, also restored pseudohyphal growth in the presence of rapamycin (Figure 4). Finally, mutation of the Sok2 repressor rendered filamentous growth resistant to rapamycin. Sok2 has previously been shown to regulate pseudohyphal differentiation via a cascade of transcription factors involving Phd1, Ash1, and Swi5. Interestingly, overexpression of the Phd1 transcription factor largely failed to restore pseudohyphal growth or agar invasion in the presence of rapamycin (Figure 4 and our unpublished results). This last finding is notable because overexpression of Phd1 dramatically enhances pseudohyphal growth of wild-type cells and restores pseudohyphal growth in many mutant strains with defects in the MAP kinase or cAMP signaling cascades (Gavrias *et al.*, 1996; Lo *et al.*, 1997; Chandarlapaty and Errede, 1998; Lorenz and Heitman, 1998a, 1998b; Pan and Heitman, 1999). Taken together, these findings illustrate that the TOR pathway regulates pseudohyphal differentiation in conjunction with the MAP kinase, cAMP, and Sok2 pathways.

The Tor Signaling Cascade Controls Filamentous Differentiation in Divergent Fungi

Many fungi differentiate into a filamentous form during their life cycle (reviewed in Madhani and Fink, 1998), and the ability to undergo this dimorphic transition is thought to be necessary for pathogenesis. Mutant strains of the human pathogen *Candida albicans* or the plant pathogen *Ustilago maydis* that are unable to adopt filamentous growth are avirulent (Banuett, 1991; Hartmann *et al.*, 1996; Lo *et al.*, 1997). In other pathogenic fungi, such as the human fungal pathogen *Histoplasma capsulatum,* a dimorphic transition from filamentous to yeast growth occurs during infection and is thought to be required for virulence (Medoff *et al.*, 1986).

In many fungi, nitrogen starvation stimulates filamentous differentiation. The human pathogen *Cryptococcus neoformans* mates in response to nitrogen limitation and forms filaments and basidia during its sexual cycle (Kwon-Chung, 1975; Alspaugh *et al.*, 2000). The emerging opportunistic yeast pathogen *Candida lusitaniae* also filaments when starved for nitrogen on SLAD medium (Young *et al.*, 2000). The most common fungal pathogen to infect humans, *C. albicans*, forms both pseudohyphae and true hyphae when grown on nitrogen-poor spider medium (Liu *et al.*, 1994). Exposure to a sublethal concentration of rapamycin (100 ng/ml in this case) completely prevented filamentous differentiation of *C. neoformans*, *C. lusitaniae*, and *C. albicans* (Figure 5). Notably, this concentration of rapamycin blocked filamentous growth but had little or no effect on vegetative growth rates (our unpublished results). We have recently isolated mutant strains of *C. neoformans* and *C. albicans* that

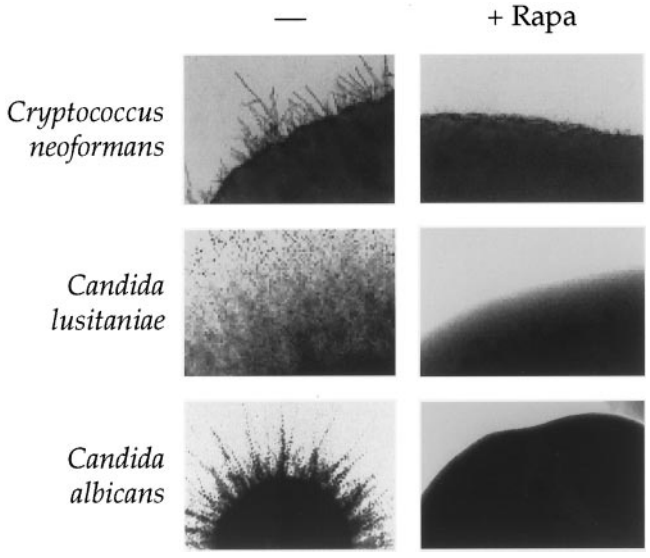

Figure 5. Filamentation of pathogenic fungi is inhibited by rapamycin. Strains of *Cryptococcus neoformans* (mating partners JEC21 and JEC20 cocultured to produce the filamentous teleomorphic form *Filobasidiella neoformans*), *Candida lusitaniae* (strain CL3), and *Candida albicans* (strain SC5314) were grown on V8 medium (top panels), SLAD medium (middle panels), or spider medium (bottom panels), respectively, lacking (−) or containing (+ Rapa) 100 ng/ml rapamycin at 30°C for 3 d. Edges of colony growth were photographed at 25× magnification.

