Psk1, an AGC kinase family member in fission yeast, is directly phosphorylated and controlled by TORC1 as S6 kinase

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Summary

Target of rapamycin (TOR), an evolutionally conserved serine/threonine protein kinase, plays pivotal roles in several important cellular processes in eukaryotes. In the fission yeast Schizosaccharomyces pombe, TOR complex 1 (TORC1) which includes Tor2 as a catalytic subunit manages the switch between cell proliferation and differentiation by sensing nutrient availability. However, little is known about the direct target of TORC1 that plays key roles in the nutrient-dependent TORC1 signaling in fission yeast. Here we report that in fission yeast, three AGC kinase family members named Psk1, Sck1, and Sck2, which exhibit high homology with human S6K1, are phosphorylated under nutrient rich conditions and are dephosphorylated by starvation conditions. Among these, Psk1 is necessary for phosphorylation of ribosomal protein S6. Furthermore, Psk1 phosphorylation is regulated by TORC1 in nutrient-dependent and rapamycin-sensitive manners in vivo. Three conserved regulatory motifs (the activation loop, the hydrophobic and the turn motifs) in Psk1 are phosphorylated and these modifications are required for Psk1 activity. Particularly, phosphorylation of the hydrophobic motif is catalyzed by TORC1 in vivo and in vitro. Ksg1, a homolog of PDK1, is also important for Psk1 phosphorylation in the activation loop and its activity. The TORC1 components, Pop3, Toc1, and Tco89, are dispensable for Psk1 regulation, but disruption of pop3Δ causes an increase in the sensitivity of TORC1 to rapamycin. Taken together, these results provide convincing evidence that the TORC1/Psk1/Rps6 constitutes a nutrient dependent signaling pathway in fission yeast.

Introduction

Eukaryotic cells operate numerous signal transduction events mediated by protein
phosphorylation to respond promptly to environmental changes. Target of rapamycin (TOR), a highly conserved serine/threonine protein kinase of the phosphatidylinositol kinase-related kinase family, plays pivotal roles in controlling various cellular processes such as cell growth, cell cycle, and protein synthesis in response to nutrient availability, growth factors, cellular energy status, and stress conditions. TOR is included as a catalytic subunit in two distinct multiprotein complexes, TOR complex 1 (TORC1) and TORC2. The TORC1 signaling promotes anabolic processes such as protein synthesis, transcription, and ribosome biogenesis, and prevents catabolic processes such as autophagy. On the other hand, TORC2 appears to be involved in actin organization and cell survival. An immunosuppressant and anticancer drug, rapamycin, preferentially inhibits TORC1 activity (Wullschleger et al., 2006; Sengupta et al., 2010).

The fission yeast Schizosaccharomyces pombe possesses two TOR genes, tor1+ and tor2+ that encode catalytic subunits of TORC2 and TORC1, respectively. TORC1, which consists of a raptor homolog Mip1, an mLST8 homolog Pop3 (also known as Wat1), Toc1, and Tco89 in addition to Tor2, plays a critical role in the switch between cell proliferation and differentiation by sensing nitrogen source availability. On the other hand, TORC2 that is composed of Tor1, a rictor homolog Ste20, Sin1, Pop3, and Bit61 participates in cell proliferation under stress conditions, entry into sexual differentiation under nitrogen starvation, as well as in leucine uptake (Alvarez and Moreno, 2006; Hayashi et al., 2007; 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). Rhb1, a fission yeast homolog of Rheb, which is a member of the Ras superfamily G-protein, and the complex of Tsc1 and Tsc2, counterpart of the mammalian TSC1-TSC2 complex that acts as a GTPase activating protein for Rheb, regulate TORC1 activity as upstream factors (Aspuria and
Tamanoi, 2008; Matsumoto et al., 2002; Nakase et al., 2006; Urano et al., 2005; van Slegtenhorst et al., 2004; Yang et al., 2001; Murai et al., 2009). It has recently been reported that the Rab-family G-protein, Ryh1, which is a homolog of human Rab6, is involved in the activity of TORC2 (Tatebe et al., 2010).

One of the important issues regarding the TOR signaling is to define direct targets of TOR complexes to gain understanding of how upstream signals can be transmitted through TOR complexes. Several serine/threonine protein kinases in the AGC (protein kinase A/protein kinase G/protein kinase C) kinase family, which include S6K, AKT (also known as PKB), SGK, and PKC in mammals and those homologs in other eukaryotes, are directly phosphorylated, and their activities are regulated by either TOR complex (Jacinto and Lorberg, 2008); in mammalian systems, S6K is phosphorylated and activated by mammalian TORC1 (mTORC1), whereas AKT, SGK, and PKC are modified by mTORC2 (Laplante and Sabatini, 2009). In fission yeast, TORC2 directly regulates the phosphorylation and function of Gad8, which is a member of the AGC kinase family (Matsuo et al. 2003; Ikeda et al. 2008). However, direct target of TORC1 has not been identified.

To gain insights into direct downstream targets of TORC1 in fission yeast, we have combined the study on AGC kinases in fission yeast with our previous study that identified Rps6 as a counterpart of ribosomal protein S6 (Nakashima et al., 2010). The latter study established that phosphorylation of Rps6 is mediated by TORC1 depending on nutrient availability such as ammonium and glucose and that the Tsc1-Tsc2 complex and Rhb1 participate in its phosphorylation (Nakashima et al., 2010). Fission yeast contains AGC kinases including Psk1, Sck1 and Sck2. There was a suggestion that Sck1 and Sck2 are counterparts of budding yeast Sch9, which has recently been characterized as S6 kinase in budding yeast (Jin et al., 1995; Fujita and Yamamoto, 1998; Urban et al., 2007). We show
here that Rps6 is a target of Psk1 based on the finding that Rps6 phosphorylation is regulated by Psk1 in a nutrient signal-dependent manner. We also show that Psk1 itself is directly phosphorylated, and its activity to phosphorylate Rps6 is regulated by TORC1 in nutrient-dependent and rapamycin-sensitive fashions. In addition, Ksg1, a kinase of PDK1 homolog, is also implicated in Psk1 phosphorylation and is critical for its activity. These results point to the significance of phosphorylation of Psk1 for nutrient-dependent signal transduction in fission yeast.

Results

Phosphorylation of S6K1 homologs in response to nitrogen source availability.

