Schizosaccharomyces pombe AGC family kinase Gad8p forms a conserved signaling module with TOR and PDK1-like kinases

Tomohiko Matsuo, Yoshiya Kubo, Yoshinori Watanabe[1] and Masayuki Yamamoto[2]

Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo and [1]SORST, JST, Hongo, Tokyo 113-0033, Japan

[2]Corresponding author
e-mail: myamamot@ims.u-tokyo.ac.jp

The TOR protein is a phosphoinositide kinase-related kinase widely conserved among eukaryotes. Fission yeast *tor1* encodes an ortholog of TOR, which is required for sexual development and growth under stressed conditions. We isolated *gad8*, which encodes a Ser/Thr kinase of the AGC family, as a high-copy suppressor of the sterility of a *tor1* mutant. Disruption of *gad8* caused phenotypes similar to those of *tor1* disruption. Gad8p was less phosphorylated and its kinase activity was undetectable in *tor1*Δ cells. Three amino acid residues corresponding to conserved phosphorylation sites in the AGC family kinases, namely Thr387 in the activation loop, Ser527 in the turn motif and Ser546 in the hydrophobic motif, were important for the kinase activity of Gad8p. Tor1p was responsible for the phosphorylation of Ser527 and Ser546, whereas Ksg1p, a PDK1-like kinase, appeared to phosphorylate Thr387 directly. Altogether, Tor1p, Ksg1p and Gad8p appear to constitute a signaling module for sexual development and growth under stressed conditions in fission yeast, which resembles the mTOR–PDK1–S6K1 system in mammals and may represent a basic signaling module ubiquitous in eukaryotes.

Keywords: AGC family kinase/PDK1-like kinase/sexual development/stress response/TOR kinase

Introduction

Organisms employ various signal transduction pathways to respond to environmental changes as well as to factors for cell-to-cell communication. The TOR (target of rapamycin) protein is a widely conserved phosphoinositide kinase-related kinase, which is thought to participate in the signal transduction of nutritional availability. TOR proteins have been found in fungi, worms, flies, plants and mammals (Schmelzle and Hall, 2000; Gingras *et al.*, 2001). The original members of the *TOR* gene family were *Saccharomyces cerevisiae TOR1* and *TOR2*, which were identified in a screen for resistant mutants against rapamycin, an immunosuppressant drug causing growth arrest in the budding yeast (Heitman *et al.*, 1991; Kunz *et al.*, 1993). It is well characterized that TOR has a function relevant to regulation of translation. However,

previous reports indicate that the TOR signaling is complex and may potentially also be involved in many biological processes (Schmelzle and Hall, 2000; Gingras *et al.*, 2001). In *S.cerevisiae*, *TOR1* and *TOR2* have been shown to participate in the regulation of G_1 progression, transcription, cytoskeletal organization, sporulation and autophagy (Schmelzle and Hall, 2000; Gingras *et al.*, 2001).

PDK1 (phosphoinositide-dependent kinase-1) is another conserved Ser/Thr protein kinase in eukaryotes. PDK1 regulates signal transduction by activating members of the AGC family kinases [protein kinases (PK) A, G and C], which include isoforms of Akt/PKB, serum and glucocorticoid induced kinase (SGK), p90 ribosomal S6 kinase (RSK) and p70 ribosomal S6 kinase 1 (S6K1) (Toker and Newton, 2000; Alessi, 2001). PDK1 activates these kinases through direct phophorylation of the activation loop located in their catalytic domain. AGC family kinases also carry conserved phosphorylation sites that are C-terminal to the catalytic domain. One site is positioned in a region called the hydrophobic motif, which exists in most AGC family kinases except PKA, and phosphorylation of this site is essential for full activation of the kinase. Some members of the AGC kinase family, including PKC, S6K1, RSK and PKA, carry another phosphorylation site in a region called the turn motif, and its phosphorylation is important for kinase activity. However, in some cases, including PKB and SGK1, phosphorylation of the turn motif does not appear to affect the activity (Newton, 2001). Although it is widely accepted that PDK1 is responsible for the phosphorylation of the activation loop of the AGC family kinases, a variety of protein kinases appear to be involved in phosphorylation of the hydrophobic motif and the turn motif, and the significance of the phosphorylation of the turn motif requires further investigation. Interestingly, mTOR (mammalian ortholog of TOR) phosphorylates both the hydrophobic and turn motifs of mammalian S6K1, a downstream target of the mTOR signaling pathway (Burnett *et al.*, 1998; Isotani *et al.*, 1999; Saitoh *et al.*, 2002).

Fission yeast arrests the mitotic cell cycle in G_1 phase and enters sexual development upon nutrient starvation. Previous studies have shown that conserved signal transduction pathways participate in the onset of sexual development, such as the cAMP–PKA pathway, the Ras–MAPK cascade and the SAPK cascade (Yamamoto, 1996). Recently, Tor1p, which is an ortholog of TOR, and Ksg1p, which is a PDK1-like kinase, have been reported as positive regulators of sexual development in fission yeast (Niederberger and Schweingruber, 1999; Kawai *et al.*, 2001; Weisman and Choder, 2001). However, no possible AGC family kinase potentially cooperating with Tor1p or Ksg1p has been identified.
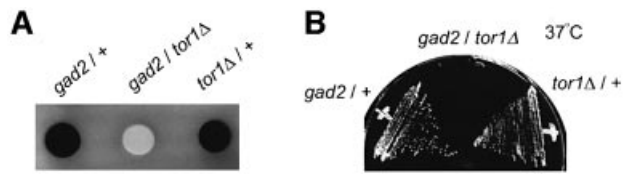
Fig. 1. The *gad2-1* (*tor1-g2*) mutation does not complement the *tor1* disruption. (**A**) Sporulation assay. Diploid strain JW958 (denoted as *gad2/tor1Δ*), constructed by crossing a *gad2-1* haploid and a *tor1Δ* haploid, was incubated on MEA medium together with control diploids heterozygous for either *gad2-1* (JW956; denoted as *gad2/+*) or *tor1Δ* (JW957; denoted as *tor1Δ/+*). After four days, patches were exposed to iodine vapor, which stains sporulated cells dark brown. (**B**) Complementation for the growth at high temperature. The same diploid strains as in (A) were streaked on the YE medium and incubated for three days at 37°C.

To search for a Tor1p target, we screened for high-copy suppressors of the sterility of the *tor1-g2* mutant, which carried a nonsense *tor1* allele and was defective in G$_1$ arrest under nitrogen starvation. We consequently identified a novel gene *gad8*, which encodes a Ser/Thr kinase of the AGC family. In this report we clarify the relationship of Gad8p with Tor1p and Ksg1p, and propose that these three protein kinases constitute a module for signaling, which may be conserved ubiquitously among eukaryotes.

Results

Identification of tor1-g2, a nonsense allele of tor1

According to the previously described screening protocol (Kanoh *et al.*, 1996), we isolated a fission yeast mutant, tentatively named *gad2-1*, that was defective in G$_1$ arrest under nitrogen starvation and was sterile. This mutant was temperature sensitive for growth and grew slowly with an elongated cell shape even at the permissive temperature 30°C (data not shown). It has been reported that *tor1* is required for sexual development and growth under stressed conditions, such as high osmolarity or high temperature (Kawai *et al.*, 2001; Weisman and Choder, 2001). We noted that the *gad2-1* mutant was phenotypically similar to the *tor1Δ* mutant, although the phenotype of *gad2-1* was somewhat weaker. To examine the allelism, we produced a diploid strain by crossing a *tor1Δ* haploid with a *gad2-1* haploid, as described in Materials and methods. This strain was sporulation-defective and exhibited temperature-sensitive growth (Figure 1), suggesting that *gad2-1* might be allelic to *tor1*. We then analyzed the *tor1* gene isolated from the *gad2-1* mutant. The wild-type *tor1* gene encodes a protein of 2335 amino acids (Kawai *et al.*, 2001; Weisman and Choder, 2001). We found that the mutant gene carried a stop codon (TGA) in place of the arginine codon (CGA) at 1071. Thus, we concluded that *gad2-1* is a new allele of *tor1*, which we call *tor1-g2* hereafter.

