

# Methylglyoxal Activates the Target of Rapamycin Complex 2-Protein Kinase C Signaling Pathway in *Saccharomyces cerevisiae*

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**Methylglyoxal is a typical 2-oxoaldehyde derived from glycolysis. We show here that methylglyoxal activates the Pkc1-Mpk1 mitogen-activated protein (MAP) kinase cascade in a target of rapamycin complex 2 (TORC2)-dependent manner in the budding yeast *Saccharomyces cerevisiae*. We demonstrate that TORC2 phosphorylates Pkc1 at Thr<sup>1125</sup> and Ser<sup>1143</sup>. Methylglyoxal enhanced the phosphorylation of Pkc1 at Ser<sup>1143</sup>, which transmitted the signal to the downstream Mpk1 MAP kinase cascade. We found that the phosphorylation status of Pkc1<sup>T1125</sup> affected the phosphorylation of Pkc1 at Ser<sup>1143</sup>, in addition to its protein levels. Methylglyoxal activated mammalian TORC2 signaling, which, in turn, phosphorylated Akt at Ser<sup>473</sup>. Our results suggest that methylglyoxal is a conserved initiator of TORC2 signaling among eukaryotes.**

All biological activities in organisms must be accompanied by an ample supply of nutrients, which warrants energy production to meet the demand for biochemical reactions in cells. Therefore, the machinery for sensing and transducing nutritional conditions inside and outside the cell plays crucial roles. Nutrients and hormones are pivotal signal initiation molecules that regulate cellular energy metabolism in animals. Although nutrients are essential for living cells, several adverse by-products, such as reactive oxygen species (ROS), are formed during energy metabolism (1). ROS are inevitably formed by oxygen respiration in accordance with ATP production and cause oxidative stress, which damages cellular components (2).

We have been focusing on methylglyoxal (MG) and its role as a metabolic stressor. MG (CH<sub>3</sub>COCHO) is a typical 2-oxoaldehyde, the main source of which in the cell is glycolysis, a ubiquitous energy-producing pathway (3, 4). Since MG is highly reactive because of its two carbonyl groups in one molecule, it reacts with DNA/RNA and proteins to make adducts that change their functions, and this change of function sometimes leads to several diseases, such as diabetes and Alzheimer's disease (5–7). MG and other carbonyl compounds produce various carbonyl compound-modified proteins through a process that is sometimes referred to as carbonyl stress. Carbohydrate-derived carbonyl compounds produce advanced glycation end products (AGEs) via spontaneous reactions with the amino groups of proteins and carbonyl compounds. However, even though the reactivity of MG with proteins is >20,000-fold higher than that of glucose, the formation of AGEs has been shown to occur on a time scale of days, weeks, or longer (8, 9). The harmful effects of MG on cellular functions have been attributed to MG-derived AGEs (4–9). We previously reported that MG causes acute cellular responses in yeasts (10, 11). For example, the exogenous addition of MG activated yeast AP-1-like transcription factors (Yap1 in *Saccharomyces cerevisiae* and Pap1 in *Schizosaccharomyces pombe*) through the reversible modification of their critical Cys residues within 45 min (10, 11). MG was also found to enhance the influx of extracellular Ca<sup>2+</sup> into the cell, thereby activating the Ca<sup>2+</sup>/calcineurin system in *S. cerevisiae* (12). In the present study, we found that MG enhances the target of rapamycin (TOR) complex 2 (TORC2)-protein kinase C (Pkc1) signaling pathway in *S. cerevisiae*.

The TOR signaling network is evolutionally conserved among

eukaryotes and is involved in diverse cellular activities (13, 14). TOR is a Ser/Thr kinase that forms two distinct protein kinase complexes, TORC1 and TORC2 (15). The TOR complex phosphorylates protein kinases involved in the AGC (named initially for cyclic AMP- and cyclic GMP-dependent protein kinases and protein kinase C) kinase family (16). *S. cerevisiae* TORC1 has been shown to phosphorylate Sch9, whereas TORC2 phosphorylates Ypk1 and Ypk2 (17–19), both of which belong to the AGC kinase family. A previous study demonstrated that TOR complexes phosphorylate conserved Thr/Ser residues within the turn motif (TM) and hydrophobic motif (HM) of AGC kinase (16). *PKC1* is the only gene that encodes protein kinase C in *S. cerevisiae* (20, 21), and Pkc1 structurally belongs to the AGC kinase family. Thr/Ser residues in the TM and HM were also found to be conserved in Pkc1 (Thr<sup>1125</sup> in TM and Ser<sup>1143</sup> in HM). Genetic interactions between *PKC1* and some genes (*TOR2*, *AVO1*, and *AVO3*), the products of which are involved in the assembly of TORC2, have been reported previously (15, 22–24); however, it still remains unclear whether Pkc1 is a direct target of TORC2 (25).

TORC1 is involved in events concerning cell growth, such as protein synthesis, while TORC2 plays a role in actin organization (14). In the present study, we demonstrate that the organization of actin is perturbed by an MG treatment, thereby inhibiting the polarized cell growth of *S. cerevisiae*. Since TORC2 is involved in actin organization, we investigated the relationship between MG and TORC2 signaling. We show that Thr<sup>1125</sup> and Ser<sup>1143</sup> in Pkc1 are phosphorylated by TORC2 both *in vivo* and *in vitro*. We also

Received 2 September 2014 Returned for modification 28 September 2014

Accepted 18 January 2015

Accepted manuscript posted online 26 January 2015

Citation Nomura W, Inoue Y. 2015. Methylglyoxal activates the target of rapamycin complex 2-protein kinase C signaling pathway in *Saccharomyces cerevisiae*. *Mol Cell Biol* 35:1269–1280. doi:10.1128/MCB.01118-14.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/MCB.01118-14>.

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found that MG enhances TORC2-Pkc1 signaling, and the downstream Mpk1 mitogen-activated protein (MAP) kinase cascade was subsequently activated following the treatment of yeast cells with MG. Furthermore, we show that MG activates TORC2 signaling in mammalian cells. A physiological trigger that has been shown to activate TORC2 signaling is insulin (and insulin-like growth factor) (26–28); however, lower eukaryotes, such as yeast, do not have a hormonal system. Therefore, it remains unclear whether the ubiquitous physiological molecules that activate TORC2 exist among eukaryotes. Our results suggest that MG is a common initiator of TORC2 signaling.

## MATERIALS AND METHODS

**Media.** The media used were synthetic dextrose (SD) medium (2% glucose, 0.67% yeast nitrogen base without amino acids) and synthetic complete (SC)-Gal medium (2% galactose, 3% glycerol, 0.67% yeast nitrogen base without amino acids). Appropriate amino acids and bases were added to SD or SC-Gal medium as necessary. Cells were cultured at 28°C unless otherwise stated.