To identify the candidates for S6K that would directly phosphorylate ribosomal protein (Rp) S6 and that should be downstream of TORC1 in fission yeast, we first searched using the BLAST program for homologs of p70 S6K1, the predominant kinase of S6 in human. This analysis showed that fission yeast has at least four potential S6K1 homologs, namely Psk1, Sck1, Sck2, and Gad8, which belong to the AGC kinase family (Fig. 1A, Supplementary material Fig. S1) (Mukai et al., 1995; Jacinto and Lorberg, 2008). Phylogenetic analysis reveals that Psk1 exhibits the highest homology with S6K1, whereas Gad8 is similar to human AKT (Fig. 1A), which is a substrate of mTORC2 (Sarbassov et al., 2005). Indeed, some phosphorylation sites in Gad8 are catalyzed by TORC2 (Matsuo et al., 2003; Ikeda et al., 2008), suggesting that Gad8 is a counterpart of AKT in fission yeast.

We next examined whether these kinases are phosphorylated in response to ammonium as the sole nitrogen source in EMM medium, because we previously showed that TORC1 is activated in ammonium rich conditions and conversely inactivated in an ammonium-free medium (Nakashima et al., 2010). For this purpose, we constructed strains
in which the epitope-tagged S6K homologs (three tandem copies of influenza hemagglutinin (HA) or 13 tandem copies of myc at their carboxy-termini) are chromosomally expressed under the control of their native promoters and detected them by immunoblotting. Mobility shifts of epitope-tagged Sck1, Sck2, and Psk1 were observed after the readdition of ammonium, whereas the migration of Gad8-3HA was not altered in media with or without ammonium (Fig. 1B). Consistent with our previous results (Nakashima et al., 2010), Rps6 (fission yeast S6) is phosphorylated under ammonium stimulation (Supplementary material Fig. S2). The \( \lambda \)-phosphatase assay revealed that the mobility shift of the three kinases, Sck1, Sck2, and Psk1, was due to phosphorylation (Fig. 1C). These results suggest that Sck1, Sck2, and Psk1 are phosphorylated in nitrogen rich conditions and oppositely dephosphorylated at least in part under nitrogen starvation, whereas Gad8 is constitutively phosphorylated. Thus, Sck1, Sck2, and Psk1 appear to be candidates for S6K.

**Psk1 is necessary for phosphorylation at Ser235/236 in ribosomal protein S6.**

To determine a bona fide kinase of Rps6, we assessed Rps6 phosphorylation in deletion mutants of those S6K candidates using an anti-phospho-Akt substrate (PAS) antibody that can recognize phosphorylation at Ser235/236 in Rps6, which is regulated by the TORC1 signaling in response to nutrient conditions (Nakashima et al., 2010). The corresponding serine residues of S6 are directly phosphorylated by S6K in other eukaryotes (Ruvinsky and Meyuhas, 2006). As shown in Fig. 2A, the Rps6 phosphorylation under nitrogen rich conditions in \( sck1\Delta \) and in \( sck2\Delta \) was comparable to that in the wild type, and its phosphorylation in both the single mutants disappeared under nitrogen starvation as with that in the wild type. Furthermore, the double disruption of \( sck1^+ \) and \( sck2^+ \) and the deletion of \( gad8^+ \) had no significant effect on the nutrient-dependent Rps6 phosphorylation (data not
shown). In contrast, Rps6 phosphorylation in psk1Δ was not detected even in nitrogen rich conditions (Fig. 2A). Fission yeast has two distinct Rps6 gene products, Rps601 and Rps602, and their phosphorylations are regulated by TORC1 (Nakashima et al., 2010). In psk1Δ, expression of myc-tagged Rps602 under control of its own promoter was comparable to that in the wild type (Fig. 2B). These results suggest that the deletion of psk1Δ results in failure to phosphorylate Rps6. To further investigate whether Psk1 phosphorylates Rps6 protein in vitro, we examined the kinase assay of Psk1 using recombinant Rps602 as a substrate. In this experiment, we utilized Psk1 (Thr415Glu) mutant, a phospho-mimetic mutant of its hydrophobic motif, as the mutant exhibited higher activity than the wild type protein. As shown in Fig. 2C, the Psk1 protein phosphorylated Rps6 in vitro. On the other hand, Rsp6 mutant that has two potential serine phosphorylation sites changed to alanine was not phosphorylated by Psk1. In addition, a kinase-dead mutant (Lys120Ala) of Psk1 did not phosphorylated Rps6. Taken together, these results suggest that Psk1 is the primary kinase responsible for Rps6 phosphorylation in response to nutrient conditions. At this point, the possibility that a Psk1-associated kinase whose activity depends on Psk1 is involved in the phosphorylation is not excluded. In the following study, we focused on Psk1 to investigate its relationship to TORC1.

Phosphorylation and activity of Psk1 are regulated by TORC1 in nutrient-dependent and rapamycin-sensitive manners.

To determine whether TORC1 participates in the ammonium-dependent Psk1 phosphorylation, we constructed a series of strains in which Psk1-13myc is expressed chromosomally in the tor2 mutant background that includes the activating, the temperature sensitive (ts), or the rapamycin-resistant mutation. Phosphorylation status of Psk1 in those
tor2 mutants was examined as in Fig. 1B by assessing its mobility shift. As shown in Fig. 3A, Psk1 phosphorylation represented as the upper band in tor2Δ wild-type cells was decreased rapidly after the shift to nitrogen starvation, whereas in the tor2\textsuperscript{L1310P} active mutant (Urano et al., 2007), its phosphorylation was sustained at least for 60 minutes after the shift to the starvation. Conversely, downregulation of tor2 function using the two different tor2-ts mutations (ts6 and ts10) (Matsuo et al., 2007) at the non-permissive temperature (35 ºC) diminished induction of Psk1 phosphorylation by ammonium readdition following nitrogen starvation (Fig. 3B). Rapamycin prevents Rps6 phosphorylation via inhibition of TORC1 (Nakashima et al., 2010), raising the possibility that this macrolide inhibits Psk1 phosphorylation. As expected, Psk1 phosphorylation in the wild-type cells was decreased by 60 minutes after adding rapamycin to a level similar to that seen in nitrogen-starved cells, whereas phosphorylation of Psk1 was sustained at least for 60 minutes in the rapamycin-resistant tor2\textsuperscript{S1837E} mutant (Nakashima et al., 2010) (Fig. 3C). These results suggest that TORC1 regulates Psk1 phosphorylation in response to nitrogen source availability and that rapamycin prevents its phosphorylation by inhibiting TORC1.