The nonsense mutation found in *tor1-g2*, which was located upstream of the kinase domain, did not appear to be very tight, because the mutant phenotype was considerably weaker than the *tor1* deletion. For instance, the *tor1-g2* mutant could grow, though slowly, on medium containing 0.4 M KCl, whereas the *tor1Δ* mutant could not. We have evidence suggesting that the *tor1-g2*
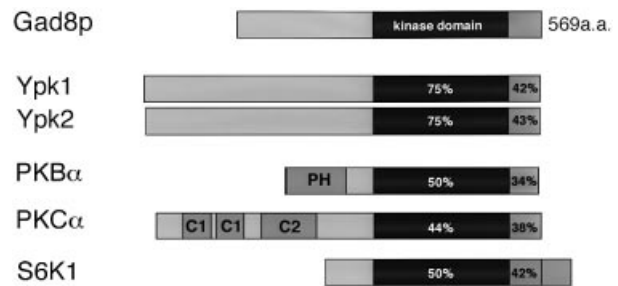

Fig. 2. The deduced *gad8* gene product, Gad8p, is a Ser/Thr kinase of the AGC family. Gad8p is aligned with some members of the AGC family, including budding yeast Ypk1 and Ypk2/Ykr2 and human PKBα, PKCα and S6K1. The kinase domain is represented by a black box and the C-terminal region conserved in AGC family kinases, by a shaded box. The percentage values indicates the amino acid identity of the respective region compared with the corresponding region in Gad8p. PH, pleckstrin homology domain; C1, diacylglycerol/phorbol ester binding domain; C2, Ca^{2+}/TPA binding domain.
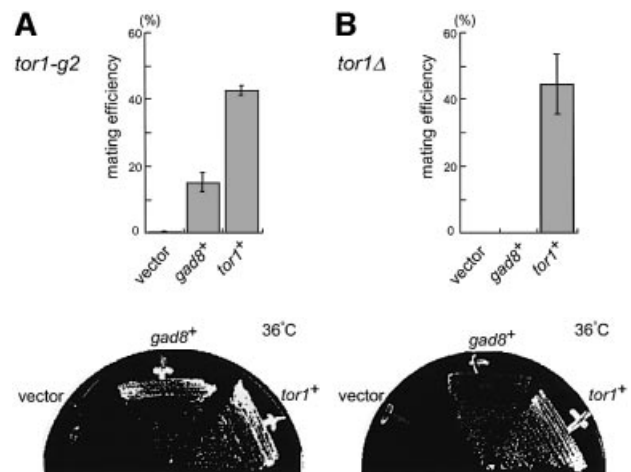

Fig. 3. Suppression of *tor1-g2* and *tor1Δ* by overexpression of *gad8*. (**A**) Suppression of the *tor1-g2* mutant. A homothallic haploid *tor1-g2* strain (JW948) was transformed with either pREP41-*gad8*, or pREP41-*tor1* or the vector pREP41. Transformants were grown on SSA medium at 30°C for four days. The mating efficiency of each transformant was scored under the microscope (top panel). Each transformant was also incubated on MM medium at 36°C for four days (bottom panel). (**B**) Suppression of the *tor1Δ* strain. A homothallic haploid *tor1Δ* strain (JW951) was examined as in (A), except that the incubation time on MM medium was three days.

mutation allows production of a small amount of the read-through gene product (data not shown).

A high-copy suppressor of tor1-g2 encodes a novel Ser/Thr kinase Gad8p

We set out to identify possible downstream targets of Tor1p by screening for high-copy suppressors of the sterility of the *tor1-g2* mutant. We transformed homothallic haploid *tor1-g2* cells with fission yeast genomic and cDNA libraries, and picked up transformants that could mate and sporulate on SSA medium. Among 370 000 transformants examined, thirteen were judged to be positive. Sequence analysis indicated that they were attributed to five genes, namely *rst2*, which encodes a transcription factor crucial for sexual development (Kunitomo *et al.*, 2000; Higuchi *et al.*, 2002), N-terminally truncated *byr2/ste8*, which encodes a MAPKKK in the pheromone-signaling pathway (Wang
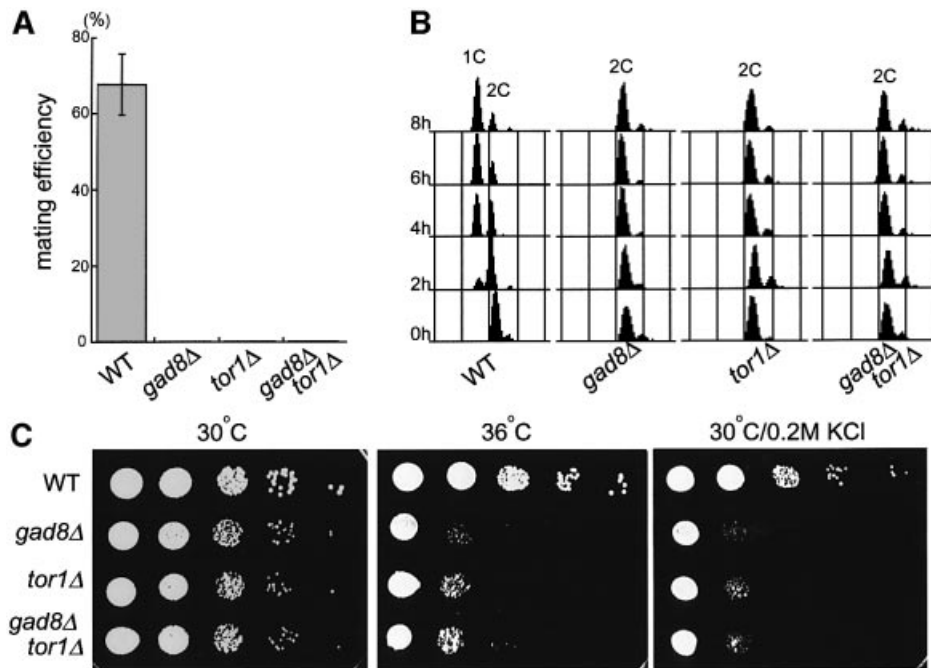
Fig. 4. Phenotypic similarities between *gad8Δ* and *tor1Δ*. (**A**) A comparison of the mating efficiencies of the wild-type (JY476), *gad8Δ* (JW945), *tor1Δ* (JW951) and *gad8Δ tor1Δ* (JW959) strains. Cells were incubated on SSA medium for four days and the mating efficiency was scored under the microscope. (**B**) Flow-cytometric analysis of the four strains subjected to nitrogen starvation. Cells were grown to logarithmic phase in MM liquid medium at 30°C, harvested, washed once and transferred to MM without a nitrogen source. Their DNA content was measured by FACS analysis at 2 h intervals. (**C**) Temperature-sensitivity and high-osmolarity sensitivity of each strain. Cells of each strain, grown to logarithmic phase at 30°C, were spotted on YE medium with sequential ten-fold dilution and incubated at either 30°C (left panel) or 36°C (middle panel) for three days. Cells were also spotted similarly on YE medium containing 0.2 M KCl and incubated at 30°C for three days (right panel).

et al., 1991) and three novel genes. In this study we characterized one of these novel genes, which we named *gad8*, and is annotated as SPCC24B10.07 in the fission yeast genomic database (Wood *et al.*, 2002).