**Strains.** The yeast strains and PCR primers used are summarized in Tables S1 and S2 in the supplemental material. The deletion allele of *MID2* with *KanMX* or *his5<sup>+</sup>* was amplified by PCR with primer sets mid2-F and mid2-R from *S. cerevisiae* BY4741-based deletion mutants (29). The corresponding loci of *S. cerevisiae* YPH250 were disrupted using PCR products. To construct *wsc1Δ* and *mpk1Δ* mutants, the *wsc1Δ::CgLEU2* allele of YOC2573 (30) and the *mpk1Δ::HIS3* allele of TNP46 (31) were amplified by PCR with primers WSC1F and WSC1R and primers MPK1FSall and MPK1REcoRI, respectively. The disruption of *MKK1*, *MKK2*, and *BCK1* was performed as described previously (32).

**Plasmids.** The plasmids used are summarized in Table S3 in the supplemental material. Details for the construction of plasmids are described in the supplemental material.

**Actin staining.** Cells were cultured in SD medium until the  $A_{610}$  reached 0.3 to 0.5. MG was then added. Cells were fixed with formaldehyde (final concentration, 4%) for 1 h. After fixation, the cells were harvested, washed twice with phosphate-buffered saline (PBS; pH 7.4), and suspended in 30  $\mu$ l of PBS. Rhodamine-phalloidin (Molecular Probes) was added to the cell suspension to a final concentration of 33 units/ml (1.1  $\mu$ M), and the cell suspension was then incubated at 4°C in the dark overnight. Cells were collected by centrifugation and washed twice with PBS, and the distribution of actin was observed using a fluorescence microscope.

**Immunoprecipitation.** Cells were cultured in SD medium until the  $A_{610}$  reached 0.5. Cells were then collected, washed with a 0.85% NaCl solution, and suspended in lysis buffer B (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween 20) containing 2.5 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and protease inhibitor cocktail (Nacalai Tesque). Cells were disrupted with glass beads using a Beads Smash 12 cell disrupter (Wakenyaku), and cell homogenates were centrifuged at 700  $\times$  g for 10 min at 4°C to remove cell debris. The protein concentrations in the cell extracts were determined using a DC protein assay (Bio-Rad Laboratories). The Avo3-13myc, Pkc1 tagged with a 3 $\times$  hemagglutinin tag (Pkc1-3HA), or Pkc1 tagged with a 3 $\times$  FLAG tag (Pkc1-3FLAG) protein was immunoprecipitated from the cell extracts (1.5 or 1 mg protein) by incubation with anti-c-myc antibodies coupled with agarose resin (Nacalai Tesque), anti-HA antibodies coupled with agarose resin (MBL), or anti-FLAG antibody-conjugated resin (Sigma) for 2 h at 4°C in lysis buffer B. After being incubated, the agarose resins were precipitated by centrifugation, washed four times with lysis buffer B, and suspended in SDS-PAGE sample buffer. SDS-PAGE was then performed, followed by Western blotting.

**Bacterial expression and purification of Ypk2.** *Escherichia coli* BL21(DE3) cells carrying pET-15b-Ypk2 were grown in LB medium containing ampicillin at 37°C until the  $A_{610}$  reached 0.3, and isopropyl- $\beta$ -D-

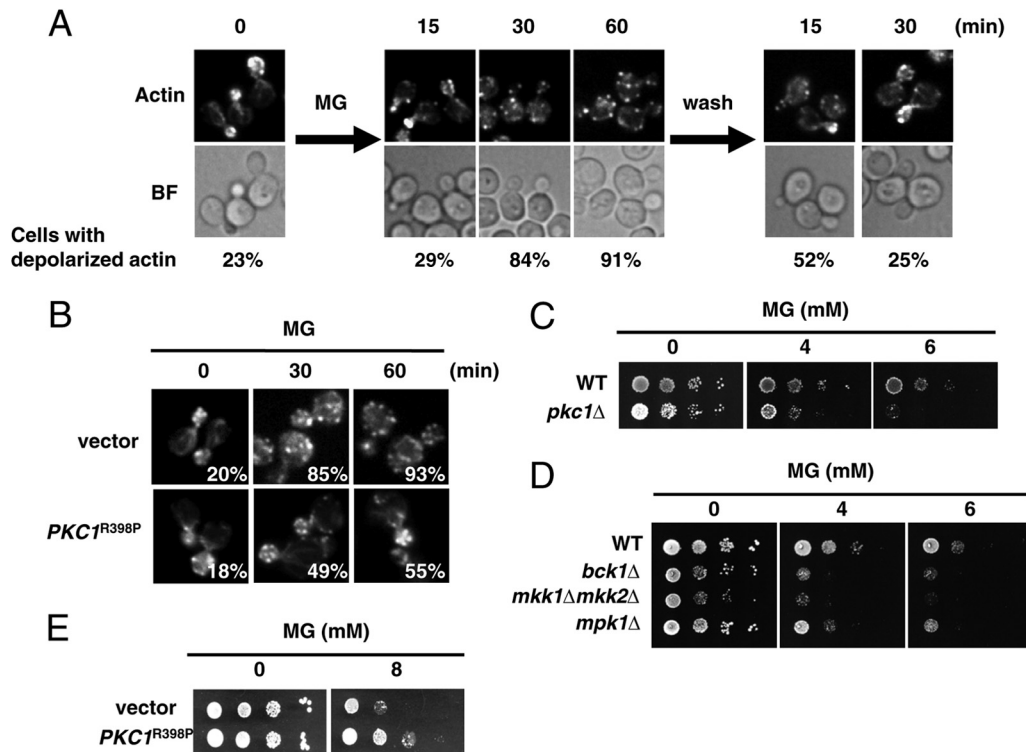
thiogalactopyranoside was added to a final concentration of 0.2 mM. Cells were incubated at 25°C overnight and then disrupted by sonication at 4°C in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 mM NaCl and 10 mM imidazole. Cell homogenates were centrifuged to remove cell debris, cell extracts were applied to a HisTrap<sup>TM</sup> HP column (GE Healthcare) equilibrated with sonication buffer, and the column was washed with the same buffer. The proteins that were adsorbed were eluted with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 mM NaCl and 250 mM imidazole. Fractions containing Ypk2 were dialyzed against 20 mM HEPES-KOH buffer (pH 7.4) containing 150 mM NaCl and 2 mM dithiothreitol.