The Tsc1-Tsc2 complex regulates Rps6 phosphorylation by negatively regulating TORC1. We further examined whether Psk1 acts as a downstream factor of the TSC1/2-TORC1 signaling pathway. Consistent with our previous results (Nakashima et al., 2010), Rps6 phosphorylation was maintained in both the tor2\textsuperscript{L1310P} active and the tsc2 null mutants even after the shift to nitrogen starvation, whereas deletion of psk1\textsuperscript{+} in those mutants abolished phosphorylation of the ribosomal protein in the presence and absence of ammonium (Supplementary material Fig. S3A). In contrast, the deletion of psk1\textsuperscript{+} did not suppress resistance of tsc2Δ cells to canavanine, a toxic arginine analog (van Slegtenhorst et al., 2004) (Supplementary Fig. S3B). These results suggest that Psk1 is at least one of the
Psk1 is phosphorylated by TORC1 in vitro.

To examine whether Psk1 is directly phosphorylated by TORC1, we performed an in vitro kinase assay using Tor2 (TORC1) as an enzyme and recombinant Psk1 as a substrate. As shown in Fig. 3D, immunopurified wild-type Tor2 (WT) strongly phosphorylated Psk1, suggesting that Psk1 is a direct substrate of TORC1. Meanwhile, only a slight radioactive Psk1 was seen in the immunopurified fraction of the Tor2 kinase-dead mutant (KD) (Fig. 3D). Because TORC1 is known to form homodimers in mammals and in budding yeast (Wullschleger et al., 2005; Takahara et al., 2006, Urano et al., 2007), exogenously expressed Tor2 kinase-dead mutant in wild-type cells probably forms a heterodimer with endogenous wild-type Tor2, thereby acquiring a weak activity to phosphorylate Psk1.

Deletion of pop3+ but not of toc1+ and tco89+, all of which are components of TORC1, increases the sensitivity of Psk1 regulation by TORC1 to rapamycin.

As described above, TORC1 consists of several proteins, such as Mip1, Pop3, Toc1, and Tco89, in addition to Tor2 (Alvarez and Moreno, 2006; Matsuo et al., 2007; Hayashi et al., 2007). Of these, Mip1 and Tor2 are essential for cell proliferation (Shinozaki-Yabana et al., 2000), implying that Mip1 plays a crucial role in TORC1 function similar to its counterparts, raptor and Kog1, in mammals and budding yeast, respectively. Pop3 is known to be included also in TORC2 and to be required for TORC2 activity to phosphorylate Gad8 (Alvarez and Moreno, 2006; Hayashi et al., 2007; Ikeda et al., 2008). However, the role of Pop3 as well as Toc1 and Tco89 in TORC1 function remains unclear. To examine whether these three TORC1 components play a role in phosphorylating TORC1 downstream factors,
we constructed deletion mutants of these genes. The *pop3* disruptant is known to be viable (Kemp et al., 1997; Ochotorena et al., 2001). The *toc1* and *tco89* genes were also dispensable for cell proliferation (data not shown). Levels of Psk1-13myc protein in the *pop3* disruptant were somewhat lower than those in the other strains, but no significant differences in the regulation of phosphorylations of Psk1 and Rps6 in response to ammonium conditions were observed in the gene disruptants of these TORC1 components compared with those in the wild type (Fig. 4A), suggesting that Pop3, Toc1, and Tco89 are dispensable for nutrient-dependent TORC1 activity at least for the modulation of Psk1 and Rps6 phosphorylation.

On the other hand, disruption of *pop3* + but not of *toc1* + and *tco89* + led to increased sensitivity of the phosphorylation of TORC1 downstream factors to rapamycin inhibition, compared with those in the wild type in both time-course and dose experiments (Fig. 4B, C). This may suggest that Pop3 influences interaction of Tor2 with the rapamycin-FKBP12 complex.

**Predicted phosphorylation sites in the conserved regulatory motifs in Psk1 are important for its kinase activity.**

In the case of mammalian S6K, its activation requires phosphorylations of serine or threonine residues in three important regulatory motifs, namely the activation loop (T-loop) in the kinase domain, the hydrophobic motif (HM) in the C-terminal tail region, and the turn motif (TM) located in the linker region close to the HM. Of these, phosphorylation in the T-loop in S6K is catalyzed by phosphoinositide-dependent protein kinase 1 (PDK1), whereas phosphorylations in the HM and the TM are mediated by mTORC1. In particular, the phosphorylated HM site is important for phosphorylation and activation of S6K by PDK1.
(Jacinto and Lorberg, 2008; Pearce et al., 2010). In fission yeast, these regulatory motifs are conserved in Psk1 as well as in the other AGC kinases described here (Fig. 5A, Supplementary material Fig. S1).

We therefore examined phosphorylation of these regulatory motifs and their role in Psk1 activity. To this end, we constructed a series of pskl mutants in which the serine or threonine residue corresponding to the predicted phosphorylation sites in the three regulatory motifs were substituted to alanine and checked phosphorylation levels and activities of these Psk1 mutants by examining mobility shifts and Rps6 phosphorylation, respectively. As shown in Fig. 5B, even under nitrogen rich conditions, mutations of Ser248Ala in the T-loop and Thr415Ala in the HM significantly decreased phosphorylation of Psk1. Furthermore, a mutation of Thr392Ala in the TM as well as double mutations of Thr392Ala and Thr415Ala (TTAA) dramatically decreased phosphorylation of the kinase, which was strikingly similar to that observed in nitrogen-starved cells. As for Psk1 activity, Rps6 phosphorylation in both the single T-loop (Ser248Ala) and the double TM (Thr392Ala)/HM (Thr415Ala) mutants was severely impaired to a level similar to that seen in the Psk1 kinase-dead (Lys120Ala) mutant. On the other hand, phosphorylation of Rps6 in both the single mutants of the TM (Thr392Ala) and the HM (Thr415Ala) was detectable but was substantially decreased compared to that in the wild type (the right panels in Fig. 5B). Conversely, a phospho-mimetic mutation of the HM (Thr415Glu) somewhat attenuated downregulation of Psk1 activity under nitrogen starvation, because Rps6 phosphorylation was detected under the starvation conditions (Fig. 5C). Taken together, these results suggest that, similar to S6K, Psk1 is phosphorylated in the three regulatory motifs depending on nitrogen source availability and that these phosphorylation events are important for its kinase activity.

