Fission yeast TORC1 regulates phosphorylation of ribosomal S6 proteins in response to nutrients and its activity is inhibited by rapamycin

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Accepted 24 November 2009
Journal of Cell Science 123, 777-786
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doi:10.1242/jcs.060319

Summary

Cellular activities are regulated by environmental stimuli through protein phosphorylation. Target of rapamycin (TOR), a serine/threonine kinase, plays pivotal roles in cell proliferation and cell growth in response to nutrient status. In Schizosaccharomyces pombe, TORC1, which contains Tor2, plays crucial roles in nutrient response. Here we find a nitrogen-regulated phosphoprotein, p27, in S. pombe using the phospho-Akt substrate antibody. Response of p27 phosphorylation to nitrogen availability is mediated by TORC1 and the TSC-Rhb1 signaling, but not by TORC2 or other nutrient stress-related pathways. Database and biochemical analyses indicate that p27 is identical to ribosomal protein S6 (Rps6). Ser235 and Ser236 in Rps6 are necessary for Rps6 phosphorylation by TORC1. These Rps6 phosphorylations are dispensable for cell viability. Rps6 phosphorylation by TORC1 also responds to availability of glucose and is inhibited by osmotic and oxidative stresses. Rapamycin inhibits the ability of TORC1 to phosphorylate Rps6, owing to interaction of the rapamycin-FKBP12 complex with the FRB domain in Tor2. Rapamycin also leads to a decrease in cell size in a TORC1-dependent manner. Our findings demonstrate that the nutrient-responsive and rapamycin-sensitive TORC1-S6 signaling exists in S. pombe, and that this pathway plays a role in cell size control.

Key words: Cell size, Nutrient response, Rapamycin, S6 phosphorylation, Signal transduction, TORC1

Introduction

Eukaryotic cells have evolutionarily conserved mechanisms by which protein-phosphorylation reactions mediate several cellular activities when cells perceive environmental stimuli such as growth factors, nutrients and stresses. Target of rapamycin (TOR), a serine/threonine kinase, plays central roles in controlling cell growth, cell-cycle progression and metabolism in response to environmental stimuli (reviewed by Chiang and Abraham, 2007; Wullschleger et al., 2006). In mammalian cells, TORC1 (mTORC1) contains mTOR, Raptor and mLST8, whereas mTORC2 consists of Rictor, mLST8 and mSin1 in addition to mTOR. The immunosuppressant and anticancer drug rapamycin forms an intracellular complex with immunophilin FKBP12, thereby inhibiting preferentially TORC1 activity. Rapamycin-sensitive TORC1 signaling regulates temporal aspects of cell growth – protein synthesis, metabolism, transcription and autophagy – whereas rapamycin-insensitive TORC2 regulates spatial aspects of cell growth, such as organization of actin cytoskeleton (Wullschleger et al., 2006). mTORC1 is positively regulated by Rheb, a member of the Ras superfamily GTP-binding protein, and is negatively regulated by a tumor-suppressor protein complex consisting of tuberous sclerosis complex (TSC)1 and TSC2, which acts as a GTPase activating protein for Rheb. The TSC-Rheb-mTORC1 pathway regulates phosphorylation of ribosomal S6 kinases (S6K), members of the AGC (protein kinase A/protein kinase G/protein kinase C) kinase family and eIF4E-binding protein (4E-BP1) to promote protein synthesis (reviewed by Huang and Manning, 2008). Phosphorylation of S6K by mTORC1 leads to activation of S6K, which subsequently phosphorylates ribosomal protein S6, whereas phosphorylation of 4E-BP1 results in dissociation of 4E-BP1 from the initiation factor eIF4E, and it results in relieving the inhibition of the initiation factor (reviewed by Hay and Sonenberg, 2004). mTORC2 phosphorylates Akt/PKB, another member of the AGC kinase family, to activate its catalytic activity (Sarbassov et al., 2005).

Recently, the fission yeast Schizosaccharomyces pombe has emerged as an ideal system to study functions of TOR (reviewed by Aspuria et al., 2007; Otsubo and Yamamoto, 2008). Fission yeast has two TOR genes, Tor1+ and Tor2+. TORC1 contains Tor2, a Raptor homolog (Mip1) and an mLST8 homolog (Wat1; also known as Pop3), whereas TORC2 consists of Tor1, a Rictor homolog (Ste20), Sin1 and Wat1 (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007). Tor2 is a pivotal controller of the switch between cell cycle and cell differentiation by sensing nitrogen availability. Tor1 participates in leucine uptake, cell proliferation under stress conditions and sexual differentiation under nitrogen starvation (Alvarez and Moreno, 2006; Hayashi et al., 2007; Ikeda et al., 2008; Kawai et al., 2001; Matsuo et al., 2003; Matsuo et al., 2007; Uritani et al., 2006; Weisman and Choder, 2001; Weisman et al., 2005; Weisman et al., 2007). In addition to the TOR complexes, fission yeast has homologous genes of mammalian TSC1 and TSC2 (tsc1+ and tsc2+, respectively), and a Rheb homolog (rhhb1+). The products of these genes are involved in vegetative growth, amino acid uptake and nitrogen starvation response (Aspuria and Tamao, 2008; Mach et al., 2000; Matsumoto et al., 2002; Nakase et al., 2006; Tabancay et al., 2003; Urano et al., 2005; van
Slegtenhorst et al., 2004; Yang et al., 2001). Rhb1 interacts with Tor2 (Uran0 et al., 2005; Uritani et al., 2006).