lack the FKBP12 protein required for rapamycin inhibition of the Tor1 homologues in these fungi (Cruz *et al.*, 1999, 2001). Importantly, *C. neoformans frr1* mutant strains mated and filamented in the presence of rapamycin (our unpublished results). Similarly, *rbp1/rbp1 C. albicans* mutant strains lacking the FKBP12 homolog Rbp1 filamented normally in the presence of rapamycin (our unpublished results). These findings indicate that the inhibitory action of rapamycin is mediated in complex with FKBP12. In summary, these observations illustrate that the Tor signaling cascade plays a highly conserved role in regulating filamentous differentiation in response to nutritional cues in diverse fungi, including several pathogens of humans.

DISCUSSION

The Tor protein kinases were first identified in *S. cerevisiae* as the targets of rapamycin in complex with the prolyl isomerase FKBP12 (Heitman *et al.*, 1991a, 1991b; Cafferkey *et al.*, 1993; Kunz *et al.*, 1993; Helliwell *et al.*, 1994). Subsequent studies identified mammalian and insect homologues of the Tor proteins, revealing the Tor protein kinases and the mechanisms of rapamycin action have been highly conserved throughout evolution. Recent studies reveal that the Tor pathway plays a conserved role in sensing nutrients, including amino acids, nitrogen sources, and possibly also carbon sources (reviewed in Gingras *et al.*, 2001; Rohde *et al.*, 2001). The Tor pathway plays a prominent role in regulating both translation and transcription, and these actions likely underlie the emerging roles for the Tor pathway in controlling a myriad of functions in response to nutrients or their absence. For example, in the yeast *S. cerevisiae* the Tor proteins regulate ribosome biogenesis, gene expression, and autophagy (Zaragoza *et al.*, 1998; Cardenas *et al.*, 1999; Powers and Walter, 1999). Recent studies reveal that rapamycin and the Tor protein kinase homologues Tor1 and Tor2 regulate cell growth, mating, and stress responses in the fission yeast *Schizosaccharomyces pombe* (Weisman *et al.*, 1997; Weisman and Choder, 2000; Kawai *et al.*, 2001). Here we demonstrate that the Tor pathway plays a novel role and regulates cellular differentiation and filamentous growth of *S. cerevisiae*.

Our studies demonstrate the Tor signaling pathway promotes yeast pseudohyphal differentiation in response to nitrogen availability. Pseudohyphal growth occurs on medium limiting for good (such as ammonium) or poor (such as proline) nitrogen sources, albeit to different extents. Thus, although yeast cells can discriminate between both the abundance and the quality of the nitrogen source, the more important feature for filamentation appears to be abundance. Rapamycin blocks filamentous growth on medium limiting for either good or poor nitrogen sources. Rapamycin effectively blocks pseudohyphal differentiation at a sublethal concentration that has little effect on colony size or growth rate in liquid SLAD medium. Thus, rapamycin inhibition of pseudohyphal growth is not the result of a decrease in vegetative growth. Mutants lacking the FKBP12 protein, or expressing dominant rapamycin resistant Tor1 or Tor2 mutant kinases, filament in the presence of rapamycin, indicating that the effects of rapamycin are mediated in complex with FKBP12 and the Tor kinases. Our studies further support a model in which the Tor1 and Tor2 proteins regulate pseudohyphal differentiation via the protein phosphatase Sit4 and its associated regulatory protein Tap42. Overexpression of Tap42 or expression of the Tap42-11 mutant protein restored pseudohyphal growth in cells treated with rapamycin. Mutants lacking the Sit4 phosphatase exhibited a severe defect in pseudohyphal growth and were rapamycin hypersensitive. These finding provide evidence that the Tor protein kinases drive pseudohyphal growth via Tap42 and Sit4.