We next examined whether TORC1 regulates phosphorylation of the HM (Thr415)
in Psk1, depending on nutrient conditions. We first found that a phosphospecific antibody against the phosphorylated HM (Thr389) in mammalian S6K, an anti-phospho-p70 S6K (Thr389) antibody, recognized phosphorylation of wild-type Psk1 immunopurified from protein extract of fission yeast cells harvested under nitrogen rich conditions, whereas this antibody did not detect non-phosphorylated HM mutant (Thr415Ala) immunopurified from cells grown under the same conditions (Supplementary material Fig. S4). These results suggest the utility of this phosphospecific antibody to monitor Thr415 phosphorylation in the HM in Psk1. Additionally, Thr415 phosphorylation in Psk1 disappeared under nitrogen starvation (Supplementary material Fig. S4), implying that Thr415 phosphorylation is regulated in response to nitrogen source availability. Using this antibody, we also found that downregulation of Thr415 phosphorylation under nitrogen starvation was markedly suppressed in the tor2^{L1310P} active mutant (Fig. 5D) and that rapamycin blocked Thr415 phosphorylation, whereas this inhibition was completely suppressed in the tor2^{S1837E} rapamycin-insensitive mutant (Fig. 5D). Furthermore, in vitro kinase assay of immunopurified Tor2 revealed that phosphorylation levels of recombinant Psk1 proteins mutated in the TM (T392A), the HM (T415A), as well as in both these residues (TTAA) were significantly less than those of the wild-type and the T-loop (S248A) mutant proteins (Fig. 5E). These findings suggest that phosphorylation of the HM (Thr415) and possibly also of the TM (Thr392) in Psk1 are regulated by TORC1 in nutrient-dependent and rapamycin-sensitive manners.

We further examined the effect of phosphorylations in other regulatory motifs on the modification in the HM in Psk1. As shown in Fig. 5F, Thr415 phosphorylation in Psk1 mutated in the TM (Thr392Ala) was markedly decreased even under nutrient rich medium, whereas its phosphorylation in the T-loop mutant (Ser248Ala) was comparable to that in the
wild type, suggesting that phosphorylation of the TM (Thr392) but not of the T-loop (Ser248), is important for the HM (Thr415) phosphorylation. We also noticed that mutation in the TM (Thr392Ala) resulted in loss of its mobility shift representing the lowest phosphorylation level as observed in nitrogen starvation (Fig. 5B). Therefore, the phosphorylation in the TM may also affect the modification of the T-loop in addition to that of the HM.

**Ksg1, a kinase homologous to PDK1, is required for phosphorylation and activity of Psk1.**

In fission yeast, Ksg1 is known as a homolog of PDK1 (Niederberger and Schweingruber, 1999). We therefore examined the role of Ksg1 in phosphorylation and activity of Psk1 using the temperature-sensitive (ts) mutants of two distinct ksg1 alleles (ts208 and ts358).

As shown in Fig. 6A, induction of Psk1 phosphorylation by readding ammonium following nitrogen starvation was markedly attenuated in both the ksg1-ts mutants not only at the non-permissive temperature (35 ºC) but also at the permissive temperature (25 ºC). Correspondingly, Psk1 activity to catalyze Rps6 phosphorylation after refeeding ammonium was abolished in both ksg1 mutants at both temperatures (Fig. 6A). We next examined whether Ksg1 phosphorylates Psk1 in vitro and found that GST-Ksg1 purified from bacteria was able to phosphorylate recombinant wild-type Psk1 protein as well as its mutant that harbors T/A mutation in both the TM and the HM (TTAA), but it could not phosphorylate the T-loop mutant (S248A) (Fig. 6B). These results suggest that Ksg1 is important for phosphorylation, particularly in the T-loop, and for activity of Psk1.

**The TORC1-dependent phosphorylation of Psk1 is regulated by glucose and glutamine as well as ammonium.**
We further examined the effect of other nutrients, such as glucose and amino acids, on the regulation of Psk1 phosphorylation and its activity, because we previously found that the TORC1-dependent Rps6 phosphorylation is downregulated by glucose starvation (Nakashima et al., 2010). Psk1 phosphorylation was substantially decreased when cells were grown in a medium containing either a low concentration of glucose (glucose starvation) or 2-deoxy-glucose (2-DG), which is a transportable, but non-metabolizable glucose analog that inhibits glucose metabolism (Fig. 7A), suggesting that the TORC1-dependent Psk1 regulation responds to glucose availability. It is of interest that the phosphorylations of Psk1 and Rps6 under glucose starvation, but not under nitrogen starvation, were increased again by 60 minutes (Fig. 7B).

It has been known that TORC1 activity to phosphorylate its substrates, such as S6K and Sch9 in mammalian cells and in budding yeast, respectively, is sustained when cycloheximide is added, which blocks protein synthesis, even under nutrient starvation (Iiboshi et al., 1999; Beugnet et al., 2003; Urban et al., 2007). As expected, cycloheximide significantly suppressed dephosphorylations of Psk1 and Rps6 under glucose or nitrogen starvation (Fig. 7B), suggesting that similar to other eukaryotes, TORC1 activity in fission yeast is also maintained by the addition of cycloheximide under nutrient starvation.

It has been well known that amino acids, especially leucine, in the cellular environment are important cues to regulate TORC1 activity (Avruch et al., 2009). We therefore examined the effect of short-term incubation with several amino acids on TORC1 activity by measuring phosphorylations of the TORC1 downstream factors. Cells were stimulated with either amino acid, glutamic acid, glutamine, proline, or leucine instead of ammonium for 15 minutes following nitrogen starvation (Fig. 7C). Glutamic acid and glutamine are used as good nitrogen sources, whereas proline is a poor one for cell
proliferation. Of these, stimulation with glutamine was sufficient to induce phosphorylations of both Psk1 and Rps6 to a level comparable to that seen with ammonium stimulation, while stimulation with the other amino acids showed little effect on those phosphorylations (Fig. 7C). L-methionine sulfoximine (MSX) is a glutamine synthetase inhibitor that provokes glutamine depletion in a cell resulting in a decrease in TORC1 activity in budding yeast (Crespo et al., 2002). Similar to the previous findings observed in budding yeast, the treatment of L-methionine sulfoximine led to dose- and time-dependent reductions in Psk1 phosphorylation in the medium containing ammonium, whereas addition of glutamine instead of ammonium suppressed these inhibitions (Fig. 7D). These results suggest that extracellular and intracellular glutamine effectively upregulates the TORC1 signaling.