The deduced *gad8* gene product was a Ser/Thr kinase. Gad8p showed high similarity to AGC family kinases in the catalytic domain (Figure 2), especially to budding yeast Ypk1 and Ypk2/Ykr2 (Maurer, 1988; Chen *et al.*, 1993) (75% amino acid identity in each case). Gad8p also showed similarity to AGC family kinases in the C-terminal region (Figure 2).

Overexpression of *gad8* suppressed both sterility and temperature-sensitive growth of the *tor1-g2* mutant (Figure 3A, top and bottom). However, overexpression of *gad8* failed to suppress the sterility of the *tor1Δ* mutant (Figure 3A, top), although it could suppress the temperature sensitivity and high-osmolarity sensitivity of this strain (Figure 3B, bottom; data not shown). This may indicate that a higher activity of Tor1p and/or Gad8p is required for sexual development than for growth under stressed conditions.

Gad8p and Tor1p are likely to be on the same regulatory pathway for sexual development and growth

We disrupted the *gad8* gene, as described in Materials and methods. Disruption of *gad8* was not lethal. However, the haploid *gad8* disruptant failed to mate on sporulation medium (Figure 4A), and apparently could not arrest the cell cycle at G_1 under nitrogen starvation (Figure 4B). Furthermore, *gad8Δ* cells could not grow under stressed

conditions, such as at 37°C or in the presence of 1 M KCl or 1.2 M sorbitol (data not shown; see below). As these *gad8Δ* phenotypes appeared very similar to those of *tor1Δ*, we constructed a *gad8Δ tor1Δ* double disruptant and compared it with the parental strains.

All of the three strains, namely *gad8Δ*, *tor1Δ* and *gad8Δ tor1Δ*, were completely mating-defective (Figure 4A), and behaved almost identically in the failure of G_1 arrest under nitrogen starvation (Figure 4B), in defective growth at 36°C (Figure 4C, middle), and in defective growth in the presence of 0.2 M KCl (Figure 4C, right). In addition, all of them grew slowly showing elongated cell shape. The doubling time in YE liquid medium was 2.8 $\pm$ 0.1 h for *gad8Δ*, 2.7 $\pm$ 0.1 h for *tor1Δ* and 2.8 $\pm$ 0.1 h for *gad8Δ tor1Δ*, while that of the wild type was 2.3 $\pm$ 0.1 h. The average length of septated cells was 17.1 $\pm$ 1.8 μm for *gad8Δ*, 17.2 $\pm$ 1.3 μm for *tor1Δ* and 17.3 $\pm$ 1.7 μm for *gad8Δ tor1Δ*, while that of the wild type was 14.2 $\pm$ 1.3 μm. These data strongly suggested that Tor1p and Gad8p should function in the same pathway. Because overexpression of *gad8* could suppress the stress sensitivity of the *tor1Δ* strain (Figure 3) but overexpression of *tor1* failed to suppress *gad8Δ* (data not shown), Gad8p was believed to be a downstream factor of Tor1p.

Tor1p affects the kinase activity and the phosphorylation state of Gad8p

To measure the kinase activity of Gad8p, we immunoprecipitated 6HA (six times tandem-repeated hemagglutinin)-tagged Gad8p from the extracts of *tor1+* and *tor1Δ* cells. We performed *in vitro* phosphorylation assay using
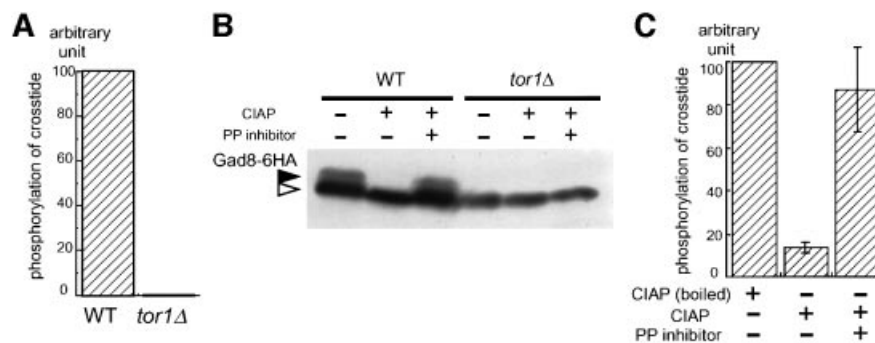
Fig. 5. Tor1p is essential for the kinase activity of Gad8p and affects its phosphorylation state. (**A**) Assay of the kinase activity of Gad8p recovered from either the wild-type (JW960) or the *tor1Δ* (JW965) background. Gad8-6HAp expressed in each strain was immunopurified from the cell extract and assayed for its ability to phosphorylate crosstide, as described in the Materials and methods. (**B**) Detection of Gad8-6HAp in wild-type and *tor1Δ* cells. Extracts of JW960 and JW965, expressing Gad8-6HAp, were separated by SDS–PAGE, and the protein was detected by western blotting using anti-HA antibody. Extracts were also treated with CIAP, with or without the addition of the phosphatase inhibitor mixture, to examine possible phosphorylation of Gad8p. The white arrowhead indicates the major band of Gad8p, and the black arrowhead indicates the minor band. (**C**) Effects of phosphatase treatment on the kinase activity of Gad8p. Gad8-6HAp immunopurified as in (A) was treated with CIAP, either native or boiled, in the presence or absence of the phosphatase inhibitor mixture. The kinase assay was performed as in (A).

'crosstide' as a substrate, which is a peptide known to be phosphorylated by AGC family kinases such as PKB (Cross *et al.*, 1995). Gad8-6HAp recovered from the *tor1Δ* background was inactive in phosphorylating crosstide, whereas Gad8-6HAp from *tor1+* was fairly active (Figure 5A). We also found that the mobility of Gad8-6HAp varied depending on the genetic background of the host from which it was derived. Gad8-6HAp from the wild type showed a small fraction that migrated slower than the majority, but this fraction was missing in the preparation from the *tor1Δ* strain (Figure 5B). Treatment of Gad8-6HAp from the wild type with calf intestine alkaline phosphatase (CIAP) diminished the slow-migrating fraction, indicating that this fraction was generated by phosphorylation (Figure 5B). Moreover, CIAP treatment of Gad8-6HAp from the wild type abolished its ability to phosphorylate crosstide, indicating that phosphorylation is crucial for the kinase activity of Gad8p (Figure 5C). These data reinforce the suggestion that Tor1p regulates the kinase activity of Gad8p via phosphorylation.

Three phosphorylation sites conserved among AGC family kinases are important for the function of Gad8p

Members of the AGC kinase family undergo phosphorylation at conserved sites, which is crucial for their activity (Figure 6A). One phosphorylation site is located in the activation loop, another in the turn motif and a third in the hydrophobic motif. Gad8p apparently carried these three sites, namely Thr387 in the activation loop, Ser527 in the turn motif and Ser546 in the hydrophobic motif. To examine whether phosphorylation of these sites was necessary for Gad8p function, we mutated each of them to alanine. The three types of mutant *gad8* alleles carried on the pR3C vector were transformed into *gad8Δ* cells, and the transformants were examined for their phenotypes (Figure 6B). The *gad8-T387A* transformant was sterile and did not grow under stressed conditions, like the *gad8Δ* host strain transformed with the vector. The *gad8-S527A* transformant was also sterile, but could grow weakly under stressed conditions. The *gad8-S546A* transformant was moderately fertile and showed no obvious growth

defect at high temperature or under high osmolarity. These results suggested that the *gad8-S527A* and *gad8-S546A* alleles were partially active, the former being less active than the latter, whereas the *gad8-T387A* allele was completely inactive. We then constructed a *gad8* allele carrying both *S527A* and *S546A* mutations. This allele, denoted *gad8-S527A/S546A*, was clearly less active than *gad8-S527A* and did not detectably complement the *gad8Δ* strain (Figure 6B).