Several genetic analyses between TSC-Rhb1 and TORC1 have shown that the TSC-Rhb1 pathway regulates TORC1 activity (Matsuo et al., 2007; Uran0 et al., 2007; Uritani et al., 2006; Weisman et al., 2007). By contrast, no downstream effectors of TORC1 have been identified. Here we screened for nitrogen-regulated phosphoproteins in fission yeast using the anti-phospho-Akt substrate (PAS) antibody and found four phosphoproteins. One of them, p27, was further characterized, and our findings showed that p27 phosphorylation is regulated by the TORC1 signaling in response to nutrient conditions and several stresses. p27 was identified as a ribosomal protein S6 (Rps6) by bioinformatic and biochemical analyses. In addition, we demonstrated that rapamycin inhibits phosphorylation of Rps6 by TORC1 and reduces cell size in a Tor2-dependent manner.

**Results**

**Phosphorylation of p27 recognized by anti-PAS antibody responds to nitrogen availability**

The phosphorylation-site-specific antibody anti-PAS was originally designed against the Akt consensus phosphorylation motif Arg-Xaa-Arg-Xaa-Xaa-[Ser(P)/Thr(P)], where Xaa represents any amino acid, and Ser(P) or Thr(P) is phosphorylated serine or threonine, respectively. The anti-PAS antibody has been used to screen for growth-factor-regulated and nutrient-regulated phosphoproteins, which include not only the actual substrates of Akt but also substrates of S6K and AMP-activated protein kinase in mammalian cells (Eguchi et al., 2009; Kane et al., 2002; Manning et al., 2002; Miyamoto et al., 2008). To examine nitrogen-regulated phosphoproteins by using the anti-PAS antibody, fission yeast cells were cultured in medium in the presence or absence of ammonium as the sole nitrogen source. The cell extracts were subjected to SDS-PAGE and immunoblotting with the anti-PAS antibody (Fig. 1A).

An intense band of approximate 27-kDa protein, which is designated as p27, was detected when the cells were incubated in a nitrogen-rich medium, but the p27 protein was not detected 15 minutes after the shift to nitrogen-starvation conditions. Furthermore, other distinct proteins designated as p150, p90 and p70 were observed in nitrogen-rich conditions, and the intensity of these bands was significantly decreased 30 minutes after the shift to nitrogen starvation (Fig. 1A). It has been known that nutrient limitation induces the phosphorylation of the α-subunit of eukaryotic initiation factor 2α (eIF2α) at Ser52 in a Gcn2-dependent manner (Zhan et al., 2004). Similarly, nitrogen depletion led to the induction of phosphorylation of Ser52 in eIF2α (Fig. 1A).

In this study, we focused on the characterization of p27. The band of p27 was not detected following phostasate treatment, suggesting that the band of p27 recognized by the anti-PAS antibody under nitrogen-rich conditions actually results from the phosphorylation of p27 (Fig. 1C). Fig. 1B shows that re-addition of ammonium to cells starved for nitrogen source rapidly induced p27 phosphorylation, with the maximal level reached by 20 minutes (Fig. 1B). Taken together, these results suggest that p27 phosphorylation recognized by the anti-PAS antibody is regulated in response to nitrogen availability.

**TORC1, but not the SAPK, CAMP-PKA or TORC2 pathways, regulates phosphorylation of p27 in response to nitrogen availability**

Recent studies in fission yeast have demonstrated that Tor2 and Tor1, which constitute TORC1 and TORC2, respectively, have been implicated in cellular responses to nitrogen availability (Aspuria et al., 2007; Otsubo and Yamamoto, 2008). Thus, we investigated whether the function of Tor2 and/or Tor1 is involved in the regulation of p27 phosphorylation in response to nitrogen status. To address this issue, we first utilized two mutants, tor2<sup>L1310P</sup> and tor2<sup>E2221K</sup>, which carry activating mutations in the Tor2 locus. These two mutants confer Rhb1-independent cell proliferation in nutrient-rich conditions and show delayed G1 arrest and decreased sporulation efficiency under nutrient starvation (Uran0 et al., 2007). In both active mutants, phosphorylation of p27 was maintained at a high level even under nitrogen starvation compared with that in the wild-type cells (Fig. 2A). Indeed, activity of the Tor2<sup>E2221K</sup> mutant was higher than that of the wild-type Tor2 in the in vitro kinase assay using 4E-BP1 as a substrate (supplementary material Fig. S1). Phosphorylation of p27 in the tor2<sup>L1310P</sup> cells was still observed by 30 minutes after the shift to nitrogen depletion, but diminished by 60 minutes (Fig. 2B). By contrast, phosphorylation of eIF2α at Ser52 was increased in the wild-type cells and the active mutants under nitrogen starvation (Fig. 2A), suggesting that the modulation of the Gen2-eIF2α pathway in response to nitrogen availability is independent of Tor2 activity. We next examined p27 phosphorylation in two distinct temperature-sensitive tor2 mutants: tor2-ts6 and tor2-ts10. These mutants arrest cell cycle in G1 phase and initiate sexual development, mimicking nitrogen starvation at the non-permissive temperature even in nitrogen-rich conditions (Matsuo et al., 2007). In both tor2-ts mutants, increase in p27 phosphorylation by the re-addition of ammonium after nitrogen starvation was markedly suppressed at the non-permissive temperature (34°C) compared with
TORC1 regulates S6 phosphorylation