**TORC1 catalyzes nutrient-dependent phosphorylation of Sck1 and Sck2.**

As shown in Fig. 1, phosphorylation levels of Sck1 and Sck2 were altered depending on nitrogen conditions. To investigate the possibility that Sck1 and Sck2 are downstream effectors of TORC1, we examined in vitro kinase assays of Tor2 using recombinant Sck1 and Sck2 proteins as substrates. Similar to Psk1 (Fig. 3D), Sck1 and Sck2 were also phosphorylated by TORC1 in vitro (Fig. 8A). The phosphorylation was much less when a kinase-dead mutant of Tor2 was used. Further support that TORC1 is involved in the phosphorylation of Sck1 is shown in Figure 8B. In this experiment, we examined Sck1 phosphorylation using *tor2* mutants. While the intensity of the top band decreases by starvation in the wild-type cells, this band remained under nitrogen depletion in the *tor2* active mutant (left panels). Phosphorylation of Sck1 was inhibited by rapamycin treatment in the wild type, whereas it showed a tolerance to the macrolide in the *tor2*
rapamycin-resistant mutant (Fig. 8B, right panels). The level of modification before rapamycin addition in the *tor2* mutant was lower than that in the wild type (right panels). Similar low activity of the rapamycin-resistant mutant was observed for Psk1 (Figs. 3C, 5D). A preliminary study with Sck2 also suggested that Sck2 phosphorylation is decreased in the wild-type cells after shifting to nitrogen starvation and that this decrease is partially suppressed in the *tor2* active mutant (unpublished results).

It has been known that *sck1*+ and *sck2*+ have a redundant function with *pka1*+, which encodes a catalytic subunit of cyclic AMP-dependent protein kinase (Jin et al., 1995; Fujita and Yamamoto, 1998). Therefore, we examined whether *psk1*+ has a redundant function with *pka1*+. Unlike overexpression of *sck1*+ or *sck2*+, exogenously expressed *psk1*+ from the thiamine-repressible *nmt81* promoter (Basi et al., 1993) failed to suppress slow growth (30°C) and cold temperature sensitivity (25°C) in *pka1Δ* on MM medium (Supplementary material Fig. S5). On SD medium where gene expressions in the plasmids were repressed, growth of *pka1Δ* cells carrying the plasmid encompassing the kinase gene was comparable to that of the cells having an empty vector (Supplementary material Fig. S5). Taken together, *psk1*+ has no redundant function with *pka1*+.

**Discussion**

In this study, we present evidence that an AGC kinase Psk1 functions as a downstream effector of TORC1 in fission yeast. This microbe has several AGC kinases homologous to human S6K1, such as Psk1, Sck1, Sck2, and Gad8. Of these, Psk1 is the closest S6K1 homolog and phosphorylated Rps6 in vivo and in vitro (Figs 1A, 2A, C). Furthermore, Psk1 itself was highly phosphorylated under nitrogen rich conditions, and its phosphorylation was at least partially catalyzed by TORC1 in vivo and in vitro (Figs. 3, 5). Rapamycin
inhibited Psk1 phosphorylation as well as Rps6 phosphorylation through blocking TORC1 activity (Fig. 3C). Taken together, Psk1 acts as S6K that mediates the nutrient-dependent TORC1 signaling to its substrate(s).

We further showed that in the TORC1 components, Toc1 and Tco89 as well as Pop3 are dispensable for cell proliferation and have little effects on the regulation of Psk1 by TORC1 at least in response to nutrient conditions (Fig. 4). In mouse studies as well as in studies using mouse embryonic fibroblasts lacking mLST8 (a homolog of Pop3), it has been shown that mLST8 is necessary for mTORC2 activity but not for that of mTORC1 (Guertin et al., 2006). Similar results were obtained in fission yeast. In contrast, there is no homolog of Tco89 in vertebrates. Unlike Tco89 in fission yeast that was dispensable for Psk1 phosphorylation, this homolog in budding yeast is required for TORC1 activity to phosphorylate Sch9, a counterpart of S6K (Binda et al., 2009). There are no Toc1 homologs in mammals and in budding yeast (Hayashi et al. 2007). Therefore, the function of each TORC1 component may have diverged during evolution.

Psk1 contains three regulatory motifs that are conserved among the AGC kinase families: the activation loop (T-loop), the hydrophobic motif (HM), and the turn motif (TM) (Supplementary material Fig. S1, Fig. 5A). Site-directed mutagenesis analyses in the regulatory motifs revealed that the conserved serine or threonine residue in these motifs was phosphorylated and that these phosphorylations are required for Psk1 activity to phosphorylate Rps6 (Fig. 5B). We also demonstrate by in vitro kinase assay of Tor2 and immunoblotting using an anti-phospho-S6K (Thr389) antibody that TORC1 catalyzes and regulates Thr415 phosphorylation in the HM of Psk1 in nitrogen-dependent and rapamycin-sensitive manners (Fig. 5D, E). Furthermore, TORC1 appears also to regulate phosphorylation in the TM, because the mutation of the TM (T392A) in the recombinant
Psk1 protein significantly decreased its phosphorylation by TORC1 in vitro (Fig. 5E). Additionally, phosphorylation status of the TM mutant in cells, which was detected by loss of its mobility shift, was very similar to that of the wild-type Psk1 under nitrogen starvation or treated with rapamycin where TORC1 is inactive (Fig. 5B). These modifications of Psk1 controlled by TORC1 are consistent with those in S6K in other eukaryotes (Jacinto and Lorberg, 2008).

We also found that Ksg1, a protein kinase homologous to PDK1, is engaged in the phosphorylation in the T-loop (Ser248) of Psk1 and is required for activity of Psk1 (Fig. 6), similar to that seen with the case of the T-loop phosphorylation in S6K by PDK1 in mammalian cells (Jacinto and Lorberg, 2008). We have previously demonstrated that phosphorylation and function of Gad8, which exhibits the highest homology with AKT, are regulated by TORC2 and Ksg1 (Matsuo et al., 2003). Taken together, like other eukaryotes, fission yeast possesses the signaling pathways involving two different AGC kinases that are separately regulated by the two TOR complexes and the PDK1 counterpart.