We assayed the kinase activities of the mutant forms of Gad8p *in vitro*. Gad8-T387Ap, Gad8-S527Ap and Gad8-S527A/S546Ap hardly phosphorylated crosstide, like the kinase-dead form Gad8-K259Rp used as a negative control (Figure 6C). In contrast, Gad8-S546Ap revealed a modest but significant kinase activity, which was about one-seventh of the wild type. These results indicate that the observed phenotypes of the mutant *gad8* alleles correlate with the kinase activity of their gene products, and that the phosphorylation sites in the activation loop, in the turn motif and in the hydrophobic motif of Gad8p are important for its full activity.

Phosphorylation of Ser527 and Ser546 depends on Tor1p

We investigated the mobility of Gad8-T387Ap, Gad8-S527Ap and Gad8-S546Ap, each tagged with 6HA, in gel electrophoresis (Figure 6D). The mobility of Gad8-T387Ap was indistinguishable from that of wild-type Gad8p, showing a slow-migrating fraction. In contrast, the slow-migrating fraction slightly shifted its position in Gad8-S527Ap, and was completely missing in Gad8-S546Ap. As wild-type Gad8p prepared from *tor1Δ* cells lacked the slow-migrating band, we suspected that Tor1p might be involved in phosphorylation of Ser527 and Ser546. To examine this hypothesis, we constructed *gad8-S527D*, *gad8-S546D*, and *gad8-S527D/S546D* alleles. The products of these mutant alleles, in which Ser527 and/or Ser546 was replaced by aspartate, were expected to mimic wild-type Gad8p phosphorylated at the respective serine residue. When we transformed the *gad8Δ* strain with the *gad8-S546D* allele carried on a pR3C vector, all the phenotypes of *gad8Δ*, including mating deficiency and
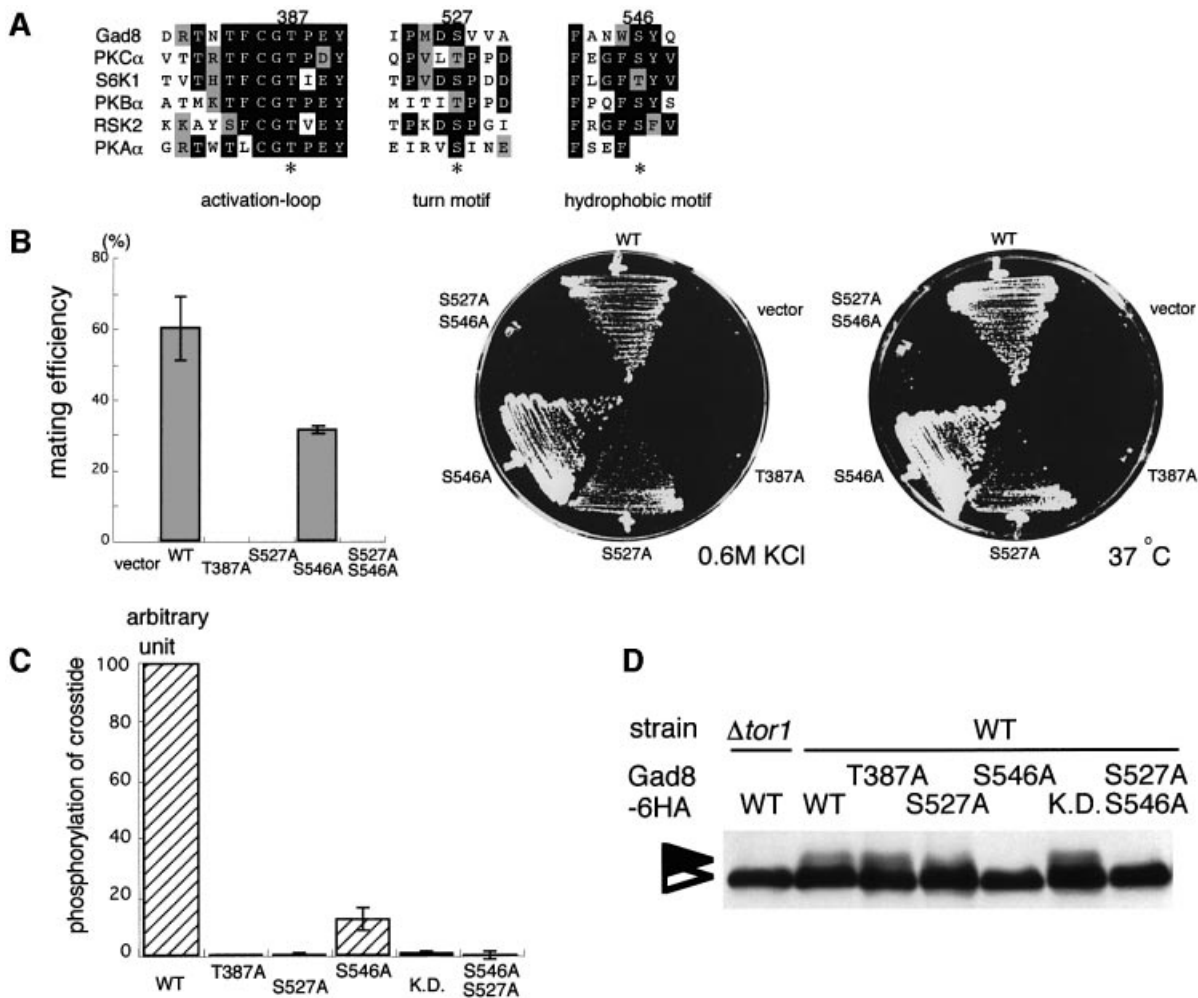
Fig. 6. Effects of mutations in the conserved phosphorylation sites on Gad8p. (**A**) Alignment of the sequences around the three possible phosphoryl-ation sites on Gad8p, in the activation loop, in the turn motif and in the hydrophobic motif, with those of human PKCα, S6K1, PKBα, PKAα and mouse RSK2. The predicted phosphorylation sites on Gad8p, namely Thr387, Ser527 and Ser546, are indicated by asterisks. (**B**) Unphosphorylatable forms of Gad8p are not functional. A homothallic haploid *gad8*Δ strain (JW945) was transformed with pR3C-*gad8*, pR3C-*gad8-T387A*, pR3C-*gad8-S527A*, pR3C-*gad8-S546A*, pR3C-*gad8-S527A S546A* or the vector pREP41. Transformants were examined for mating efficiency (left panel), growth at high osmolarity (middle panel) and growth at high temperature (right panel). Their mating efficiency was scored after incubation on SSA medium at 30°C for three days. Growth was examined on MM medium containing 0.6 M KCl at 30°C for six days, or on MM medium at 37°C for six days. (**C**) The kinase activity of the unphosphorylatable forms of Gad8p. Gad8-6HAp immunopurified from each strain, namely the *gad8-T387A* (JW962), *gad8-S527A* (JV107), *gad8-S546A* (JW963) and *gad8-S527A S546A* (JV108) mutants was subjected to the *in vitro* kinase assay, together with the control wild-type (JW960) and the *gad8-K259R* mutant (JW961) supposed to generate a kinase-dead product. (**D**) Detection of mutant forms of Gad8-6HAp in SDS–PAGE followed by western blot analysis.

defects in growth at high temperature (37°C) or under high osmolarity (0.6 M KCl), were suppressed as efficiently as by the wild-type *gad8* allele (Figure 7), suggesting that the S546D mutation indeed mimicked phosphorylated Ser546. Interestingly, *gad8-S546D* could suppress the *tor1*Δ *gad8*Δ double mutant weakly, while wild-type *gad8* could not. This observation was consistent with the idea that phosphorylation of Ser546 was regulated by Tor1p, but at the same time implied the possibility that Ser546 was not the sole target of Tor1p.