In mammalian cells, loss of either of the TSC genes or overexpression of Rheb suppresses, at least in part, downregulation of the mTORC1 activity under nutrient starvation (Gao et al., 2002; Garami et al., 2003; Inoki et al., 2002; Tee et al., 2003). Several lines of evidence from genetic studies in fission yeast have demonstrated that the TSC1-TSC2 complex negatively regulates TORC1, and conversely, Rhl1, a Rheb homolog, activates TORC1 activity (Aspuria et al., 2007; Otsubo and Yamamoto, 2008). Thus, the strains carrying the leucine auxotrophy used in this study were transformed with a plasmid having a leucine marker. As shown in Fig. 2D, the response of p27 phosphorylation in the tor1Δ or ste20Δ cells to nitrogen availability was comparable to that in the wild-type strain. These results suggest that TORC2 is not implicated in the regulation of p27 phosphorylation.

The stress-activated protein kinases (SAPKs) and cyclic AMP (cAMP)-dependent protein kinase (PKA) pathways are also involved in cellular responses to nitrogen starvation. Disruption of phh1Δ, which is also known as stylΔ and spc1Δ and encodes a SAPK, interferes with cell-cycle arrest in G1 and sexual differentiation in response to nitrogen starvation (Kato et al., 1996; Shiozaki and Russell, 1996; Wilkinson et al., 1996). However, null mutation of cgs1Δ, which encodes the regulatory subunit of PKA, leads to constitutive activation of PKA and prevents sexual differentiation in response to nitrogen starvation (Kunitomo et al., 2000). We therefore examined the role of the SAPKs and PKA pathways in the regulation of p27 phosphorylation. Similar to the wild type, phosphorylation of p27 in phh1Δ and cgs1Δ cells was decreased in response to nitrogen depletion (Fig. 2E). These results suggest that downregulation of p27 phosphorylation after the shift to nitrogen starvation is independent of the SAPKs and PKA pathways.

The TSC1-TSC2 complex and Rhl1 are involved in the regulation of p27 phosphorylation

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nitrogen starvation. Mutation of Ser16 to His in human Rheb causes a hyperactivation of the mTORC1 pathway (Yan et al., 2006). In fission yeast, overexpression of wild-type rhb1 and rhb1S16H did not activate TORC1 activity in p27 phosphorylation (supplementary material Fig. S3). There seems to be a difference in the strength of TORC1 activity in p27 phosphorylation (supplementary material Fig. S4) (Ruvinsky and Meyuhas, 2006). In addition, Ser235 and Ser236 in human rpS6 are highly conserved in fission yeast and budding yeast. Moreover, residues adjacent to S6K-phosphorylation sites Ser235 and Ser236 in human rpS6 are highly conserved in fission yeast and budding yeast rpS6 (supplementary material Fig. S4) (Ruvinsky and Meyuhas, 2006). In addition, Ser235 in both of the fission yeast RpS6 proteins is consistent with the phosphorylated serine residue within the consensus phosphorylation motif recognized by the anti-PAS antibody.

Identification of ribosomal protein S6 as a TORC1-regulated phosphoprotein, p27

The anti-PAS antibody used in this study mainly recognizes the consensus phosphorylation motif, [Arg/Lys]-Xaa-[Arg/Lys]-Xaa-Xaa-[Ser(P)/Thr(P)]. We next used the Scansite program (http://scansite.mit.edu) (Yaffe et al., 2001) to identify candidates for p27, which are predicted to have the sites [Arg/Lys]-Xaa-[Arg/Lys]-Xaa-Xaa-[Ser(P)/Thr(P)]. The most likely candidates found in the S. pombe SWISS-PROT database in the 26-28-kDa molecular-weight range are listed in Table 1. Because p27 phosphorylation was regulated by the TORC1 pathway in response to nitrogen availability as described above, we focused on two ribosomal protein (rp) S6s, RpS601 and RpS602, among all the candidates. In the mammalian system, mTORC1 regulates rpS6 phosphorylation by activation of S6K. S6K can phosphorylate the Akt consensus phosphorylation motif (reviewed by Ruvinsky and Meyuhas, 2006). In fact, the anti-PAS antibody recognizes the mTORC1-S6K-dependent phosphorylation of S6 (Kane et al., 2002; Manning et al., 2002). Both RpS6 proteins in fission yeast show high homology with those in human and budding yeast. Moreover, residues adjacent to S6K-phosphorylation sites Ser235 and Ser236 in human rpS6 are highly conserved in fission yeast and budding yeast RpS6 (supplementary material Fig. S4) (Ruvinsky and Meyuhas, 2006). In addition, Ser235 in both of the fission yeast RpS6 proteins is consistent with the phosphorylated serine residue within the consensus phosphorylation motif recognized by the anti-PAS antibody.