It has been known that phosphorylations of mammalian S6K occur in a stepwise manner; namely, phosphorylation of the TM in S6K is required for phosphorylation in the HM and then the modified HM participates in the phosphorylation of the T-loop by PDK1 (Jacinto and Lorberg, 2008; Pearce et al., 2010). In fission yeast, it seems likely that phosphorylations of the three regulatory motifs in Psk1 are also closely related to one another and arise in a stepwise manner; namely, mutation of Thr392Ala in the TM caused a marked decrease in Thr415 phosphorylation in the HM (Fig. 5F). In addition, phosphorylation in the T-loop in Psk1 may require the phosphorylated HM by TORC1, because phosphorylation status of the T-loop mutant (Ser248Ala) resembled that of the HM mutant (Thr415Ala) (Fig. 5B).
Similar to phosphorylation of Rps6 as previously described (Nakashima et al., 2010), Psk1 phosphorylation was sensitive to glucose availability (Fig. 7A). On the other hand, cycloheximide attenuated dephosphorylation of Psk1 and Rps6 by nitrogen or glucose starvation (Fig. 7B). Similarly, in mammalian cells, cycloheximide as well as other protein synthesis inhibitors, puromycin and anisomycin, attenuates the decrease in mTORC1 activity to phosphorylate its substrates (Iiboshi et al., 1999; Beugnet et al., 2003). Those translation inhibitors are likely to suppress consumption of amino acids by protein synthesis, thereby maintaining the pools of intracellular amino acids. In budding yeast, cycloheximide causes hyperphosphorylation of Sch9, which is catalyzed by TORC1. In this case, elevated intracellular amino acid pools are observed under amino acid depletion (Urban et al., 2007; Binda et al., 2009). We further showed that the TORC1-dependent Psk1 phosphorylation is controlled by extracellular and intracellular glutamine (Fig. 7C, D). Similarly, a subset of TORC1 function is regulated in response to intracellular glutamine levels in budding yeast (Crespo et al., 2002). Therefore, TORC1, at least in part, may perceive intracellular storage of amino acids directly or indirectly. It has recently been reported that in mammalian systems, mTORC1 is translocated in response to amino acid availability by Rag G-proteins that interact with raptor, which is a component of mTORC1, to the surface of lysosomes where the kinase is activated by Rheb G-protein. This translocation of mTORC1 appears to be necessary for its activation by amino acid (Sancak et al., 2008). In fission yeast, there are two homologous proteins of the Rag G-proteins as Gtr1 and Gtr2. Most recently, it has been reported that these Rag proteins appear also to participate in regulation of TORC1 by amino acids (Valbuena et al., 2012).

We have shown here that TORC1 controls phosphorylation and function of Psk1 and possibly of Sck1 and Sck2. We have further shown that Psk1 is the major kinase
downstream of TORC1 phosphorylating Rps6 (fission yeast S6) and that Sck1 and Sck2 have no effect on Rps6 phosphorylation. It has been shown that the loss of Tor2 (TORC1) function leads to entry into sexual differentiation (Alvarez and Moreno, 2006; Matsuo et al., 2007; Uritani et al., 2006). In a preliminary study, we have detected increased mating efficiency by disruption of psk1+, although this was seen in the background of disruption of sck1+ and sck2+ (data not shown). Further work is needed to clarify how these AGC kinases function downstream of TORC1 to affect various physiological aspects of fission yeast cells.

In the current study, the direct substrate of the fission yeast TORC1 was identified. We also showed that those phosphorylations catalyzed by TORC1 could be easily monitored, providing a convenient assay to examine the activation of the TORC1 signaling. Employing these tools should be helpful to investigate molecules in the nutrient-TORC1 signaling in fission yeast.

Materials and Methods

Yeast strains, growth media, and general methods

Fission yeast strains used in this study are listed in supplementary material Table S1. Cells were grown exponentially at 30°C, except in the experiments using temperature-sensitive mutants, in yeast extract with supplements (YES) medium, 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), SD or minimal medium (MM) (Watanabe et al., 1988). EMM-N, a nitrogen free version, and EMM low glucose (Low G) (3% glycerol and 0.1% glucose), a glucose depletion version (Kohda et al., 2007), were employed as starvation media. EMM+2-DG contains 2% 2-deoxyglucose (Sigma) instead of glucose. EMM+glutamic acid, EMM+glutamine,
EMM+proline, or EMM+leucine, contains 20 mM of each amino acid instead of ammonium, which were used within a week after preparation. General and molecular genetic techniques followed standard protocols (Moreno et al., 1991).

**Antibodies and reagents**

Both polyclonal antibodies recognizing phospho-Akt substrates (PAS) and phospho-p70 S6K (Thr389) were purchased from Cell Signaling Technology. Anti-GST polyclonal, anti-FLAG (M2), and anti-α-Tubulin (B5-1-2) antibodies, and L-methionine sulfoximine (MSX) were purchased from Sigma. Anti-myc (9E10) antibody was purchased from Santa Cruz Biotechnology and Covance. 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 rapamycin were purchased from Pierce and Calbiochem, respectively.

**Construction of modified strains and gene expression plasmids**

Integration of either the 3xHA-hphMX or 13xmyc-hphMX cassette before the terminal codons of genes encoding the AGC kinases and gene disruptions by replacing the individual open reading frames (ORF) with either the kanMX or hphMX cassette were performed using the direct chromosomal integration methods (Bahler et al., 1998; Sato et al., 2005). To construct the psk1 mutation alleles, DNA fragment containing psk1\(^+\)-13xmyc-hphMX and its 5’ and 3’ UTR regions was amplified by PCR employing the genomic DNA of the psk1\(^+\)-13xmyc-hphMX strain as a template and then cloned into pCR2.1. Substitutions of the regulatory motif sites in psk1\(^+\) with alanine (K120A, S248A, T392A, and T415A) or with glutamic acid (T392E and T415E) were generated by site-directed mutagenesis using
pCR2.1-psk1\textsuperscript{+}-13xmyc-hphMX, and the sequence was confirmed. The substituted DNA fragments were amplified by PCR and integrated by homologous recombination.

To construct expressing plasmids of psk1\textsuperscript{+}, sck1\textsuperscript{+}, and sck2\textsuperscript{+}, ORFs of these kinases were amplified by PCR and cloned into pREP81-HA, which possesses the thiamine repressible nmt1 promoter (Basi et al. 1993). To construct plasmids expressing GST-rps602\textsuperscript{+} and the GST-rps602\textsuperscript{SS235236AA}, DNA fragment containing rps602\textsuperscript{+} or rps602\textsuperscript{SS235236AA} was amplified by PCR and cloned into pGEX. ORFs encoding psk1\textsuperscript{+}, the psk1 mutants, sck1\textsuperscript{+}, and sck2\textsuperscript{+} were amplified by PCR and cloned into pGEX-KG.