In the case of the S527D mutation, Gad8p function appeared to be compromised by this substitution, although the mutant allele could suppress the mating deficiency of the *gad8*Δ strain to some extent and was obviously more active than *gad8-S527A* (Figure 7, also see Figure 6B). The *gad8-S527D* allele weakly suppressed growth defects of the *tor1*Δ *gad8*Δ double mutant at high temperature or

under high osmolarity, but apparently failed to suppress its mating deficiency (Figure 7). Notably, however, the *gad8-S527D/S546D* allele could suppress the mating deficiency of the *tor1*Δ *gad8*Δ strain at considerably high efficiency, clearly more effectively than either the *gad8-S527D* or the *gad8-S546D* allele (Figure 7). Furthermore, the *gad8-S527D/S546D* allele suppressed the *tor1*Δ *gad8*Δ strain and the *gad8*Δ strain at nearly the same efficiency, while the wild-type and single mutant alleles of *gad8* suppressed *tor1*Δ *gad8*Δ much less efficiently than *gad8*Δ (Figure 7). A simple interpretation of these observations is that the S527D mutation mimics the phosphorylated state of Ser527, though only to a limited extent. Also, the major targets of Tor1p on Gad8p appear to be Ser527 and Ser546, because replacement of both of them with aspartate suppresses *gad8*Δ effectively even in the absence of Tor1p. This is consistent with the aforementioned
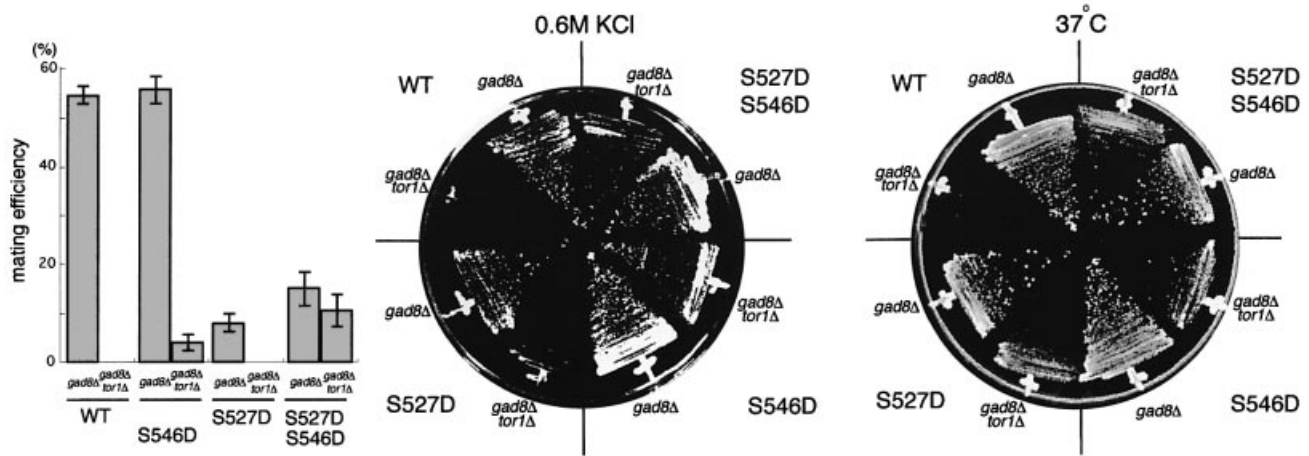
Fig. 7. Effects of the *tor1* disruption were alleviated by *gad8-S527D S546D*. Homothallic haploid *gad8Δ* (JW945) or *gad8Δ tor1Δ* (JW959) strains were transformed with pR3C-*gad8*, pR3C-*gad8-S527D*, pR3C-*gad8-S546D* and pR3C- *gad8-S527D S546D*. Each transformant was tested for mating efficiency (left panel), growth at high osmolarity (middle panel) and growth at high temperature (right panel). The mating efficiency was scored after incubation on SSA medium at 30°C for five days. Growth was examined on MM medium containing 0.6 M KCl at 30°C for six days, or on MM medium at 37°C for five days.

observation that the *gad8-S527A/S546A* double mutant allele cannot detectably rescue the *gad8Δ* strain, leaving all the phenotypes similar to those of the *tor1Δ* strain as they are.

The kinase activity of Gad8p is also regulated by Ksg1p, a PDK1-like kinase

The activation loop of most AGC family kinases undergoes phosphorylation by PDK1 or PDK1-like kinase in mammals and budding yeast (Casamayor *et al.*, 1999; Inagaki *et al.*, 1999; Toker and Newton, 2000). It has been previously shown that fission yeast *ksg1* encodes a PDK1-like kinase that is required for both vegetative growth and sexual development (Niederberger and Schweingruber, 1999). The *ksg1-208* mutant is temperature sensitive for cell growth, and is impaired in mating and sporulation at the temperature permissive for growth (Niederberger and Schweingruber, 1999). We found that overexpression of *gad8* could suppress these defective phenotypes of *ksg1-208* (Figure 8A), suggesting that Gad8p might function downstream of Ksg1p. This possibility was examined using an *in vitro* phosphorylation assay. Gad8p was prepared from either the wild-type strain or the temperature-sensitive *ksg1-208* mutant and its kinase activity was measured. As shown in Figure 8B, the kinase activity of Gad8p recovered from the *ksg1-208* mutant was considerably low, whether the cells were cultured at the permissive temperature (26.5°C) or the restrictve temperature (34°C). These results indicate that Ksg1p is a determinant of the kinase activity of Gad8p.

Overexpression of the *gad8-T387A* allele did not suppress the *ksg1-208* mutant (Figure 8A). Although this observation was consistent with Thr387 being a target of Ksg1p kinase, it was not conclusive, because overexpressed *gad8-S546A*, or *gad8-S527A* or *gad8-S527D* could not suppress *ksg1-208*, while *gad8-S546D* could, suggesting that overexpression of any weak *gad8* allele generally fails to suppress *ksg1-208* (Figure 8A; data not shown). We also examined mutation of Thr387 into aspartate or glutamate, which might mimic the state of

Gad8p activated by Ksg1p, but these mutants failed to complement *gad8Δ*, suggesting that neither *gad8-T387D* nor *gad8-T387E* was functional. As we could not establish genetic evidence for phosphorylation of Thr387 by the Ksg1p kinase, we carried out a direct phosphorylation assay as described below.

Ksg1p phosphorylates Gad8p on Thr387

To see whether Ksg1p might directly phosphorylate Gad8p, we prepared and purified Ksg1p tagged with glutathione *S*-transferase (GST) and Gad8p tagged with maltose binding protein (MBP) from *Escherichia coli*. Using these proteins we performed an *in vitro* phosphorylation assay. Ksg1p was found to phosphorylate Gad8p and Ksg1p itself, but Ksg1-K128Rp, a kinase-dead form, was not (Figure 8C). Gad8-S527Ap, Gad8-S546Ap or Gad8-S527A/S546Ap were phosphorylated by Ksg1p, but phosphorylation of Gad8-T387Ap was very weak, if any, indicating that Ksg1p was likely to phosphorylate Gad8p on Thr387. These data indicate that Ksg1p activates Gad8p via direct phosphorylation of Thr387, and activated Gad8p in turn controls cell growth and sexual development.