**In vitro protein kinase assay.** An *in vitro* protein kinase assay for TORC2 was performed as described previously (17), with some modifications. Briefly, TORC2 was immunopurified using myc-tagged Avo3 (Avo3-myc) instead of HA-tagged Tor2 (HA-Tor2), which was used in a previous study (17). Immunopurified TORC2 was incubated with 5  $\mu$ g of Pkc1 peptides (for wild-type [WT] Pkc1 [Pkc1<sup>WT</sup>], APPTLTPLPSVLTTTSQQEEFRGFSFMPDDL; for Pkc1 with the T1125A and S1143A mutations [Pkc1<sup>T1125A/S1143A</sup>], APPTLAPLPSVLTTTSQQEEFRGFAMPDDL) or 4  $\mu$ g of recombinant Ypk2 protein. Synthetic Pkc1 peptides were produced by the GenScript Corporation (Piscataway, NJ). The reaction was initiated by adding [ $\gamma$ -<sup>32</sup>P]ATP (10  $\mu$ Ci). After being incubated for 30 min at 30°C, the reaction was terminated by the addition of SDS-PAGE sample buffer, and the samples were then incubated for 5 min at 65°C. Samples were subjected to Tricine-SDS-PAGE (33) or SDS-PAGE, and phosphorylated peptides were detected by autoradiography.

**Western blotting of Mpk1.** Cells were collected and washed with lysis buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 mM NaF, 2  $\mu$ g each of pepstatin A and leupeptin per ml), and cell pellets were frozen with liquid nitrogen. Cell pellets were suspended in lysis buffer A and agitated with glass beads using a Beads Smash 21 cell disrupter (Wakenyaku). Cell homogenates were centrifuged for 5 min at 15,000  $\times$  g and 4°C, and the protein concentration in clear supernatants was determined using the DC protein assay (Bio-Rad Laboratories). Samples were subjected to SDS-PAGE, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore). The blots were incubated with appropriate dilutions of the primary antibodies (anti-phospho-p44/42 MAP kinase [Thr<sup>202</sup>/Tyr<sup>204</sup>], Cell Signaling) and anti-Mpk1 [ $\gamma$ C-20; catalog number sc-6803; Santa Cruz Biotechnology]). Immunoreactive bands were visualized with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium solution kit for alkaline phosphatase (Nacalai Tesque) or Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore) using a LAS-4000 mini-imaging system (Fujifilm).

**Western blotting of Pkc1.** Total protein extracts were prepared using the trichloroacetic acid extraction method (12). The protein concentrations of the samples were determined using an RC DC protein assay kit (Bio-Rad Laboratories). The antibodies used for Western blotting were anti-Pkc1 antibodies.

**Anti-Pkc1, anti-p-T1125, and anti-p-S1143 antibodies.** Anti-Pkc1 polyclonal antibodies were raised by immunizing rabbits with a peptide corresponding to amino acid residues 470 to 488 in Pkc1. Antisera were used to detect Pkc1 in Western blots. Anti-phospho-Pkc1 polyclonal antibodies were raised by immunizing rabbits with Pkc1 containing a phosphorylated Thr<sup>1125</sup> peptide (p-T1125) in the turn motif [NH<sub>2</sub>-C+APPTL(pT)PLPSVLTTTSQQEE-COOH] or a phosphorylated Ser<sup>1143</sup> peptide (p-S1143) in the hydrophobic motif [NH<sub>2</sub>-C+EFRGF(pS)FMPD-COOH]. Antisera were loaded onto a column packed with the resin conjugated with each of the phosphorylated peptides. After the adsorbed fractions were recovered, they were passed through a column packed with the resin conjugated with each of the unphosphorylated peptides. The fractions were recovered and used as anti-phospho-Pkc1 at Thr<sup>1125</sup> (anti-p-T1125) or anti-phospho-Pkc1 at Ser<sup>1143</sup> (anti-p-S1143) antibodies.



**FIG 1** MG depolarizes the actin cytoskeleton. (A) Cells (YPH250) were cultured in SD medium until the  $A_{610}$  was 0.3 and were then treated with 10 mM MG for 60 min. Cells were stained for actin with rhodamine-phalloidin and observed using a fluorescence microscope. The proportion of cells with depolarized actin was determined by counting the cells in which actin had not accumulated in the bud. More than approximately 200 cells were counted in each experiment. BF, bright field. (B) Cells (YPH250) carrying YCp50 (vector) or YCp50-*PKC1<sup>R398P</sup>* were cultured in SD medium until the  $A_{610}$  was 0.3 and were then treated with 10 mM MG for the times indicated. Cells were stained for actin with rhodamine-phalloidin. The proportion of cells with depolarized actin is indicated for each image. (C) Wild-type (DL100) and *pkc1Δ* (DL376) cells were cultured in SD medium containing 0.5 M sorbitol until the log phase of growth and serially diluted (1:10) with 0.5 M sorbitol solution. An aliquot (4  $\mu$ l) of each cell suspension was spotted onto SD agar plates containing 0.5 M sorbitol with or without MG. (D) Cells (YPH250) defective in the components of the Mpk1 MAP kinase cascade (*bck1Δ*, *mkk1Δ mkk2Δ*, and *mpk1Δ* cells) were cultured in SD medium until the log phase of growth and serially diluted (1:10) with 0.85% NaCl solution, and 4  $\mu$ l of each cell suspension was then spotted onto SD agar plates containing MG. (E) Cells (YPH250) carrying YCp50 (vector) or YCp50-*PKC1<sup>R398P</sup>* were cultured in SD medium until the log phase of growth. The cell suspension was diluted as described in the legend to panel D, and 4  $\mu$ l of each suspension was spotted onto SD agar plates containing MG.

**Culture conditions for mammalian cells.** Mouse 3T3-L1 cells were maintained in maintenance medium (10% fetal bovine serum and 10 mg/ml penicillin-streptomycin in Dulbecco modified Eagle medium [DMEM]) at 37°C with 5% CO<sub>2</sub>. Preadipocytes were differentiated to adipocytes by the established protocol (34) as briefly described below. 3T3-L1 preadipocytes were grown for 2 days until postconfluence, and medium was switched to a differentiation medium consisting of the maintenance medium supplemented with 0.25  $\mu$ M dexamethasone, 10  $\mu$ g/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine. After 48 h, the medium was replaced with a postdifferentiation medium consisting of the maintenance medium supplemented with 5  $\mu$ g/ml insulin. Medium was exchanged every 2 days with a fresh postdifferentiation medium until the cells were ready for experimentation.