**Protein preparation, phosphatase treatment, and immunoblotting**

Cultures were mixed with trichloroacetic acid (TCA) (final concentration 6%) and put on ice for at least 5 minutes. After centrifugation, cell pellets were washed twice with cold ethanol, and dried. Cells were disrupted 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 Na\textsubscript{3}VO\textsubscript{4}, 10 mM p-nitrophenyl phosphate (p-NPP), 10 mM NaF, 1mM dithiothreitol (DTT), 1mM phenylmethysulfonyl fluoride (PMSF), and protease inhibitor cocktail (Complete EDTA-free; Roche)] with glass beads. Whole cell extracts were mixed with 3xSDS sample buffer, and boiled for 5 minutes.

For phosphatase treatment, TCA-quenched cells were broken in the urea buffer [50 mM Tris-HCl (pH 7.5), 6 M urea, 5 mM EDTA, 1% SDS, 10 mM NaN\textsubscript{3}, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 0.1 mM Na\textsubscript{3}VO\textsubscript{4}, 10 mM p-NPP, 10 mM NaF, 1mM DTT, 1mM PMSF, and protease inhibitor cocktail] with glass beads by repeating three times of vortex for 5 minutes at RT and incubation for 10 minutes at 65°C. After centrifugation, supernatants were diluted 20-fold into a reaction buffer with 2 mM MnCl\textsubscript{2} and
then incubated with ⁵-phosphatase (400U) (New England Biolabs) at 30°C for 40 minutes with or without the phosphatase inhibitors (50 mM EDTA, 10 mM Na₃VO₄, and 50 mM NaF).

To assess phosphorylation at Thr415 in Psk1 with the anti-phospho-p70 S6 kinase (Thr389) antibody, cells in 40 ml cultures were quenched with TCA and were broken in 140 μl of the urea buffer with glass beads as described above. Cell extracts were diluted gradually with 1100 μl of buffer A and centrifuged at room temperature. Supernatants were incubated with anti-myc antibody and protein G-Sepharose at 4°C for 2.5 hours. Immunoprecipitates were washed three times with buffer A without protease inhibitors.

Cell extracts or immunoprecipitates were separated by SDS-PAGE and then immunoblotted with the primary antibodies. After incubation with HRP-conjugated secondary antibodies, detection of proteins was carried out using the ECL plus detection system (GE Healthcare Bio-Sciences).

**In vitro kinase assay**

GST-fused proteins were expressed in the *Escherichia coli* BL21 (DE3) strain and purified. Cells expressing *psk1⁴¹⁵E-13xmyc* or *psk1¹²⁰A-13xmyc* were broken with glass beads in buffer A with 20 μg/ml leupeptin. After centrifugation at 10,000 g for 15 minutes and subsequently at 14,000 g for 20 minutes at 4°C, supernatants were incubated with anti-myc antibody and protein G-Sepharose at 4°C for 2.5 hours, and immunoprecipitates were washed twice with buffer A without protease inhibitors and then washed twice with washing buffer [50 mM MOPS-KOH (pH 7.2), 10 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 10 mM MgCl₂]. The Psk1 kinase assay was carried out as follows: the immunoprecipitates were incubated with 1.5 μg of GST-Rps602 wild-type or -Rps602⁵⁸²₃⁵²₃⁶AA as a substrate in buffer
Kpsk1 [50 mM MOPS-KOH (pH 7.2), 10 mM β-glycerophosphate, 0.1 mM Na$_3$VO$_4$, 10 mM MgCl$_2$, 100 μM ATP] for 1 hour at 30ºC.

For the Tor2 kinase assay in vitro, cells were transformed with either pREP41-His6Flag2-tor2$^+$ (Matsuo et al., 2007) or pREP41-His6Flag2-tor2KD (Kinase Dead; D2140A). Tor2 or Tor2KD was immunoprecipitated as described previously (Matsuo et al., 2007). After washing three times with buffer B [50 mM Tris-HCl (pH 7.6), 150 mM KCl, 5 mM EDTA, 1 mM DTT, 10% glycerol, 0.2% NP-40, 20 mM β-glycerophosphate, 0.1 mM Na$_3$VO$_4$, 15 mM p-NPP and 1 mM PMSF], immunoprecipitates were washed twice with buffer Ktor [20 mM HEPES-KOH (pH 7.5), 1 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na$_3$VO$_4$, 15 mM p-NPP, 10 mM MnCl$_2$]. Then, in vitro kinase assay was carried out as follows: the immunoprecipitates were incubated with 2 μg of GST-Psk1, -Psk1 mutants, -Sck1, or -Sck2 as substrates and 2 μg BSA in buffer Ktor. The reaction was initiated by adding 25 μM cold ATP and 5 μCi [$\gamma$-$^{32}$P] ATP and terminated by adding SDS sample buffer after 35min incubation at 32ºC.

In vitro kinase assay of Ksg1 using GST-Psk1 wild type and its mutants as substrates was carried out essentially as described (Matsuo et al., 2003).

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phosphorylation of ribosomal S6 proteins in response to nutrients and its activity is inhibited

*ksg1*, that shows structural homology to the human phosphoinositide-dependent protein

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**Figure legends**

**Fig. 1.** Nitrogen source-dependent phosphorylation of S6K homologs in fission yeast.

(A) Phylogenetic tree of fission yeast homologs of human S6K1 was generated using ClustalW program. (B) Cells of JUp1204 (non-tagged), AN0151 (Sck1-3HA), AN0176 (Gad8-3HA), AN0153 (Sck2-3HA), and AN0180 (Psk1-13myc) were cultured in EMM, then washed and starved in EMM-N for 1 hour. After the starvation, ammonium (final concentration, 0.5%) (+) or water (-) was added to cells and incubated for 30 minutes. (C) Protein extracts from ammonium-stimulated cells as described in B were incubated with $\lambda$-phosphatase in the presence or absence of its inhibitors. (B, C) Protein extracts were subjected to immunoblotting with the indicated antibodies.

**Fig. 2.** Identification of Psk1 as the major kinase to phosphorylate Rps6. (A-C)

Proteins were probed with the indicated antibodies. (A) Cells of JUp1204 (WT), AN0170 (sck1Δ), AN0203 (sck2Δ), and AN0133 (psk1Δ) were cultured in EMM (+), washed, and then incubated in EMM-N for 30 minutes (-). (B) Cells of JUp1204 (WT), AN0129 (rps601Δmyc-rps602), and AN0168 (psk1Δrps601Δmyc-rps602) were washed and incubated in EMM with (+) or without (-) ammonium for 20 minutes. (C) Cells of AN0216 (T415E) and AN0219 (K120A) were grown in EMM and harvested. Myc-tagged Psk1 was
immunoprecipitated and Psk1 kinase assay in vitro was carried out using GST-Rps602 proteins as substrates as described in Materials and Methods. Phosphorylation of Rps602 was evaluated by immunoblotting. Arrowhead denotes a band corresponding to phosphorylated Rps602 probed with the PAS antibody.