The Tor kinase-Tap42–Sit4 pathway plays a role in regulating both translation and the nitrogen catabolite repression (NCR) transcriptional response (Barbet *et al.*, 1996; Di Como and Arndt, 1996; Beck and Hall, 1999; Bertram *et al.*, 2000). As outlined in Figure 6, we propose that rapamycin inhibits pseudohyphal differentiation by causing both constitutive expression of the NCR genes and by inhibiting translation. In the NCR transcriptional pathway, nitrogen-rich conditions promote binding of the Ure2 repressor to the Gln3 transcription factor, preventing nuclear import of Gln3 and blocking activation of Gln3-dependent genes. In response to either limiting quantities of a good nitrogen source (such as ammonium) or poor nitrogen sources (such as proline), Gln3 is released from Ure2 inhibition and gene induction occurs. Interestingly, *ure2* mutations result in constitutive expression of NCR-regulated genes and block pseudohyphal growth, whereas *gln3* mutations prevent expression of NCR-regulated genes and also block pseudohyphal growth (Lorenz and Heitman, 1998a, 1998b). Furthermore, either overexpression or mutation of the Ure2 inhibitor Mks1 prevents pseudohyphal growth (Edskes *et al.*, 1999). The sim-

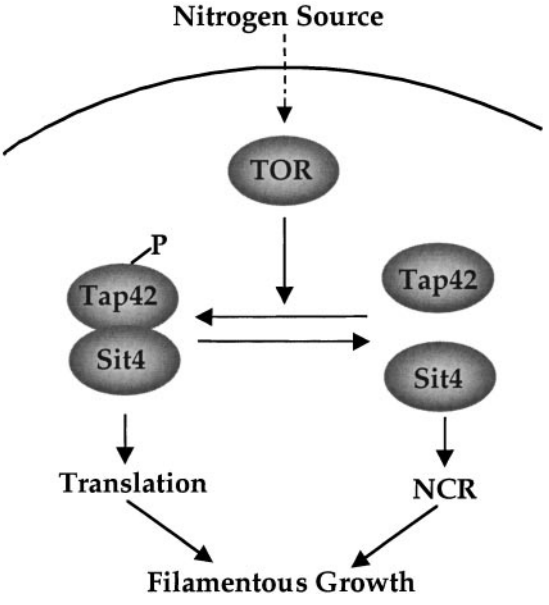

Figure 6. Model of how the Tor signaling cascade controls filamentous growth via the Tap42–Sit4 phosphatase complex. The Tor pathway plays a role in sensing nitrogen sources and regulating physiological responses. Evidence presented here reveals a novel role for the Tor pathway in controlling the dimorphic transition from yeast to filamentous growth. Epistasis analysis indicates that the effects of Tor are mediated via the Tap42–Sit4 phosphatase complex and independent of other pathways (MAP kinase, cAMP, and Sok2) known to regulate pseudohyphal growth. We propose that Tor controls filamentous growth by regulating both translation and the nitrogen catabolite repression (NCR) transcriptional response that allows cells to sense and respond to limiting nitrogen sources.

plest model is that when cells are shifted to limiting nitrogen medium, the NCR genes are induced. These cells can then sense and import the limiting nitrogen source and, as a consequence, the NCR response is repressed. We propose that this cycle of NCR gene induction and repression is required for filamentous differentiation. In this model, rapamycin causes constitutive activation of the NCR response and thereby prevents filamentous differentiation in response to nitrogen source sensing.

Two findings suggest that the known role of Tor in translation may also be involved in regulating pseudohyphal differentiation. First, we find that sublethal concentrations of the translation inhibitor cycloheximide also inhibit pseudohyphal growth (unpublished results). Second, at concentrations that inhibit pseudohyphal growth, rapamycin causes a modest decrease in methionine incorporation into protein in low-nitrogen medium (unpublished results). A certain level of Tor activity may be required to translate transcripts encoding proteins necessary for pseudohyphal growth. Epistasis experiments indicate that inhibition of pseudohyphal differentiation by rapamycin and cycloheximide is distinct. For example, overexpression of Tap42 restored pseudohyphal growth in the presence of rapamycin but not cycloheximide, whereas overexpression of the Phd1 transcription factor restored pseudohyphal growth in the presence of cycloheximide but not in the presence of rapamycin (Figure 4 and unpublished results). Thus, the ability of rapamycin to interfere with Tor regulation of both translation and transcription likely underlies its ability to inhibit pseudohyphal differentiation.