To determine whether p27 is identical to RpS6, we first constructed null mutants of either rpS601 or rpS602 and assessed the phosphorylation level of p27 in those mutants. We were unable to obtain a double disruptant of rpS601 and rpS602 in approximately $8 \times 10^5$ spores from heterozygous diploid cells, thus the double disruption is probably lethal (data not shown). Level of p27

Table 1. Candidates for p27

| Gene identifier | Gene symbol | Predicted mass (kDa) | Description |
|-----------------|-------------|----------------------|--------------|
| SPAC13D6.05     | alp11       | 26.6                 | Cell-polarity protein |
| SPAC27D7.12c    | but1        | 27.4                 | Uba3-binding protein |
| SPAC4G9.11c     | cnb1        | 26.3                 | Mismatch-binding protein |
| SPAC31G5.16c    | dpm1        | 26.6                 | Dolichol-phosphate mannosyltransferase catalytic subunit |
| SPAC17A5.13     |             | 26.4                 | GTP cyclohydrolase I |
| SPAPB24D3.04c   | mag1        | 26.3                 | DNA-3-methyladenine glycosylase 1 |
| SPAP10F6.12c    | man4        | 26.5                 | Protein-S-isoprenylcysteine O-methyltransferase |
| SPBC11G11.03    |             | 26.6                 | mRNA turnover protein 4 homolog |
| SPAC1F7.13c     | rpl801      | 27.1                 | 60S ribosomal protein L2 |
| SPAC3A12.04c    |             | 26.7                 | Probable ribonuclease P protein subunit 3 |
| SPAC3G9.10c     | ski6        | 26.8                 | Putative exosome complex exonuclease RRP41 |
| SPBC16G5.14c    | rps3        | 27.5                 | 40S ribosomal protein S3 |
| SPAC13G6.07c    | rps601      | 27.4                 | 40S ribosomal protein S6-A |
| SPAPB1E7.12c    | rps602      | 27.4                 | 40S ribosomal protein S6-B |
| SPAC13G6.05c    |             | 27.5                 | Hypothetical protein |
| SPAC22G7.03     |             | 26.8                 | Hypothetical protein |
| SPAC15F9.01c    |             | 26.2                 | Hypothetical protein |
| SPAC1D4.09c     |             | 27.0                 | Hypothetical protein |
| SPBC582.09      | pec11       | 26.4                 | Peroxisomal biogenesis factor 11 |
| SPBC651.04      |             | 27.2                 | Hypothetical protein |
| SPAC15A10.05c   | mag182      | 26.4                 | YjE family protein |
| SPAC4C5.01      |             | 27.3                 | Halocacid dehalogenase-like hydrolase |
| SPAC17H9.03c    | rdi1        | 26.0                 | RAD51D-like protein |
| SPAC23H3.12c    |             | 26.1                 | Hypothetical protein |
| SPAC22H12.01c   | mag35       | 27.5                 | Hypothetical protein |
| SPBP23A10.12    |             | 27.6                 | FRG1 family protein |
| SPBCX39.14c     |             | 27.0                 | Methyltransferase (predicted) |
| SPBC1921.07c    | ssg29       | 27.5                 | SAGA complex subunit (predicted) |
| SPCC297.06c     |             | 27.6                 | Hypothetical protein |
phosphorylation in the \textit{rps602\Delta} mutant cells was significantly decreased compared with that in the wild-type cells in the presence of ammonium (Fig. 4A) and, in the \textit{rps601\Delta} cells, the phosphorylation was slightly decreased. We next replaced chromosomal \textit{rps602} with a \textit{myc-rps602} allele, which expresses an N-terminal tagged myc-Rps602 under the control of the endogenous \textit{rps602} promoter, in an \textit{rps601\Delta} background. The \textit{rps601\Delta myc-rps602} cells grew similarly to the \textit{rps601\Delta} cells; therefore, the myc epitope did not interfere with the function of the ribosomal protein. In the \textit{rps601\Delta myc-rps602} cells, only phosphorylation of myc-Rps602, of which the molecular weight is increased by the addition of the myc tag compared with that of p27, was observed under nitrogen-rich conditions (Fig. 4B). This suggests that phosphorylation of p27 is substituted by that of myc-Rps602 in \textit{rps601\Delta myc-rps602}. Nitrogen depletion abolished the phosphorylation of the tagged protein (Fig. 4B). In Fig. 4C, myc-Rps601 or -Rps602 was expressed from the \textit{nmt1} promoter in pREP1 vector and immunoprecipitated with anti-myc antibody. The immunoprecipitated myc-Rps601 and -Rps602 were phosphorylated, whereas phosphorylation of the immunoprecipitates from nitrogen-starved cells was not observed. Taken together, p27 is identical to the two Rps6 proteins, and phosphorylation of Rps6 is modulated by the TORC1 pathway in response to nitrogen availability.

In previous reports, expression of \textit{rps6} mRNA is greatly reduced by nitrogen depletion (Bonnet et al., 2000; Nakashima et al., 2002). We examined the effects of nitrogen deprivation on protein level of Rps602 in the \textit{rps601\Delta myc-rps602} strain. As shown in Fig. 4D (left), the protein level of myc-Rps602, which was expressed under the control of its own promoter as mentioned above, did not change for at least 90 minutes under nitrogen depletion, although phosphorylation of myc-Rps602 was abolished. These findings suggest that the decrease in Rps6 phosphorylation under nitrogen starvation is not caused by a decrease in the amount of protein.