3T3-L1 adipocytes were incubated in the maintenance medium for 20 h in DMEM for Western blotting and then starved of serum for 5 h in DMEM. Cells were lysed with SDS-PAGE sample buffer, and the protein concentrations of the samples were determined using the RC DC protein assay kit (Bio-Rad Laboratories). The antibodies used for Western blotting were anti-Akt antibodies (Cell Signaling) to detect the bulk protein levels of Akt, anti-phospho-Akt (Ser<sup>473</sup>) antibodies (Cell Signaling) to detect the phosphorylation of Ser<sup>473</sup> in the HM of Akt, and anti-phospho-PKC $\alpha$ / $\beta$ II (Thr<sup>638/641</sup>) antibodies (Cell Signaling) to detect the phosphorylation of Thr<sup>450</sup> in the TM of Akt (35). Immunoreactive bands were visualized with a kit (Immobilon Western chemi-

luminescent HRP substrate; Millipore) and an LAS-4000 mini-imaging system (Fujifilm).

## RESULTS

**MG perturbs actin organization.** We previously demonstrated that a moderate concentration of MG reversibly inhibits the growth of *S. cerevisiae* cells without decreasing cell viability (12). *S. cerevisiae* exhibits polarized growth, and actin is known to play crucial roles in determining cellular polarity (36). To gain insight into the MG-induced inhibition of growth, we examined the organization of actin. Actin patches accumulated in the buds of logarithmically growing cells; however, actin patches were almost completely (84 to 91%) and uniformly dispersed in both the mother cell and bud 30 to 60 min after the MG treatment (Fig. 1A). The depolarization of actin patches by the MG treatment was reversible; i.e., the patches returned to the bud 30 min after the removal of MG from the medium (Fig. 1A).

*PKC1* with the R398P mutation (*PKC1<sup>R398P</sup>*) is a constitutively active allele of *PKC1* that has been shown to suppress the depolarization of actin patches in a *rho1-2* temperature-sensitive mutant at nonpermissive temperatures when *PKC1<sup>R398P</sup>* is expressed using its own promoter in a single-copy plasmid (23, 37). Therefore,

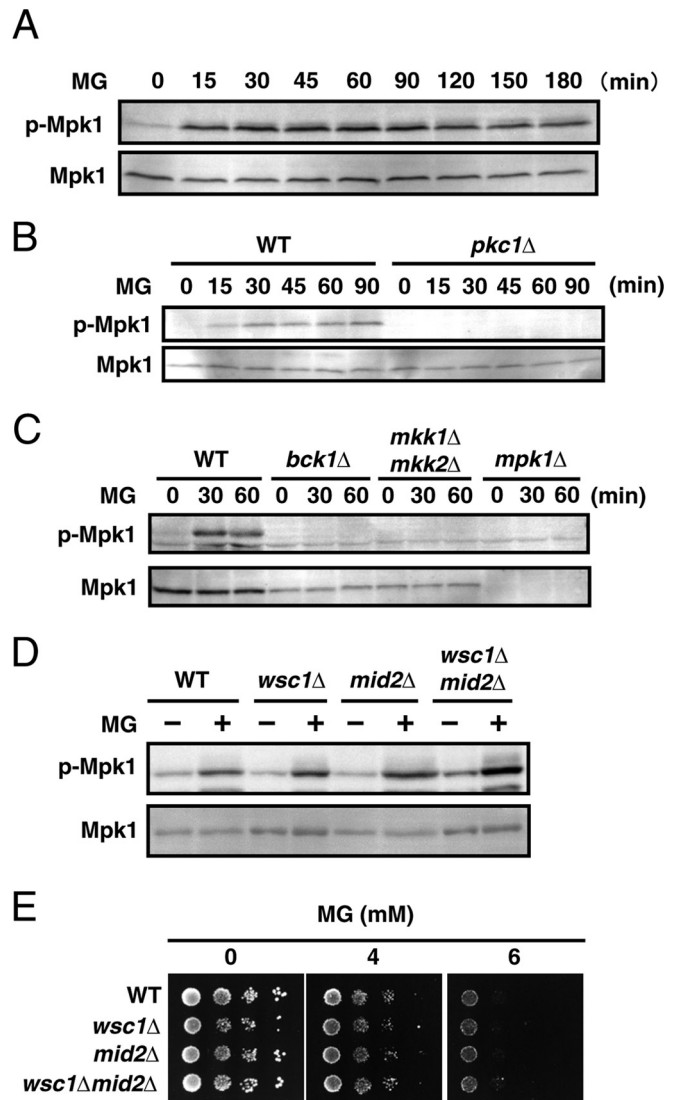
we investigated whether the depolarization of actin patches in the presence of MG is alleviated by the expression of *PKC1<sup>R398P</sup>*. As shown in Fig. 1B, *Pkc1<sup>R398P</sup>* partially repressed the depolarization of actin patches in cells treated with MG.

**Pkc1-Mpk1 MAP kinase cascade mutants are sensitive to MG.** The organization of actin was previously shown to be regulated by the Pkc1-Mpk1 MAP kinase cascade (38, 39). To verify whether MG influences the growth of mutants defective in the Pkc1-Mpk1 MAP kinase cascade, we conducted spot assays on SD agar plates containing MG. *pkc1Δ* cells are not able to grow without sorbitol (38) because synthesis of the cell wall is defective in this mutant and cell lysis occurs without an osmoprotectant. Therefore, spot assays were performed using SD agar plates containing sorbitol. As shown in Fig. 1C, the *pkc1Δ* mutant exhibited susceptibility to MG. Similarly, *bck1Δ*, *mkk1Δ mkk2Δ*, and *mpk1Δ* mutants, defective in the MAP kinase cascade, also showed susceptibility to MG (Fig. 1D). Wild-type cells displayed an increased tolerance toward MG when *PKC1<sup>R398P</sup>* was expressed from its own promoter using a single-copy plasmid (Fig. 1E).

**MG activates the Mpk1 MAP kinase cascade.** Since mutants defective in the Pkc1-Mpk1 MAP kinase cascade were sensitive to MG (Fig. 1C and D), we speculated that MG may activate this signaling pathway to combat MG-induced cellular stress. We monitored the phosphorylation levels of Mpk1. As shown in Fig. 2A, Mpk1 was phosphorylated following treatment with MG, whereas no phosphorylated band was detected in *pkc1Δ* cells or in any of the Mpk1 MAP kinase cascade mutants (Fig. 2B and C). These results indicate that MG activates the Pkc1-Mpk1 MAP kinase cascade.