It was noted that Ksg1p phosphorylated Gad8p more intensively when Ser546 was mutated to aspartate (Figure 8C). The phosphorylated threonine or serine in the hydrophobic motif of S6K1, SGK, PKB or RSK2 is known to be captured by the PIF (PDK1-interacting fragment)-binding pocket of PDK1, which stimulates phosphorylation of these AGC family kinases in the activation loop (Frodin *et al.*, 2000; Biondi *et al.*, 2001; Frodin *et al.*, 2002). To examine whether the observed hyperphosphorylation of Gad8-S546Dp by Ksg1p was due to a similar mechanism, we used PIF-tide, which is known to bind to the PIF-binding pocket on PDK1 and inhibit its ability to phosphorylate substrates (Biondi *et al.*, 2001). As shown in Figure 8D, the addition of PIF-tide reduced phosphorylation of both Gad8p and Gad8-S546Dp by Ksg1, showing more prominent reduction with Gad8-S546Dp. Thus, it appears likely that Tor1p-dependent phosphorylation of Gad8p on Ser546 facilitates the
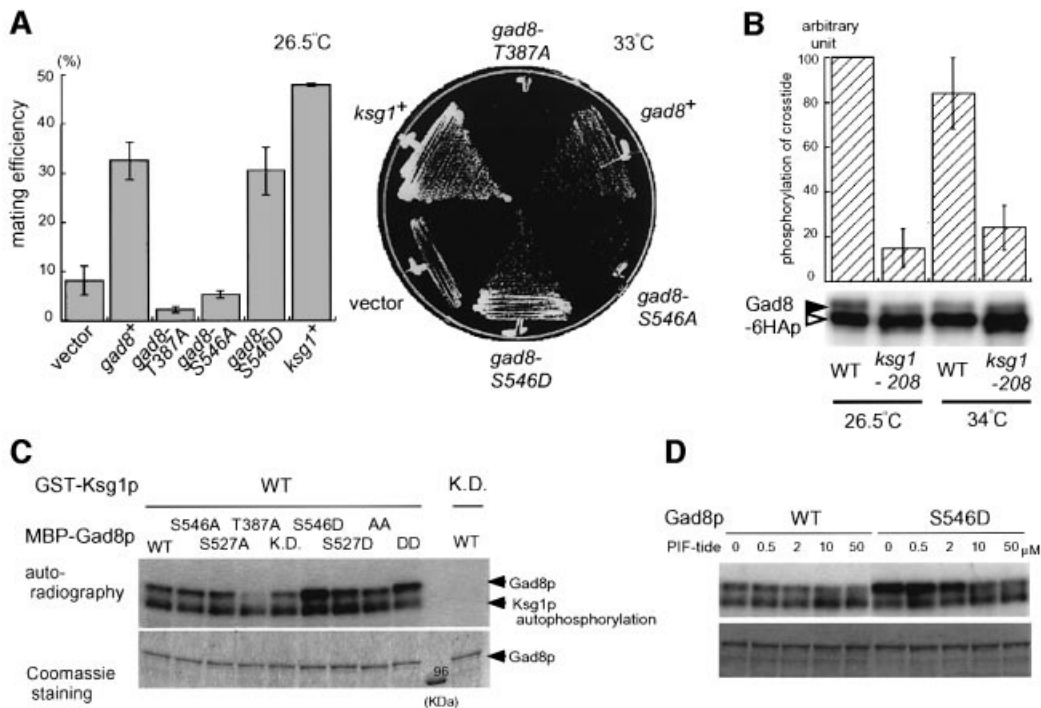
Fig. 8. Functional relationship of Gad8p with Ksg1p. (**A**) Suppression of *ksg1-208* by overexpression of *gad8*. The *ksg1-208* mutant (JW237) was transformed with pREP41-*gad8*, pREP41-*gad8-T387A*, pREP41-*gad8-S546A*, pREP41-*gad8-S546D*, pREP41-*ksg1* and the vector pREP41. Each transformant was examined for mating efficiency (left panel) and growth at the restrictive temperature (right panel). Transformants were cultured on SSA at 26.5°C for six days before scoring mating efficiency, and on MM medium at 33°C for three days to estimate temperature sensitivity. (**B**) The *ksg1-208* mutation affects the kinase activity of Gad8p. Cells expressing Gad8-6HAp in either the wild-type (JW960) or the *ksg1-208* (JW967) background were grown to logarithmic phase in YE liquid medium at 26.5°C and shifted to 34°C. Cells were harvested before and 4.5 h after the shift, and Gad8-6HAp was immunopurified from each sample. The kinase activity of Gad8-6HAp from each sample was assayed *in vitro* using crosstide. Immunoprecipitated Gad8-6HAp, detected by western blotting, is also shown. (**C**) Ksg1p phosphorylates Gad8p *in vitro*. Wild-type and mutant Gad8p fused to MBP, expressed bacterially and immunopurified, was incubated with GST-tagged Ksg1p in the presence of [γ-^{32}P]ATP. Phosphorylation of MBP–Gad8p was detected by autoradiography. Ksg1-K128Rp, a kinase-dead form of Ksg1, was also examined as a negative control. An autoradiograph is shown in the upper panel and a Coomassie staining pattern in shown in the lower panel. (**D**) Inhibition of the Ksg1p kinase activity by PIF-tide. Phosphorylation reaction of Gad8p and Gad8-S546Dp was conducted as described in (C), in the presence of PIF-tide at the concentration indicated.

interaction of Ksg1p with Gad8p via the PIF-binding pocket and enhances phosphorylation of Gad8p by Ksg1p on Thr387.

Discussion

The fission yeast *gad8* gene encodes a novel Ser/Thr kinase of the AGC family. Disruption of *gad8* results in the same phenotypes as disruption of *tor1*, both strains being defective in sexual development, in proper G$_1$ arrest under nitrogen starvation, and in growth at high temperature or high osmolarity. Importantly, both the kinase activity of Gad8p and its phosphorylation state change depending on the function of *tor1*, with strong suggestions that Tor1p is responsible for the phosphorylation of Gad8p on Ser527 and Ser546. Replacement of both Ser527 and Ser546 with alanine abolishes Gad8p function almost completely, highlighting the importance of the dual phosphorylation in the turn and hydrophobic motifs for Gad8p activity. Although it is probable that Tor1p might have a third target site on Gad8p in addition to Ser527 and Ser546, the extensive similarity between the phenotypes of the *gad8Δ* and the *gad8Δ tor1Δ* strains each transformed with *gad8-S527D/S546D* indicates that Ser527 and Ser546 are the major target sites for Tor1p. We have seen that Tor1p

prepared from fission yeast cells can phosphorylate Gad8p *in vitro* (our unpublished results), but this phosphorylation is very weak and it is as yet inconclusive as to whether the observed phosphorylation is physiologically meaningful or merely artefactual. Comparison of the Tor1p/Gad8p system to the mammalian system in which mTOR has been shown to phosphorylate S6K1 on Ser371 in the turn motif and on Thr389 in the hydrophobic motif *in vitro* (Burnett *et al*., 1998; Isotani *et al*., 1999; Saitoh *et al*., 2002), may suggest that Tor1p is likely to phosphorylate the two residues directly. However, it should be noted that the experiments on mTOR have not completely excluded the possibility that phosphorylation of S6K1 is due to an intermediate kinase co-immunoprecipitated with mTOR. Thus, it remains inconclusive whether Tor1p directly phosphorylates Gad8p on Ser527 and Ser546.