### Ser235 and Ser236 of Rps6 are crucial for its phosphorylation

To investigate whether the conserved Ser235 and Ser236 residues in Rps6 are important for the phosphorylation of Rps6, we constructed a series of mutations in both \textit{rps6} genes, in which either or both the serine residues were replaced by alanine. These mutants tagged with the myc epitope at their N-termini were expressed under the control of the \textit{nmt1} promoter in pREP1 in an \textit{rps602} background. Because the \textit{rps601} gene exists, the band of endogenous Rps601 phosphorylation was observed below that of myc-Rps6 in immunoblotting with the anti-PAS antibody (top panels in Fig. 5A,B). Unlike wild type, both myc-Rps601\textit{SSAA} and -Rps602\textit{SSAA} failed to be phosphorylated even in the presence of ammonium (Fig. 5A). Furthermore, as shown in Fig. 5B, phosphorylation of myc-Rps601\textit{SSAA} and -Rps602\textit{SSAA} under nitrogen-rich conditions was markedly diminished, and the phosphorylation of myc-Rps601\textit{SSAA} and -Rps602\textit{SSAA} was hardly detected. These results suggest that both Ser235 and Ser236 in Rps6 play a key role in phosphorylation of this ribosomal protein in nitrogen-rich conditions and that the TORC1 pathway would regulate phosphorylation of Rps6 at Ser235 and Ser236. To further examine the biological role of phosphorylations of Ser235 and Ser236 in Rps6, we replaced both the serine residues of the chromosomal \textit{rps602} gene by alanine in an \textit{rps601\Delta} background. Whereas the
Characterizations of regulation of Rps6 phosphorylation

The above studies establish that the phosphorylation of Rps6 can be used to monitor effects of a variety of conditions on TORC1 signaling. We have examined a number of conditions that might influence TORC1. In mammalian cells, glucose depletion, which induces low energy status in the cells, causes downregulation of mTOR activity through the activation of AMPK, an energy sensor (reviewed by Kimball, 2006). We first tested the effect of glucose starvation on TORC1 activity. Glucose starvation (0.1% glucose and 3% glycerol as carbon sources) quickly reduced phosphorylation of Rps6 in the wild-type cells similar to what was observed following nitrogen starvation, whereas downregulation of the protein phosphorylation by glucose starvation was partially attenuated by a tor2 activating mutation (tor2L1310P) (Fig. 6A). However, this resistance to glucose depletion in tor2L1310 was somewhat weak compared with that after nitrogen depletion in the tor2 active mutant. The amount of myc-Rps602 in rps601A myc-rps602 cells was not altered during glucose depletion (data not shown). Taken together, TORC1 activity is regulated by both nitrogen and glucose availability.

In mammalian cells and budding yeast, TORC1 activity is inhibited by several kinds of stress, including oxidative and osmotic stresses, as well as nitrogen starvation induces phosphorylation of Ser52 in eIF2α (Cherkesova and Hinnebusch, 2003; Kabota et al., 2003). In fission yeast, phosphorylation of Ser52 in eIF2α exhibited no increase during the rapamycin treatment (Fig. 7A) (Petersen and Nurse, 2007), suggesting that rapamycin does not affect eIF2α phosphorylation.

To further determine whether the decline in Rps6 phosphorylation by rapamycin is due to inhibition of Tor2 activity by the drug, we employed two distinct mutants, fkh1Δ, which is a null mutant of FKBP12, and tor2S1837E, in which chromosomal wild-type tor2 was replaced with the tor2S1837E mutation. The latter mutant has a mutation of the conserved serine residue in the FRB domain; this residue is crucial for the binding between the FRB domain in TOR and the FKBP12-rapamycin complex in other eukaryotes (Chen et al., 1995; Hellwigel et al., 1994; Lorenz and Heitman, 1995; Stan et al., 1994). In fission yeast, a mutation of Ser1834 to Glu in Tor1 shift to high osmotic medium (Fig. 6C). When rps601A myc-rps602 cells were subjected to either oxidative or osmotic stress, the amount of the chromosomally expressed myc-Rps602 was unaltered (data not shown). These results suggest that TORC1 activity is inhibited by oxidative and osmotic stresses, although the inhibition of TORC1 activity by these stresses is slower than the inhibition by nutrient starvation.