The membrane-integrated proteins Wsc1 and Mid2 act as sensors for the heat shock-induced activation of the Mpk1 MAP kinase cascade (see Fig. 8) (38–40). To explore whether these proteins function as sensors for MG-triggered stress, we disrupted *WSC1* and *MID2* and determined the phosphorylation of Mpk1 in the resultant mutants. However, as shown in Fig. 2D, Mpk1 was phosphorylated following treatment with MG in *wsc1Δ*, *mid2Δ*, and *wsc1Δ mid2Δ* cells. Subsequently, susceptibility to MG was not increased by the deletion of *WSC1* and/or *MID2* (Fig. 2E). Hence, the MG-induced activation of the Pkc1-Mpk1 MAP kinase cascade occurred irrespective of the Wsc1/Mid2 pathway. Besides Wsc1/Mid2, Wsc2 and Mtl1 have also been reported to act as upstream sensors for the Pkc1-Mpk1 pathway (41, 42). Therefore, we determined whether these proteins are involved in the MG-induced phosphorylation of Mpk1. As shown in Fig. S1A in the supplemental material, the phosphorylation of Mpk1 occurred in *wsc2Δ* and *mtl1Δ* mutants following treatment with MG, and these mutants did not subsequently exhibit increased sensitivity to MG (see Fig. S1B in the supplemental material).

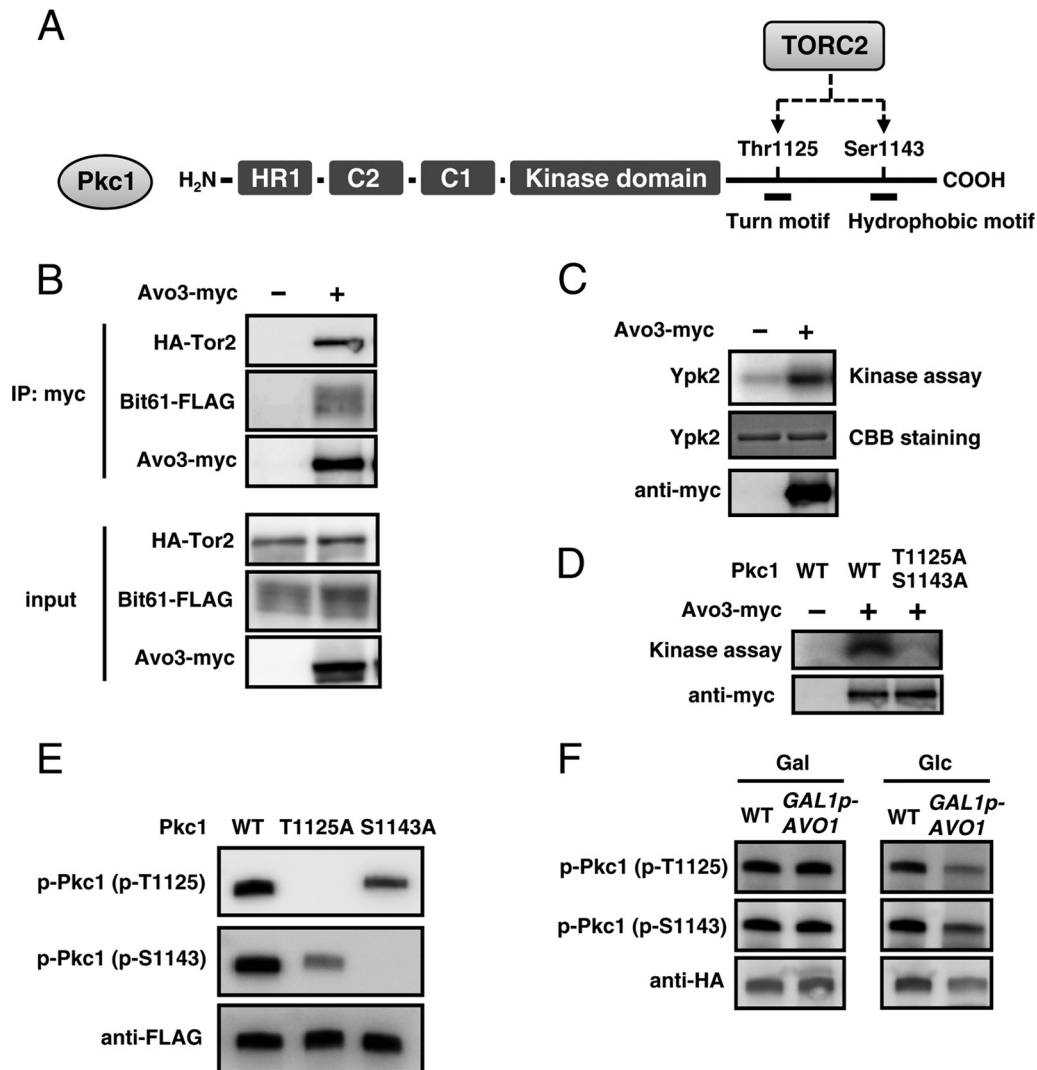
**Yeast TORC2 phosphorylates Pkc1.** Previous studies suggested that genetic interactions may occur between TORC2 and the Pkc1-Mpk1 signaling pathway (22–24, 43). For example, the temperature sensitivity of a temperature-sensitive (*ts*) *tor2* (*tor2<sup>ts</sup>*) mutant was suppressed by the overexpression of *RHO1*, *PKC1*, and *MPK1* (22, 23, 43). Pkc1 belongs to the AGC kinase family, and AGC kinases are generally the targets of TOR kinases (16). Potential phosphorylation sites (Thr<sup>1125</sup> in TM and Ser<sup>1143</sup> in HM) are conserved in Pkc1 (Fig. 3A) (16, 35, 44); however, it currently remains unknown whether yeast TORC2 directly phosphorylates Pkc1. To gain insight into the MG-induced activation of the Pkc1-



**FIG 2** Effects of MG on activation of the Mpk1 MAP kinase cascade. (A) Cells (YPH250) were cultured in SD medium until the  $A_{610}$  was 0.3, and 10 mM MG was added. The levels of phosphorylation of Mpk1 (p-Mpk1) and the Mpk1 protein (Mpk1) were determined after incubation for the prescribed times. (B) Wild-type (DL100) and *pkc1Δ* (DL376) cells were cultured in SD medium containing 1 M sorbitol until the  $A_{610}$  was 0.3 to 0.5 and were treated with 10 mM MG for the prescribed times. (C) Cells (YPH250) defective in the components of the Mpk1 MAP kinase cascade (*bck1Δ*, *mkk1Δ mkk2Δ*, and *mpk1Δ* cells) were cultured in SD medium containing 1 M sorbitol until the  $A_{610}$  was 0.3 to 0.5, and 10 mM MG was added. (D) Wild-type (YPH250), *wsc1Δ*, *mid2Δ*, and *wsc1Δ mid2Δ* cells were cultured in SD medium until the  $A_{610}$  was 0.3 to 0.5 and treated with 10 mM MG for 30 min, and the phosphorylation of Mpk1 was then determined. (E) The cells of each mutant in the YPH250 background were cultured in SD medium until the log phase of growth and serially diluted (1:10) with a 0.85% NaCl solution, and 4  $\mu$ l of each cell suspension was then spotted onto SD agar plates containing MG.