Our analysis has demonstrated that Ksg1p is responsible for the phosphorylation of Gad8p on Thr387. From the results of the *in vitro* study using bacterially expressed Ksg1p (Figure 8A), it appears plausible that Ksg1p phosphorylates Thr387 directly. Furthermore, it is also evident that Ksg1p has a target protein(s) other than Gad8p, which is essential for vegetative growth, as *ksg1Δ* is lethal and *gad8Δ* is not. Consistently, while overexpression of *gad8* can recover the growth of *ksg1-208* at

Table I. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype
JV107	h^{90} ade6-M210 leu1 ura4-D18 gad8S527A-6HA<< kan^r gad8::ura4$^+$
JV108	h^{90} ade6-M210 leu1 ura4-D18 gad8S527A/S546A-6HA<< kan^r gad8::ura4$^+$
JW237	h^{90} ade6-M216 leu1 ksg1-208
JW945	h^{90} ade6-M210 leu1 ura4-D18 gad8::ura4$^+$
JW948	h^{90} ade6-M216 leu1 gad2-1
JW951	h^{90} ade6-M210 leu1 ura4-D18 tor1::ura4$^+$
JW956	h^{90}/h^{90} ade6-M210/ade6M216 leu1/leu1 gad2-1/+
JW957	h^{90}/h^{90} ade6-M210/ade6M216 leu1/leu1 tor1::ura4$^+$/+ ura4-D18/+
JW958	h^{90}/h^{90} ade6-M210/ade6M216 leu1/leu1 tor1::ura4$^+$/gad2-1 ura4-D18/+
JW959	h^{90} ade6-M210 leu1 ura4-D18 gad8::ura4$^+$ tor1::ura4$^+$
JW960	h^{90} ade6-M210 leu1 ura4-D18 gad8-6HA<<kan^r gad8::ura4$^+$
JW961	h^{90} ade6-M210 leu1 ura4-D18 gad8K259R-6HA<< kan^r gad8::ura4$^+$
JW962	h^{90} ade6-M210 leu1 ura4-D18 gad8T387A-6HA<< kan^r gad8::ura4$^+$
JW963	h^{90} ade6-M210 leu1 ura4-D18 gad8S546A-6HA<< kan^r gad8::ura4$^+$
JW965	h^{90} ade6-M210 leu1 ura4-D18 tor1::ura4gad8-6HA<< kan^r gad8::ura4$^+$
JW967	h^{90} ade6-M210 leu1 ura4-D18 ksg1-208gad8-6HA<< kan^r gad8::ura4$^+$
JY476	h^{90} ade6-M210 leu1
JY878	h^{90} ade6-M216 leu1 ura4-D18
JZ489	h^{90}/h^{90} ade6-M210/ade6M216 leu1/leu1 ura4-D18/ura4-D18

Fig. 9. A model for the activation of Gad8p by Tor1p and Ksg1p. Gad8p is an essential factor required for sexual development and growth under stressed conditions. Tor1p is responsible for phosphorylation of Ser546 in the hydrophobic motif and Ser527 in the turn motif. Ksg1p phosphorylates Thr387 in the activation loop. Phosphorylation of these three residues is essential for the full activation of Gad8p. Phosphorylation of Ser546 by Tor1p may facilitate the access of Ksg1p to Gad8p and hence enhance the phosphorylation of Thr387.

high temperature (Figure 8), it cannot rescue the lethality of *ksg1Δ* (our unpublished results).

Gad8p recovered from the *ksg1-208* mutant is partially active, irrespective of the culture temperature (Figure 8). This suggests that the product of the *ksg1-208* allele is unlikely to be a thermo-labile protein but likely to have reduced Ksg1p activity at all temperatures, which is insufficient to support cell growth at a high temperature. The phenotypes of the *ksg1-208* mutant, namely sterility and temperature-sensitive growth, may be largely attributed to limited Gad8p activity in this strain due to the reduced Ksg1p kinase activity, as overproduction of Gad8p suppresses *ksg1-208* fairly well (Figure 8A and B).

Altogether, phosphorylation of Gad8p by Tor1p and Ksg1p appears to play a crucial role in the regulation of its kinase activity (schematically illustrated in Figure 9). The three phosphorylation sites on Gad8p, namely Thr387 in the activation loop, Ser527 in the turn motif and Ser546 in the hydrophobic motif, correspond to the conserved residues in AGC family kinases that are important for their activity (Toker and Newton, 2000; Newton, 2001). There are great similarities between the Tor1p–Ksg1p–Gad8p system we have identified in fission yeast and the mTOR–PDK1–S6K1 system found previously in mammals and *Drosophila*. S6K1, an AGC family kinase in mammalian cells, has been identified as a downstream effector of mTOR (Chung *et al.*, 1992; Price *et al.*, 1992). Rapamycin, which disrupts the function of mTOR, diminishes the kinase activity and the phosphorylation of S6K1. Thr229 in the activation loop of S6K1 is phosphorylated by PDK1 (Alessi *et al.*, 1998; Pullen *et al.*, 1998), whereas Ser371 in the turn motif and Thr389 in the hydrophobic motif are targeted by mTOR (Burnett *et al.*,
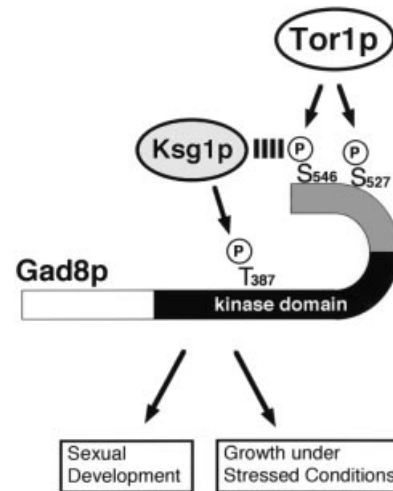
1998; Isotani *et al.*, 1999; Saitoh *et al.*, 2002). Substitution of each phosphorylation site with alanine results in a reduction in the S6K1 kinase activity (Pearson *et al.*, 1995; Moser *et al.*, 1997). *Drosophila* has a homologous signaling system, although less characterized than the one in mammals. The *Drosophila* ortholog of S6K1; named dS6K, is regulated by dTOR via phosphorylation of the hydrophobic motif (Oldham *et al.*, 2000; Zhang *et al.*, 2000; Radimerski *et al.*, 2002). It is also regulated by dPDK1 (Rintelen *et al.*, 2001; Radimerski *et al.*, 2002), however, at an unknown site and phosphorylation in the turn motif is unclear. In summary, although the upstream signals and downstream effectors may not be entirely the same, the system composed of the three kinases is apparently conserved in mammals, fly and fission yeast. We suspect that this three kinase system, involving TOR, PDK1-like and AGC family kinases, may be a basic module for signal transduction, which might compare with the ubiquitous MAP kinase cascade.

In budding yeast, Ypk1 and Ypk2/Ykr2 (Maurer, 1988; Chen *et al.*, 1993) are highly similar to Gad8p in the kinase domain and the C-terminal region. They are activated by PDK1-like kinases Pkh1 and Pkh2 in the sphingolipid-mediated signaling pathway, and regulate endocytosis and cell wall integrity (Sun *et al.*, 2000; Friant *et al.*, 2001; deHart *et al.*, 2002; Roelants *et al.*, 2002), with Pkh1 phosphorylating the activation loop of Ypk1 (Casamayor *et al.*, 1999). Some observations have implied that Ypk1 and Ypk2/Ykr2 are relevant to the TOR pathway (Gelperin *et al.*, 2002; Schmelzle *et al.*, 2002), presumably through Tor1 and Tor2 regulating Ypk1 and Ypk2/Ykr2 by phosphorylation of the apparently conserved turn and hydrophobic motifs.

Finally, the significance of the three kinase module in fission yeast physiology is considered. TOR proteins are thought to positively regulate translation in response to nutrient availability (Schmelzle and Hall, 2000; Gingras

et al., 2001). As shown in this study, fission yeast *tor1Δ* and *gad8Δ* cells grow slowly in rich medium. A previous study reported that *tor1Δ* cells grow as fast as wild-type cells (Weisman and Choder, 2001); we suspect that this discrepancy may have stemmed from the fact that *tor1Δ* as well as *gad8Δ* cells readily produce fast-growing pseudo-revertants (our unpublished results). Thus, it appears that *tor1* and *gad8* function positively for growth, as *ksg1* does. The three kinase module of fission yeast may have a role in harmonizing the balance of nutrients in the environment with cell growth and sexual development. A decrease in the Gad8p kinase activity apparently affects sexual development more severely than growth under stressed conditions. It is possible that cells may keep a certain level of Gad8p kinase activity under nutrient starvation in order to complete mating and meiosis, the energy-consuming processes that should proceed in the absence of fuel supply. The three kinase module may monitor the level of environmental nutrition or intracellular ATP and assure completion of sexual development.