Rapamycin inhibits Rps6 phosphorylation in an Fkh1-Tor2-dependent manner

In mammalian cells and budding yeast, rapamycin inhibits TORC1 activity when the drug-FKBP12 complex interacts with the FRB domain in TOR (Chen et al., 1995; Lorenz and Heitman, 1995). Examination of Rps6 phosphorylation provides a convenient assay to examine whether rapamycin inhibits TORC1 function in fission yeast. As shown in Fig. 7A, phosphorylation of Rps6 was markedly diminished by 30 minutes after the addition of rapamycin and was not detected by 60 minutes. Fig. 4D shows that the phosphorylation of myc-Rps602 in rps601A myc-rps602 was abolished by 60 minutes after the addition of rapamycin. The amount of the tagged protein was unaffected for at least 90 minutes after the addition of the drug. We also found that 200 nM rapamycin is required to abolish Rps6 phosphorylation (Fig. 7B). In budding yeast, rapamycin as well as nitrogen starvation induces phosphorylation of Ser52 in eIF2α (Cherkasova and Hinnebusch, 2003; Kabota et al., 2003). In fission yeast, phosphorylation of Ser52 in eIF2α exhibited no increase during the rapamycin treatment (Fig. 7A) (Petersen and Nurse, 2007), suggesting that rapamycin does not affect eIF2α phosphorylation.
results in loss of interaction with the Fkh1-rapamycin complex and suppression of the decreased leucine uptake by rapamycin (Weisman et al., 2005). In the case of tor2\(^{\text{S1837E}}\), the tor2\(^{\text{S1837E}}\) mutation is predicted to confer rapamycin resistance by preventing interaction with the Fkh1-rapamycin complex. Indeed, phosphorylation of Rps6 in the tor2\(^{\text{S1837E}}\) mutant was shown to be completely resistant to rapamycin (Fig. 7C). Similarly, phosphorylation of Rps6 in fkh1\(^{\Delta}\) was not impaired by rapamycin (Fig. 7C). By contrast, Rps6 phosphorylation in the tor2\(^{\text{S1837E}}\) mutant as well as in fkh1\(^{\Delta}\) was abolished under nitrogen-depletion conditions (supplementary material Fig. S5A; data not shown). In addition, Rps6 phosphorylation in the tor2\(^{\text{L1310P}}\) active mutant was sensitive to rapamycin (supplementary material Fig. S5B), similar to the inhibition of the mTOR active mutants by rapamycin in mammalian cells (Uran et al., 2007). These biochemical results suggest that rapamycin inhibits TORC1 activity to regulate Rps6 phosphorylation owing to an interaction of the Fkh1-rapamycin complex with the FRB domain of Tor2.

**Rapamycin decreases cell size through Tor2 inhibition**

Loss of Tor2 function causes growth defect because of the cell-cycle arrest in G1 phase and promotion of sexual development (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007; Uritani et al., 2006; Weisman et al., 2007). Although rapamycin inhibits TORC1 activity to regulate Rps6 phosphorylation as demonstrated in this study, rapamycin did not interfere with cell proliferation of the wild-type cells as previously reported (data not shown) (Weisman et al., 1997). To further investigate the effect of rapamycin on cell cycle and cell size, we performed flow cytometric analysis to measure DNA content and forward scatter analysis to measure cell size of wild-type and tor2\(^{\text{S1837E}}\) cells after 14 hours incubation in medium with or without rapamycin. Interestingly, the cell size of the wild-type cells was significantly reduced by rapamycin, whereas the reduction by the drug was not observed in tor2\(^{\text{S1837E}}\) (Fig. 8, right panels and bar graph), suggesting that rapamycin decreases cell size via TORC1 inhibition in fission yeast as well as the effect in mammalian cells (Fingar et al., 2002; Kim et al., 2002). Rapamycin did not affect cell cycle in both wild-type or tor2\(^{\text{S1837E}}\) cells (Fig. 8, left panels). By contrast, nitrogen starvation for 14 hours led to accumulation of G1-phase cells (denoted as 1C; Fig. 8, bottom in left panels). Taken together, rapamycin inhibits a subset of the TORC1 functions that are involved in regulation of S6 phosphorylation and cell size but not in cell-cycle progression or cell proliferation.

**Discussion**

In mammalian cells, the TSC-Rheb-mTORC1 signaling pathway regulates phosphorylation of S6 through activation of S6K in response to several stimuli such as growth factors, nutrients and energy status (Hay and Sonenberg, 2004; Huang and Manning, 2008). We demonstrated here that a similar signal-transduction system is conserved in fission yeast. TORC1 regulates phosphorylation of Rps6 (p27) by sensing nitrogen availability. Phosphorylation of Rps6 in the tor2 active mutants was maintained at a high level even after the shift to nitrogen depletion. Analogous activating mutations of mTOR confer hyperphosphorylation of S6 when overexpressed in human embryonic kidney (HEK)293 cells starved for nutrients (Uran et al., 2007). Conversely, inactivation of Tor2 in the tor2-ts mutants at non-permissive temperature decreased Rps6 phosphorylation in nitrogen-rich conditions. We further showed that TSC-Rhb1 signaling is involved in the TORC1-Rps6 signaling. Downregulation of the TORC1-dependent Rps6 phosphorylation under nitrogen starvation was significantly suppressed either by disruption of tsc2\(^{\text{tsz}}\), which would cause Rhb1 to be maintained as a GTP-bound form, or by expressing the rhb1 active mutants.

The Ser235 and Ser236 residues, which are directly phosphorylated by S6K, in S6 are conserved from yeasts to mammal (Ruvinsky and Meyuhas, 2006) (supplementary material Fig. S4). We further demonstrated that these serine residues in Rps6 would be the sites of phosphorylation in response to TORC1 activity. We also indicated that phosphorylation of both the serine residues is dispensable for cell viability in fission yeast. Similarly, in budding yeast, phosphorylation of the conserved serine residues is not necessary for cell survival and protein synthesis (Johnson and Warner, 1987). In the case of mouse, phosphorylation of S6 is also dispensable for cell viability, whereas phosphorylation of the ribosomal protein is involved in cell size and glucose homeostasis (Ruvinsky et al., 2005).