Mpk1 MAP kinase cascade, we investigated whether Pkc1 is a substrate of TORC2.

TORC2 consists of Tor2, Avo1, Avo2, Avo3, Bit61, and Lst8. Tor2 and Lst8 have also been shown to be involved in TORC1 (14, 15). We carried out the immunoprecipitation of TORC2 from yeast cells that simultaneously expressed Avo3-myc, Tor2-HA, and FLAG-tagged Bit61 (Bit61-FLAG). The immunoprecipitation of

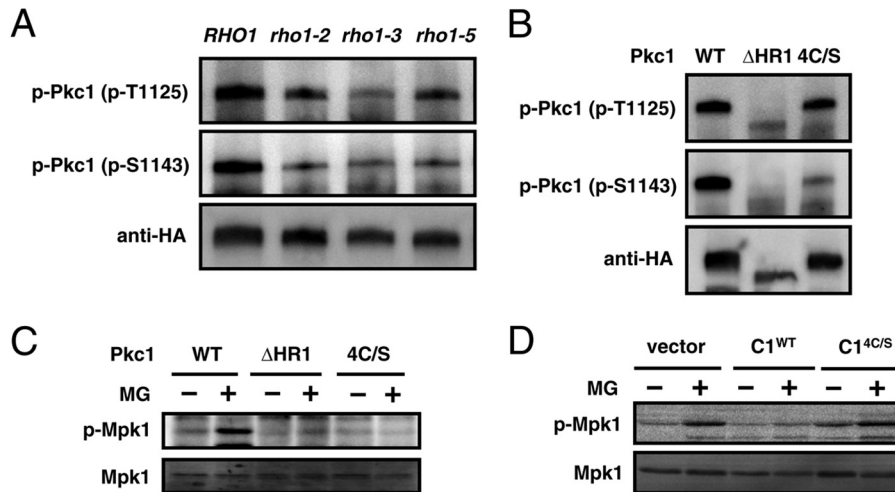


**FIG 3** TORC2 phosphorylates Pkc1. (A) Diagram of Pkc1. The HR1 domain, C1 domain, and C2 domain represent homology region 1, the cysteine-rich domain, and the lipid-binding domain, respectively. AGC kinases contain conserved Ser/Thr in the turn motif (TM) and the hydrophobic motif (HM), which are phosphorylated by TOR kinases. Pkc1 also contains Thr<sup>1125</sup> in TM and Ser<sup>1143</sup> in HM. Both are potential phosphorylation sites of TORC2; however, TORC2-dependent phosphorylation of these sites has not yet been biochemically confirmed. (B) TORC2 components were immunoprecipitated (IP) using anti-myc antibodies from wild-type (YPH250) cells with or without Avo3-myc carrying HA-Tor2 and Bit61-FLAG. Each TORC2 component was determined using the respective antibodies for each tag. (C) Immunoprecipitated TORC2 containing Avo3-myc from wild-type (YPH250) cells was incubated with [ $\gamma$ -<sup>32</sup>P]ATP and recombinant Ypk2. The phosphorylation of Ypk2 was detected by autoradiography. CBB, Coomassie brilliant blue. (D) The WT Pkc1 peptide or an Ala-substituted variant (T1125A/S1143A) was incubated with immunopurified TORC2 and [ $\gamma$ -<sup>32</sup>P]ATP. After Tricine-SDS-PAGE, the phosphorylation of each Pkc1 peptide was detected by autoradiography. (E) Cells (YPH250) carrying *PKC1*-3FLAG (WT), *PKC1*<sup>T1125A</sup>-3FLAG (T1125A), or *PKC1*<sup>S1143A</sup>-3FLAG (S1143A) were cultured in SD medium until the  $A_{610}$  was 0.4 to 0.6. After the immunoprecipitation of FLAG-tagged Pkc1 using anti-FLAG antibody-conjugated resin, samples were subjected to SDS-PAGE followed by Western blotting using anti-phosphorylated Pkc1<sup>T1125</sup> antibodies [p-Pkc1 (p-T1125)], anti-phosphorylated Pkc1<sup>S1143</sup> antibodies [p-Pkc1 (p-S1143)], or an anti-FLAG monoclonal antibody (Sigma). (F) Wild-type (TB50a) and *GAL1p-AVO1* (RL25-1c) cells carrying pFL39-PKC1-3HA were cultured overnight in SC-Gal medium (Gal). After being transferred to SD medium (Glc), cells were cultured for 17 h to impair TORC2 activity. After the immunoprecipitation of HA-tagged Pkc1 using the anti-HA antibody-conjugated resin, samples were subjected to SDS-PAGE followed by Western blotting using anti-phosphorylated Pkc1<sup>T1125</sup> antibodies [p-Pkc1 (p-T1125)], anti-phosphorylated Pkc1<sup>S1143</sup> antibodies [p-Pkc1 (p-S1143)], or the anti-HA monoclonal antibody (Cell Signaling).

Avo3-myc was performed using anti-myc monoclonal antibody-conjugated resin, and we verified that Tor2-HA and Bit61-FLAG were coimmunoprecipitated with Avo3-myc (Fig. 3B). Since Tor2 is involved not only in TORC2 but also in TORC1 (15), we performed immunoprecipitation with myc-tagged Avo3, which was previously shown to be specifically involved in TORC2 (18, 45), in order to reduce the probability of contamination of TORC1. We then determined whether immunopurified TORC2 actually pos-

sessed TORC2 activity. Since the substrate of yeast TORC2 has been identified to be Ypk2 (17), we carried out an *in vitro* kinase assay using a 6 $\times$ His-tagged Ypk2 protein purified from recombinant *E. coli* cells as a substrate. As shown in Fig. 3C, immunopurified TORC2 was able to phosphorylate 6 $\times$ His-Ypk2. Therefore, the *in vitro* kinase assay system that we constructed was confirmed to work properly.