Materials and methods

Yeast strains, media and genetic methods
Schizosaccharomyces pombe strains used in this study are listed in Table I. Yeast media YEA, SD, MM, SSA and MEA were used for routine culture of *S.pombe* strains (Sherman *et al.*, 1986; Moreno *et al.*, 1990). General genetic methods for *S.pombe* were described previously (Gutz *et al.*, 1974). Specific mutations were introduced into the *gad8* and *ksg1* genes according to a standard method (Kunkel, 1985). When a cross of a sterile haploid strain was desired, the strain was made fertile by introducing a plasmid carrying the respective wild-type gene, and the plasmid was removed after the cross.

Gene disruption
One-step gene disruption of *gad8* was carried out as follows. A 0.4 kb *Hinc*II–*Hinc*II fragment within the *gad8* open reading frame (ORF) corresponding to the kinase domain was replaced by a *ura4+* cassette. A diploid strain JZ489 (*ura4-D18/ura4-D18*) was transformed with a 3.4 kb *Eco*RI–*Pst*I fragment carrying this *gad8::ura4+* construct, and cells were spread on SD medium without uracil. Stable Ura4+ transformants were selected and proper disruption of *gad8* was confirmed in some of the transformants by PCR. To disrupt *tor1*, we employed the direct chromosomal integration method described previously (Bahler *et al.*, 1998), with some modifications. We first constructed a template vector for PCR, named pKS(FAura4), which was designed for the disruption of a target gene with *ura4+* instead of *kan^r* employed in the original protocol. We then used the following primers to generate a fragment for gene replacement. Forward: CATTGTGATGAATGCCTAAGTGGAAGAAT-TGAACACCGCGACTATTAGAAAGTCTATCGTTTCACTCGCTCT-CTTTGATTCCGGATCCCCGGGTTAATTAA. Reverse: CATTTTAA-AAAAAGGTAAAAGAGAAGTCTCTTTGAAATTTTTGATGAGTA-TGAGAAATAAAATAGTCATCCAGGAAAAGAGTTTAAACGAG-CTCGAATTC.

The PCR-generated fragment, in which the entire *tor1* ORF was substituted by *ura4+*, was transformed into JY878 (*ura4-D18*). We selected and confirmed *tor1* disruptants in the same way as *gad8* disruptants.

Construction of strains producing wild-type or mutant Gad8p tagged with 6HA
We followed a standard integration method using the integration vector int8, which was derived from int2 (Hirota *et al.*, 2001) by replacing the green fluorescent protein (GFP) ORF with 6HA. To connect the *gad8+* or each mutant *gad8* ORF, which was cloned in the vector pR3C (Matsuyama *et al.*, 2000), to the 6HA ORF in frame, a fragment flanked by the *Sal*I site on the vector and the *Bgl*II site created at the C-terminus of each *gad8* ORF was inserted between the *Sal*I and *Bam*HI sites of the int8 vector. The resultant plasmid was cleaved at the *Kpn*I site in the N-terminal region of the *gad8* ORF to boost integration efficiency and

transformed into JW945 (*gad8::ura4+*). The correct integration of the fusion gene was confirmed by PCR.

Detection of Gad8p by western blotting
Harvested cells were boiled, and then disrupted with glass beads in buffer A [50 mM Tris–Cl pH 7.6, 150 mM KCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol]. Each suspension containing 6 μg of protein was separated by SDS–PAGE. We used 8% separating acrylamide gel with the mono/bis ratio of 29.8:0.2 to detect Gad8p. Mouse anti-HA antibody 12CA5 (Sigma) was used as the primary antibody, and sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham-Pharmacia) was used as the secondary antibody. Enhanced chemiluminescence (Amersham-Pharmacia) was used for immunodetection on the membrane. For phosphatase treatment, cells were disrupted in buffer A containing 1% SDS. The supernatant containing 15 μg of protein was incubated with 20 U of CIAP (Takara Biomedicals) at 37°C for 30 min, either with or without the inhibitor mixture [10 mM EGTA, 0.1 mM Na₃VO₄, 20 mM β-glycerophosphate, 15 mM *p*-nitrophenyl phosphate (PNPP)]. Immunodetection of Gad8p was performed similarly to that described above.

Assay of the kinase activity of Gad8p
Schizosaccharomyces pombe cells producing 6HA-tagged Gad8p were disrupted with glass beads in buffer B [50 mM Tris–Cl 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 Na₃VO₄, 15 mM PNPP, 1 mM phenylmethyl-sulfonyl fluoride and the protease inhibitor cocktail (Roche)]. Gad8p was immunoprecipitated with monoclonal anti-HA antibody 16B12 (Berkeley Antibody Co.) and protein G–Sepharose (Amersham-Pharmacia) from each crude lysate containing 750 μg protein. The Sepharose beads were washed three times with buffer B and then twice with buffer C (20 mM HEPES–KOH pH 7.5, 10 mM MgCl₂, 1 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 15 mM PNPP). The reaction mix, adjusted to 25 μl with buffer C, contained 25 μM cold ATP, 5 μCi of [γ-³²P]ATP (Amersham-Pharmacia) and 100 μM crosstide (GRPR-TSSFAEG) (Upstate Biotechnology). After a 30 min incubation at 32°C, the reaction was stopped by boiling. Ten microliter of the mixture was spotted onto phosphocellulose paper (Whatman P81), which was washed five times by 75 mM phosphate buffer for 1 min, followed by an acetone wash for 5 min. The radioactivity was measured in a Scintillation Counter (LSC-5100; Aloka) and normalized by the quantum of Gad8p after subtracting radioactivity in the control specimen.

To evaluate the effect of phosphatase treatment on the kinase activity of Gad8p, Gad8p was immuprecipitated as described above. The Sepharose beads were washed three times with buffer B containing 500 mM NaCl instead of 150 mM KCl, and then washed three times by alkaline phosphatase buffer (Takara Biomedicals). The beads were incubated with 20 U of either natural or boiled CIAP (Takara Biomedicals) at 32°C for 30 min, with or without the inhibitor mixture. After the reaction, the beads were washed five times with buffer C, and then the activity of Gad8p was measured as described above.

Phosphorylation assay of Ksg1p in vitro
GST-fused Ksg1p and MBP-fused Gad8p were produced in *E.coli* BL21 by using expression vector pGEX 4T-3 (Pharmacia Biotech) and pMAL-cRI (New England Biolabs), respectively, together with the mutant variants of these fusion proteins. Affinity purification of the fusion proteins was carried out according to the protocol described previously (Guan and Dixon, 1991) or provided by the manufacturer, except that buffer D (50 mM Tris–Cl pH 7.6, 0.1 mM EGTA, 10 mM β-mercaptoethanol) was used as the solvent for elution. MBP–Gad8p (0.5 μg) was incubated with 0.05 μg of GST–Ksg1p in buffer D containing 4 μCi of [γ-³²P]ATP, 100 μM cold ATP and 10 mM MgCl₂ for 30 min at 30°C. In some experiments, PIF-tide (PILTPPREPRI-LSEEEQEMFRDFAYL) (Biondi *et al.*, 2001), supplied by a custom peptide synthesis service (BEX Corporation), was added to the reaction mix. The reaction was stopped by boiling the mix with SDS–PAGE sample buffer, and proteins were separated by 10% SDS–PAGE, followed by Coomassie Brilliant Blue staining and autoradiography.

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