![Fig. 8. Rapamycin leads to reduction of cell size in wild-type but not tor2\(^{\text{S1837E}}\) cells.](image-url) Exponentially growing cells of 972 (WT) and AN0083 (tor2\(^{\text{S1837E}}\)) were diluted to OD\(_{600}\)=0.06 and then incubated in EMM with either DMSO (control) or 200 nM rapamycin at 30°C. After 14 hours incubation, the cells were stained with propidium iodide to analyze cellular DNA content and cell size (measured as forward scatter) by flow cytometry. The upper half shows a representative experiment. The green line overlaid on the profile (purple) of cell size from the rapamycin-treated cells indicates the profile outline for control DMSO-treated cells. Profile of DNA content from the wild-type cells that were starved for nitrogen source for 14 hours is shown at the bottom. The bar graph shows the average size as a mean FSC-H ± s.e.m. (n=4). **P<0.01.
Although both alleles of the tor2-ts mutants exhibited decreased Rps6 phosphorylation at a non-permissive temperature, there was a difference between the two mutants, tor2-ts6 and tor2-ts10, in the status of Rps6 phosphorylation at a permissive temperature. Whereas Rps6 phosphorylation in the tor2-ts6 mutants was abolished under nitrogen starvation, Rps6 phosphorylation in the tor2-ts10 mutant was maintained even under nitrogen-starvation conditions at a permissive temperature. This particular property of tor2-ts10 might be due to the fact that the mutant carries two mutations, A1399E in the FAT domain and F2198L in the kinase domain (Matsuo et al., 2007). Therefore, one or both of the mutations in tor2-ts10 might confer resistance to nitrogen starvation in addition to its temperature sensitivity. In fact, we have found that the tor2 activating mutations are clustered mainly within or close to the FAT and kinase domains, which are close to the mutation sites in tor2-ts10 (Urano et al., 2007).

Our demonstration of Rps6 phosphorylation raises the question: what gene encodes S6K? Similar to other eukaryotes, fission yeast has a number of potential AGC kinases, including Sck1, Sck2 and Gad8 (reviewed by Jacinto and Lorberg, 2008). Sck1 and Sck2 are predicted to be homologs of budding yeast SC19, which has recently been shown to have functional resemblance to S6K (Jacinto and Lorberg, 2008; Urban et al., 2007). In addition, we found by using the BLAST program that another candidate Psk1 has high homology with S6K1. As with other S6K candidates, Psk1 has a conserved hydrophobic motif in which a threonine residue of S6K is directly phosphorylated by mTORC1 in the mammalian system. The mobility shift of these S6K candidates was observed under nitrogen-rich conditions (supplementary material Fig. S6) and phosphatase treatment showed that the mobility shift of the S6K candidates was due to phosphorylation (data not shown). Therefore, phosphorylation of Sck1, Sck2 or Psk1 would be regulated in response to nitrogen availability. These results point to the presence of S6K in fission yeast. Gad8 is a homolog of Akt in mammal and of YPK1 and YPK2 in budding yeast, which are known substrates of TORC2 (Jacinto and Lorberg, 2008; Kamada et al., 2005; Sarbassov et al., 2005), suggesting that Gad8 functions downstream of TORC2. In fact, Gad8 is phosphorylated by TORC2 (Ikeda et al., 2008; Matsuo et al., 2003) and the mobility shift of Gad8 was not affected by nitrogen conditions (supplementary material Fig. S6). By contrast, Hartmuth and Petersen have recently raised the possibility that Gad8 also acts downstream of TORC1, which consists of Tor1 but not of Tor2 (Hartmuth and Petersen, 2009).

TORC1-Rps6 signaling is regulated by glucose status in addition to nitrogen status. In mammalian cells, energy stress caused by glucose depletion activates AMPK. The activated AMPK phosphorylates TSC2 and raptor, thereby inhibiting mTORC1 signaling (reviewed by Dunlop and Tee, 2009; Kimball, 2006). As with Tsc2 and a raptor homolog, Mip1, AMPK homologs exist as Ppk9 and Sp52 in fission yeast (Hanyu et al., 2009). These AMPKs might act as energy sensors and mediate the energy signaling to TORC1 via phosphorylation of Tsc2 and/or Mip1. Further investigation is needed to gain insights into the regulation of the TORC1-Rps6 signaling by glucose.

We have established that rapamycin inhibits the activity of TORC1 to regulate Rps6 phosphorylation. This inhibition occurred in a manner that the Fkh1-rapamycin complex binds to the FRB domain of Tor2. These results agree with several reports that suggest that rapamycin at least partially inhibits Tor2 functions in fission yeast. Namely, rapamycin prevents proliferation of the tor1Δ cells, and this is restored by disruption of fkh1Δ (Kawai et al., 2001; Weisman et al., 2005). A tor2 temperature-sensitive mutant, tor2-287 shows growth defect and cell-cycle arrest in G1 phase in the presence of rapamycin (Hayashi et al., 2007). Furthermore, we showed that TORC1 inhibition by rapamycin decreases cell size. Petersen and Nurse have shown that short-term treatment with rapamycin (up to 150 minutes) for synchronized cells promotes mitotic onset that causes the accumulation of small cells (Petersen and Nurse, 2007). In our case, prolonged treatment with rapamycin (14 hours) for asynchronous cells decreases cell size probably without promoting mitotic onset. This is consistent with the observation that rapamycin decreases the size of mammalian cells in an mTORC1-dependent manner (Fingar et al., 2002; Kim et al., 2002).