Using this assay system, we conducted an *in vitro* kinase assay



**FIG 4** Role of Rho1 in MG-triggered TORC2-Pkc1 signaling. (A) *RHO1* (YOC764), *rho1-2* (YOC752), *rho1-3* (YOC729), and *rho1-5* (YOC755) cells carrying pFL39-PKC1-3HA were cultured in SD medium at 25°C until the  $A_{610}$  was 0.3 to 0.5 and then shifted to 37°C for 3 h. After the immunoprecipitation of HA-tagged Pkc1 using anti-HA antibody-conjugated resin, samples were subjected to SDS-PAGE followed by Western blotting using anti-phosphorylated Pkc1<sup>T1125</sup> antibodies [p-Pkc1 (p-T1125)], anti-phosphorylated Pkc1<sup>S1143</sup> antibodies [p-Pkc1 (p-S1143)], or an anti-HA monoclonal antibody (Cell Signaling). (B) Cells (YPH250) carrying pFL39-PKC1-3HA (WT), pFL39-PKC1<sup>ΔHR1</sup>-3HA (ΔHR1), or pFL39-PKC1<sup>4C/S</sup>-3HA (4C/S) were cultured in SD medium until the  $A_{610}$  was 0.4 to 0.6. Immunoprecipitation and Western blotting were performed as described in the legend to panel A. (C) *pkc1Δ* (DL376) cells carrying pFL39-PKC1-3HA (WT), pFL39-PKC1<sup>ΔHR1</sup>-3HA (ΔHR1), or pFL39-PKC1<sup>4C/S</sup>-3HA (4C/S) were cultured in SD medium until the  $A_{610}$  was 0.3 to 0.5, and 10 mM MG was added. The phosphorylation of Mpk1 was determined after incubation for 30 min. (D) Cells (YPH250) carrying the vector (pRS426), ADH1 p-PKC1 C1 WT (C1<sup>WT</sup>), or ADH1 p-PKC1 C1<sup>4C/S</sup> (C1<sup>4C/S</sup>) were cultured in SD medium until the  $A_{610}$  was 0.3 to 0.5, and 10 mM MG was added. The phosphorylation of Mpk1 was determined after incubation for 30 min.

to determine whether Pkc1 is phosphorylated by TORC2. As shown in Fig. 3D, the wild-type Pkc1 peptide (the C-terminal region of Pkc1 [amino acid residues 1120 to 1149] including Thr<sup>1125</sup>, Ser<sup>1143</sup>, and some other Ser and Thr residues) was phosphorylated, while the Pkc1<sup>T1125A/S1143A</sup> peptide, an Ala-substituted variant of Thr<sup>1125</sup> and Ser<sup>1143</sup>, was not. In addition, TORC2 containing kinase-dead Tor2 did not phosphorylate the Pkc1<sup>WT</sup> peptide (see Fig. S2 in the supplemental material). These results indicate that TORC2 phosphorylates Pkc1 *in vitro*.

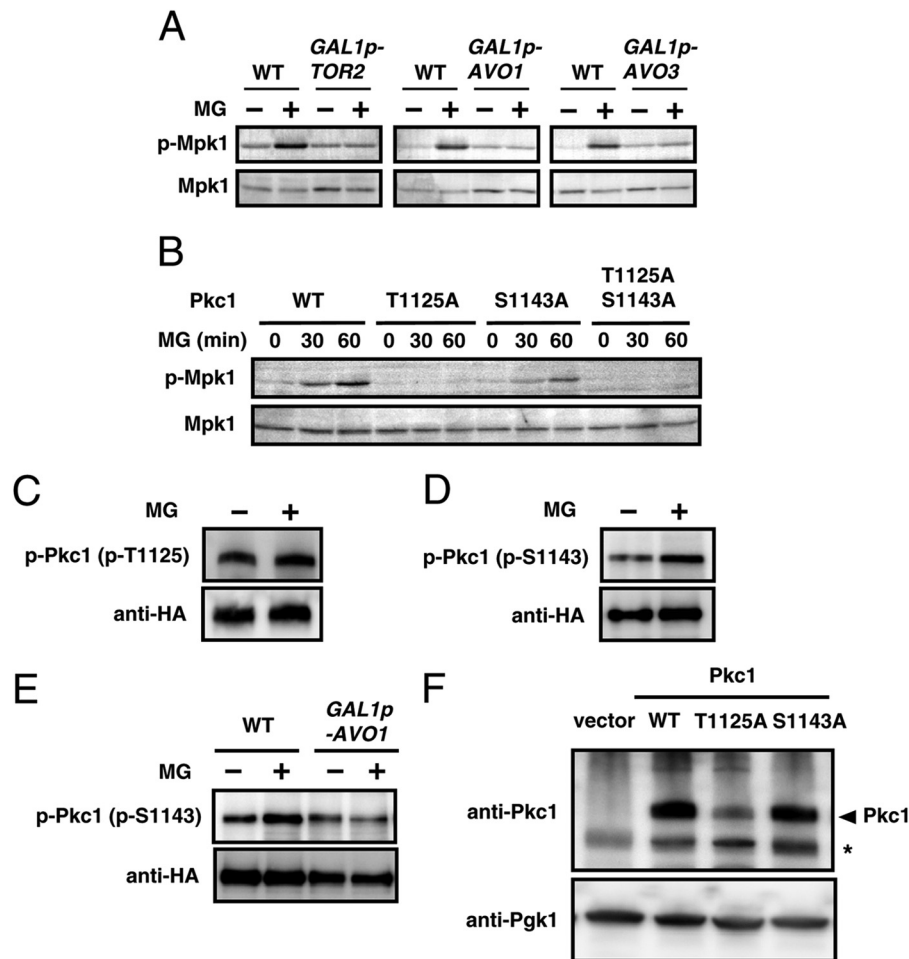
We then raised anti-phospho-Pkc1<sup>T1125</sup> and anti-phospho-Pkc1<sup>S1143</sup> antibodies to examine whether Pkc1 is a target of TORC2 in yeast cells. To verify the specificity of these antibodies, Pkc1 variants with Ala substitutions at Thr<sup>1125</sup> (Pkc1<sup>T1125A</sup>) and Ser<sup>1143</sup> (Pkc1<sup>S1143A</sup>) were constructed. As shown in Fig. 3E, each antibody specifically crossed with the respective phosphorylation site. To determine whether these amino acid residues are phosphorylated by TORC2, we used a strain carrying the *AVO1* gene, which encodes an essential component of TORC2 and which is driven by the *GAL1* promoter (45), and shifted the cell from a galactose medium to a glucose medium to stop the expression of *AVO1*, which impaired TORC2 activity. The impairment of TORC2 activity in yeast cells in the glucose medium was verified by observing the depolarization of actin (see Fig. S3 in the supplemental material). As shown in Fig. 3F, Thr<sup>1125</sup> and Ser<sup>1143</sup> were phosphorylated in the basal state in both wild-type and *GAL1p-AVO1* cells in the galactose medium; however, the phosphorylation levels of these amino acids decreased in *GAL1p-AVO1* cells in the glucose medium. These results indicate that Pkc1 is phosphorylated in a TORC2-dependent manner in yeast cells.