Rapamycin has been previously shown to simulate the growth of yeast cells in a poor nitrogen source or limiting amounts of a good nitrogen source. If this is the case and pseudohyphal differentiation is a response to limiting nitrogen, why then does rapamycin inhibit rather than enhance filamentation? Tor is part of a signal transduction pathway dedicated to sensing nitrogen. Several recent studies indicate that growth on low-nitrogen medium, which is conducive to filamentous growth, may lead to a decrease in Tor activity. Exposure of cells to rapamycin in low-nitrogen medium may further decrease Tor activity below a threshold level required for pseudohyphal growth. This correlates with the different effects on transcription we observed when cells were exposed to rapamycin in low- versus high-ammonium medium. In rich medium, rapamycin has a dramatic effect on the transcription of many genes (Cardenas *et al.*, 1999). In contrast, we see little effect of rapamycin on transcription of the *MEP2* and *GAP1* NCR-regulated genes in low-ammonium medium, likely because these genes are already induced (unpublished results). Thus, although treatment of cells with rapamycin in rich medium may decrease Tor activity to a level similar to that in cells grown in low-nitrogen medium, addition of rapamycin to cells in low-nitrogen medium has a different effect. Such cells may have an even lower level of Tor signaling, possibly similar to complete nitrogen deprivation, which does not support pseudohyphal growth. Rapamycin did not induce pseudohyphal differentiation at any concentration tested on medium with different levels of nitrogen source (not shown). Although under these conditions rapamycin may decrease Tor activity to the level associated with pseudohyphal differentiation, other signaling pathways likely operate that sense high levels of nitrogen and prevent filamentation.

Recent studies using whole genome array analysis have implicated the Tor proteins in sensing the quality of both the carbon and the nitrogen source (Shamji *et al.*, 2000). Could rapamycin therefore block filamentous growth by impairing both carbon and nitrogen sensing? Although this is possible, the role for the Tor proteins in carbon source sensing is considerably more modest than its prominent role in sensing nitrogen sources. In addition, the cAMP–PKA pathway plays a central role in sensing carbon sources during both pseudohyphal growth and the control of sporulation (Pan and Heitman, 1999; Lorenz *et al.*, 2000a, 2000b). It has been previously reported that rapamycin increases sporulation of some diploid strains of *S. cerevisiae* (Zheng and Schreiber, 1997); however, it has not been examined whether this reflects a role for the Tor proteins in sensing nitrogen source, carbon source, both, or neither. In addition, the effects of rapamycin on sporulation may be strain specific because sublethal concentrations of rapamycin do not enhance sporulation of the Σ1278b or JK9–3da/α diploid strains (unpublished results). Finally, little if any induction of meiotic specific genes by rapamycin was observed by whole genome array analysis (Hardwick *et al.*, 1999). In summary, rapamycin impairs the ability of yeast cells to differentiate and form pseudohyphal filaments and does not induce

Table 1. Strains used in this study

Strain	Genotype	Reference
Saccharomyces cerevisiae		
MLY40α	Σ1278b *MATα ura3-52*	Lorenz and Heitman (1997)
MLY41a	Σ1278b *MAT***a** *ura3-52*	Lorenz and Heitman (1997)
MLY61a/α	Σ1278b *MAT***a**/α *ura3-52/ura3-52*	Lorenz and Heitman (1997)
MLY88a/α	Σ1278b *MAT***a**/α *ura3-52/ura3-52 fpr1/fpr1*	This study
MLY88α	Σ1278b *MATα ura3-52 fpr1*	This study
MLY109a	Σ1278b *MAT***a** *ura3-52 tor1Δ::G418*	This study
MCY46a/α	Σ1278b *MAT***a**/α *leu2::URA3/leu2::URA3 ura3-52/ura3-52 tap42Δ::G418/tap42Δ::G418* [*tap42-11 LEU2 CEN*]	This study
SCY65a/α	Σ1278b *MAT***a**/α *ura3-52/ura3-52 TOR1-4/TOR1*	This study
SCY94a	Σ1278b *MAT***a** *ura3-52 sit4Δ::G418*	This study
SCY94a/α	Σ1278b *MAT***a**/α *ura3-52/ura3-52 sit4Δ::G418/sit4Δ::G418*	This study
SCY105a	Σ1278b *MAT***a** *ura3-52 pph21Δ::G418 pph22Δ::HYG*	This study
SCY105a/α	Σ1278b *MAT***a**/α *ura3-52/ura3-52 pph21Δ::G418/pph21Δ::G418 pph22Δ::HYG/pph22Δ::HYG*	This study
SCY98a/α	Σ1278b *MAT***a**/α *ura3-52 pph21Δ::G418/pph21Δ::G418*	This study
SCY100a	Σ1278b *MAT***a** *ura3-52 pph3Δ::G418*	This study
SCY100a/α	Σ1278b *MAT***a**/α *ura3-52/ura3-52 pph3Δ::G418/pph3Δ::G418*	This study
SCY95a	Σ1278b *MAT***a** *ura3-52 srk1Δ::G418*	This study
SCY95a/α	Σ1278b *MAT***a**/α *ura3-52/ura3-52 srk1Δ::G418/srk1Δ::G418*	This study
SCY91a	Σ1278b *MAT***a** *ura3-52 cdc55Δ::G418*	This study
SCY92a	Σ1278b *MAT***a** *ura3-52 tpd3Δ::G418*	This study
XPY80a/α	Σ1278b *MAT***a**/α *ura3-52/ura3-52 sok2Δ::HygB/sok2Δ::HygB*	Pan and Heitman (1999)
Cryptococcus neoformans		
JEC20	Serotype D *MAT***a**	Moore and Edman (1993)
JEC21	Serotype D *MATα*	Moore and Edman (1993)
Candida albicans		
SC5314		Fonzi and Irwin (1993)
Candida lusitaniae		
CL3	MAT**a**	Young *et al.* (2000)