**Fig. 3.** **Psk1 is a downstream target of TORC1.** (A-C) Proteins were probed with the indicated antibodies. (A) Cells of AN0182 (tor2+) and AN0184 (tor2L1310P) (0 minute) were washed and incubated in EMM-N for the indicated time points. (B) Cells of AN0180 (WT), AN0217 (tor2-ts6), and AN0218 (tor2-ts10) were grown in EMM at 25°C. For treatments at the non-permissive temperature, cell culture was shifted to 35°C for 1 hour before cells were transferred to pre-warmed EMM-N. After incubation in EMM-N for 1 hour at either 25°C or 35°C (0 minute), ammonium was added to cells to 0.5% and incubated for 15 minutes. (C) Cells of AN0181 (tor2+) and AN0185 (tor2S1837E) were treated with 200 nM rapamycin for the indicated time points. (D) In vitro kinase assay of Tor2 was carried out as described in Materials and Methods in detail. The gel was dried and autoradiographed (the right panel). The left panel (input); the coomassie brilliant blue staining.

**Fig. 4.** **Effect of gene disruptions of the TORC1 components on phosphorylation of the TORC1 downstream factors.** (A-C) Proteins were probed with the indicated antibodies. (A) Cells of AN0179 (WT), AN0233 (pop3Δ), AN0237 (toc1Δ), and AN0238 (tco89Δ) in EMM (+) were washed and cultured in EMM-N for 15 minutes (-). (B) Cells as described in A were treated with 150 nM rapamycin for the indicated time points. (C) Cells of AN0179 (WT) and AN0233 (pop3Δ) were cultured with the indicated concentrations of rapamycin for 1 hour. (B and C) The relative reduction of Psk1 phosphorylation (%) was estimated by densitometry using ImageJ software and normalized to tubulin expression. The amount of Psk1 phosphorylation in wild type before addition of rapamycin (0 minutes)
was set as 100.

**Fig. 5.** Role of the predicted phosphorylation sites of Psk1 in its kinase activity. (A) Schematic diagram of Psk1 that has a kinase domain in the middle region and an AGC-kinase C-terminal domain in the carboxy-terminal region. The predicted amino acid residues at the bottom indicate Lys120 at the ATP-binding site, Ser248 in the activating loop (T-loop), Thr392 in the turn motif (TM), and Thr415 in the hydrophobic motif (HM) as shown in Fig. S1. (B-F) Proteins were probed with the indicated antibodies. (B) Cells of AN0179 (WT), AN0219 (K120A), AN210 (S248A), AN211 (T392A), and AN0212 (T415A) were harvested as +N (+). Another portion of AN0179 culture was washed and cultured in EMM-N for 20 minutes. (C) Cells of AN0179 (WT), AN0213 (T392E), and AN0216 (T415E) in EMM (+) were washed and cultured in EMM-N for 20 minutes (-). (D) The left panels; cells of AN0182 (WT) and AN0184 (L1310P) in EMM (+) were washed and cultured in EMM-N for 30 minutes. The right panels; cells of AN0181 (WT) and AN0185 (S1837E) in EMM were treated with DMSO (-) or 200 nM rapamycin (+) and incubated for 1 hour. Psk1-13myc was immunoprecipitated and subjected to immunoblotting. (E) In vitro phosphorylations of GST-Psk1 proteins as indicated by Tor2 carried out as described in Fig. 3D. (F) Cells of AN0179 (WT), AN210 (S248A), AN211 (T392A), and AN0212 (T415A) were harvested as +N (+). Another portion of AN0179 culture was washed and cultured in EMM-N for 20 minutes.

**Fig. 6.** Ksg1, a PDK1 homolog, is required for phosphorylation and activity of Psk1.

(A) Thermal treatments of cells of AN0180 (WT), AN0243 (ksg1-ts208), and AN245 (ksg1-ts358) were carried out as described in Fig. 3B. After incubation in EMM-N for 1 hour at 25°C or 35°C (0 minute), ammonium was added to cells to 0.5% and incubated for 15 minutes. Proteins were probed with the indicated antibodies. (B) In vitro
phosphorylations of GST-Psk1 proteins as indicated by GST-Ksg1 carried out as described in Materials and Methods. The gel was dried and autoradiographed (the upper panel). The lower panel (input); the coomassie brilliant blue staining.

**Fig. 7.** The TORC1 signaling responds to the availability of glucose and glutamine. (A-D) Proteins were probed with the indicated antibodies. (A) Cells of AN0179 were washed and incubated in EMM, EMM low glucose (Low G), or EMM+2-DG for 15 minutes. (B) Cells of AN0180 (0 minute) were washed and incubated with either EMM low glucose or EMM -N with DMSO or cycloheximide (CHX) (50 μg/ml) for the indicated time points. (C) After incubation in EMM-N for 1 hour (-), cells of AN0179 were incubated in EMM (+) or EMM containing either 20 mM glutamic acid (Glu), glutamine (Gln), proline (Pro), or leucine (Leu) instead of ammonium for 15 minutes. (D) Left panel; cells of AN0179 were treated in EMM in the absence or presence of 5 or 10 mM L-methionine sulfoximine (MSX) for 30 minutes. Gln, EMM plus 20 mM Glutamine instead of ammonium with 5 mM MSX. Right panel; cells of AN0179 were transferred to EMM (+NH4) or EMM plus 20 mM Glutamine (Gln) with 5 mM MSX and incubated for the indicated time points.

**Fig. 8.** Sck1 and Sck2 might also be partly involved in the TORC1 signaling. (A) In vitro phosphorylations of Sck1 and Sck2 by Tor2 carried out as described in Fig. 3D. (B) Cells of AN0163 (*tor2*+), AN0164 (*tor2*+), AN0166 (*tor2*L1310P), AN0167 (*tor2*S1837E) were nitrogen-starved and treated with rapamycin as described in Fig. 3A, C. Proteins were probed with the indicated antibodies. The relative reduction of Sck1 phosphorylation (%) was estimated by densitometry using ImageJ software and normalized to tubulin expression. The amount of Sck1 phosphorylation in wild type before treatment of nitrogen starvation or of rapamycin (0 minutes) was set as 100 (B).
Fig. 3

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