Unlike the effect of rapamycin on Rps6 phosphorylation and cell size, the drug does not affect cell-cycle progression and cell proliferation. These phenomena by rapamycin are different from those in budding yeast. By contrast, loss of Tor2 function due to temperature-sensitive mutations leads to growth inhibition in fission yeast (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007; Uritani et al., 2006; Weisman et al., 2007). Thus, the rapamycin effects are more limited than the effects of Tor2 inactivation. In addition, decrease in cell size by rapamycin is less dramatic than that caused by the loss of Tor2 function (data not shown). These observations suggest that rapamycin inhibits a subset of TORC1 functions and that rapamycin-resistant function of TORC1 exists. Recently, studies using ATP-competitive mTOR inhibitors in mammalian cells have shown the existence of rapamycin-resistant functions of mTORC1 in the control of cell proliferation, cell size etc. (Feldman et al., 2009; Thoren et al., 2009). Those cellular responses are mimicked cellular responses, including cell-cycle arrest in G1 phase and alteration of gene expression (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007; Uritani et al., 2006; Weisman et al., 2007). Those cellular responses are not affected by rapamycin (Matsuo et al., 2007; Weisman et al., 1997). Our discovery of S6 in fission yeast provides a new avenue to examine TORC1 activity. Importantly, inactivation of TORC1 occurred rapidly, within 15 minutes after the shift to starvation conditions, and occurred following rapamycin treatment, suggesting that our method enables characterization of early and direct alteration of TORC1 activity during nitrogen starvation. In the mammalian system, a large number of molecules that associate with mTORC1 signaling have been established by assessing function of the molecules in phosphorylation of the TORC1 downstream players such as S6K1, 4E-BP1 and S6. In the future, assessment of Rps6 phosphorylation will be useful in studying the molecules that associate with the TORC1 signaling in fission yeast.

Materials and Methods

Yeast strains, growth media and general methods

The fission yeast strains used in this study are listed in supplementary material Table S1. Cells were grown in yeast extract with supplements (YES) medium or Edinburgh minimal medium (EMM) supplemented with 200 mg/l adenine, when necessary, which contains 2% glucose as a carbon source and 0.5% ammonium chloride as a nitrogen source (Moreno et al., 1991). EMM-N, a nitrogen-free version, and EMM-C (3% glycerol and 0.1% glucose), a carbon-depletion version, were employed as starvation.
Antibodies and reagents
Anti-PAS and anti-phospho-eIF2α (Ser51) polyclonal antibodies were purchased from Cell Signaling Technology. Anti-FI2α (K24) and anti-α-tubulin (B5-1-2) antibodies were provided by Sigma. Anti-Myc (9E10) antibody was purchased from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody and protein-G–Sepharose were purchased from GE Healthcare Bio-Sciences. HRP-conjugated donkey anti-rabbit IgG antibody and HRP-conjugated protein A were purchased from Pierce and Invitrogen, respectively. Rapamycin was purchased from Calbiochem.

Gene disruption and construction of strains and the gene expression plasmids
Disruption of pgs11 or pgs2 mutations was performed using the direct homologous recombination method as described previously (Love et al., 1998; Sato et al., 2005). The entire open reading frames (ORFs) of pgs11 or pgs2 were substituted by the kanMX or hphMX cassette. The 3′XHA-hphMX or 13′myc-hphMX cassette was integrated into the C-terminus of SK1, SK2, ga20, or pki1 via the similar PCR-base method. To construct a myc-rps11 allele that expresses an N-terminal tagged myc-rps11 under the control of its own promoter, the myc-rps11DNA fragment (880 bp) including 470 bp of 5′ UTR region of pgs11 and one copy of the myc-epitope tag before the start codon of pgs11 were introduced by two-step PCR using genomic DNA as the template and cloned to generate the pUG6-myc-rps11 plasmid. The DNA fragment containing the myc-rps20NT and the kanMX cassette was inserted by homologous recombination after digesting the plasmid with a restriction enzyme (AgeI). To construct the pgs11 or pgs2, we first created a pREP1-myc vector in which the NTAP tag in pREP1-NTAP (Tasto et al., 2001) is replaced with one copy of the myc-epitope tag. The pREP1-myc vector was constructed by homologous recombination as described previously (Urano et al., 2007).

For construction of a series of the expression plasmids of pgs11 or pgs2, we first created a pREP1-myc vector in which the NTAP tag in pREP1-NTAP (Tasto et al., 2001) is replaced with one copy of the myc-epitope tag. The pREP1-myc vector was constructed by homologous recombination as described previously (Urano et al., 2007).

Protein preparation and immunoprecipitation
For detecting phosphorylated proteins, harvested cells were boiled for 5 minutes and disrupted with glass beads in buffer A [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.2% NP-40, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 10 mM p-nitrophenyl phosphate (p-NPP), 10 mM NaF, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Complete EDTA-free; Roche)]. The whole-cell extracts were added with 3X SDS sample buffer and then boiled for 5 minutes. The samples were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20, and then incubated at room temperature for 2 hours with antibodies that were diluted in 5% skim milk. After washing with TBS containing 0.1% Tween-20, the membranes were incubated with appropriate secondary antibodies (IRDye 800 or IRDye 680) for 1 hour. Finally, the membranes were scanned with an Odyssey infrared imaging system (Li-Cor Biosciences).
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