sporulation under these conditions in the Σ1278b strain background.

Previous studies of pseudohyphal growth have identified several signaling cascades that sense nutrient availability and regulate bud site selection, mother-daughter cell adhesion, and cell elongation. Pseudohyphal differentiation is regulated by both the MAP kinase signaling cascade and the cAMP–PKA cascade (Liu *et al.*, 1993; Lorenz and Heitman, 1997; Robertson and Fink, 1998; Pan and Heitman, 1999). The MAP kinase cascade is activated in response to nitrogen starvation through an unknown mechanism (Mösch *et al.*, 1996). The cAMP pathway is regulated by the G-protein–coupled receptor Gpr1 and the Gα protein Gpa2 and plays a role in sensing both fermentable carbon sources and nitrogen availability (Kübler *et al.*, 1997; Xue *et al.*, 1998; Kraakman *et al.*, 1999; Lorenz *et al.*, 2000a, 2000b). Recent studies have revealed a third pathway involving a transcription factor cascade comprised of Sok2, Phd1, Ash1, and Swi5 that controls cell–cell adhesion and separation (Pan and Heitman, 2000). Other proteins that do not appear to function in any of these pathways also regulate filamentous growth, including the Mep2 ammonium permease, a glutamine tRNA, and nitrogen catabolism regulators including Gln3, Ure2, Dal80, Npr1, and Npi1 (Gimeno and Fink, 1994; Lorenz and Heitman, 1998a, 1998b; Murray *et al.*, 1998). Previous studies revealed that Tor activity represses expression of many of the nitrogen utilization and regulatory genes (Beck and Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999). Our epistasis analysis reported here reveals that Tor acts in parallel with the MAP kinase, cAMP pathways, and Sok2–Phd1 pathways to elicit pseudohyphal differentiation in response to nitrogen limitation. The nitrogen regulators Mep2, Ure2, and Gln3 likely function together with Tor in a distinct pathway that controls pseudohyphal growth.

Filamentous differentiation plays an important role in the life cycle of diverse fungi (reviewed in Madhani and Fink, 1998). We have found that the Tor signaling pathway acts in parallel with other signaling pathways to promote pseudohyphal growth in *S. cerevisiae*. We also found that the Tor pathway regulates filamentation in three other divergent fungi, indicating that the role of the Tor pathway in filamentation in response to nitrogen is widely conserved among fungi. Because filamentation has been shown to be

Table 2. Plasmids used in this study

Name	Genotype	Reference
pRS426	2μ *URA3*	Christianson *et al.* (1992)
CB2516	2μ *URA3 TAP42*	Di Como and Arndt (1996)
CY5755	*CEN LEU2 tap42-11*	Di Como and Arndt (1996)
p3.8.6	2μ *URA3 PHD1*	Lorenz and Heitman (1998)
p2.5.2	2μ *URA3 TEC1*	Lorenz and Heitman (1998)
pMW2	*CEN URA3 RAS2-Val19*	Ward *et al.* (1995)
pXP3	2μ *URA3 TPK2*	Pan and Heitman (1999)

important for pathogenesis in a number of human and plant fungal pathogens, rapamycin or its analogs may prove useful as potential antifungal agents via their ability to inhibit both filamentous and vegetative growth.

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