**Involvement of Rho1 in the phosphorylation of Pkc1 at Thr<sup>1125</sup> and Ser<sup>1143</sup>.** Rho1 is a small G protein that is crucially involved in the heat shock-triggered phosphorylation of Mpk1

(37, 38) (see Fig. 8). Rho1 also physically interacts with Pkc1 (37, 38). Therefore, we investigated whether Rho1 is involved in TORC2-Pkc1 signaling. Since *RHO1* is an essential gene, we used temperature-sensitive *rho1* mutants. Saka et al. isolated several *rho1<sup>ts</sup>* mutants, of which *rho1-2*, *rho1-3*, and *rho1-5* were impaired in heat shock-induced phosphorylation of Mpk1 (46). These findings prompted us to examine Pkc1 phosphorylation levels in these mutants. As shown in Fig. 4A, the basal phosphorylation levels of Thr<sup>1125</sup> and Ser<sup>1143</sup> of Pkc1 were decreased in the temperature-sensitive *RHO1* mutants at nonpermissive temperatures.

We then constructed Pkc1 mutants defective in the domains for the physical interaction with Rho1. Two domains in the N-terminal region of Pkc1, the homology region 1 (HR1) domain and the Cys-rich C1 domain, have been shown to contribute to the physical interactions with Rho1 (37, 47, 48) (Fig. 3A). Previous studies reported that the HR1 domain was deleted in the Pkc1<sup>ΔHR1</sup> mutant and also that the Pkc1<sup>4C/S</sup> mutant was a variant with Cys-to-Ser substitutions of the four Cys residues (Cys<sup>434</sup>, Cys<sup>437</sup>, Cys<sup>514</sup>, and Cys<sup>517</sup>) necessary for the zinc finger motif in the C1 domain (47–49). The basal phosphorylation levels of Thr<sup>1125</sup> and Ser<sup>1143</sup> were markedly decreased in the Pkc1<sup>ΔHR1</sup> mutant (Fig. 4B). The phosphorylation level of Ser<sup>1143</sup> was also markedly decreased in the Pkc1<sup>4C/S</sup> mutant, whereas the phosphorylation level of Thr<sup>1125</sup> was similar to that of wild-type Pkc1 (Pkc1<sup>WT</sup>) (Fig. 4B). The MG-induced phosphorylation of Mpk1 did not occur in either mutant (Fig. 4C). Additionally, overproduction of the C1 domain, which may have competed with endogenous Pkc1, inhibited the MG-induced phosphorylation of Mpk1, whereas overproduction of the C1 domain in the mutant with Cys-to-Ser substitution mutations (C1<sup>4C/S</sup>) did not (Fig. 4D).

**MG facilitates TORC2 signaling in yeast.** We demonstrated that MG activates the Pkc1-Mpk1 MAP kinase cascade. To assess



**FIG 5** MG functions as an initiator of the TORC2-Pkc1 signaling pathway. (A) *GAL1p-TOR2* (JM340), *GAL1p-AVO1* (RL25-1c), and *GAL1p-AVO3* (RS61-5b) cells were cultured overnight in SC-Gal medium. After being transferred to SD medium, cells were cultured for 16 h (*GAL1p-TOR2*), 9 h (*GAL1p-AVO1*), or 10 h (*GAL1p-AVO3*) to impair TORC2 activity and were then treated with 10 mM MG for 30 min. (B) *pkc1Δ* (DL376) cells carrying YEp352-PKC1 (pFR22), YEp352-PKC1<sup>T1125A</sup>, YEp352-PKC1<sup>S1143A</sup> (pFR74), or YEp352-PKC1<sup>T1125A/S1143A</sup> were cultured in SD medium until the  $A_{610}$  was 0.3 to 0.5 and were then treated with 10 mM MG for the prescribed times. (C) Cells (YPH250) carrying pFL39-PKC1-3HA were cultured in SD medium until the  $A_{610}$  was 0.4 to 0.6, and 10 mM MG was added. After the immunoprecipitation of HA-tagged Pkc1 using the anti-HA-antibody-conjugated resin, samples were subjected to SDS-PAGE followed by Western blotting using anti-phosphorylated Pkc1<sup>T1125</sup> antibodies [p-Pkc1 (p-T1125)] or an anti-HA monoclonal antibody (Cell Signaling). (D) Cells (YPH250) carrying YCplac111-PKC1-3HA were cultured in SD medium. Immunoprecipitation and Western blotting were performed as described in the legend to panel C, except that anti-phosphorylated Pkc1<sup>S1143</sup> antibodies [p-Pkc1 (p-S1143)] were used. (E) Wild-type (TB50a) cells and *GAL1p-AVO1* (RL25-1c) cells carrying YCplac111-PKC1-3HA were cultured overnight in SC-Gal medium. After being transferred to SD medium, cells were cultured for 15 h to impair TORC2 activity and were then treated with 10 mM MG for 60 min. Immunoprecipitation and Western blotting were performed as described in the legend to panel C. (F) *pkc1Δ* (DL376) cells carrying YEp352-PKC1 (pFR22), YEp352-PKC1<sup>T1125A</sup>, or YEp352-PKC1<sup>S1143A</sup> (pFR74) were cultured in SD medium until the  $A_{610}$  was 0.4 to 0.6. DL376 cells carrying the vector (YEp352) were cultured in SD medium containing 1 M sorbitol. Cell extracts were subjected to SDS-PAGE followed by Western blotting to determine the protein levels of Pkc1 using anti-Pkc1 antibodies. As the loading control, Pgk1 protein levels were determined using an anti-Pgk1 monoclonal antibody (Molecular Probes). The asterisk indicates a nonspecific band.

whether TORC2 is involved in this signaling pathway triggered by MG, we first monitored the phosphorylation of Mpk1 using a *tor2-21<sup>ts</sup>* mutant. Although Tor2 was shown to be involved in both TORC1 and TORC2, the *tor2-21<sup>ts</sup>* allele was specifically defective in TORC2 function (22). The MG-induced phosphorylation of Mpk1 was observed at 28°C in *tor2-21<sup>ts</sup>* cells, whereas its level was greatly reduced at a nonpermissive temperature (see Fig. S4A in the supplemental material). The MG-induced phosphorylation of Mpk1 also rarely occurred in cells defective in TORC2 activity (Fig. 5A), which was achieved by transferring cells carrying the essential components of TORC2 (*AVO1* or *AVO3*) with the *GAL1* promoter from the galactose medium to the glucose medium. The

same result was obtained when cells with *GAL1* promoter-driven *TOR2* were used (Fig. 5A). We confirmed that TORC2 function was impaired under these conditions by observing the depolarization of actin (see Fig. S4B in the supplemental material).

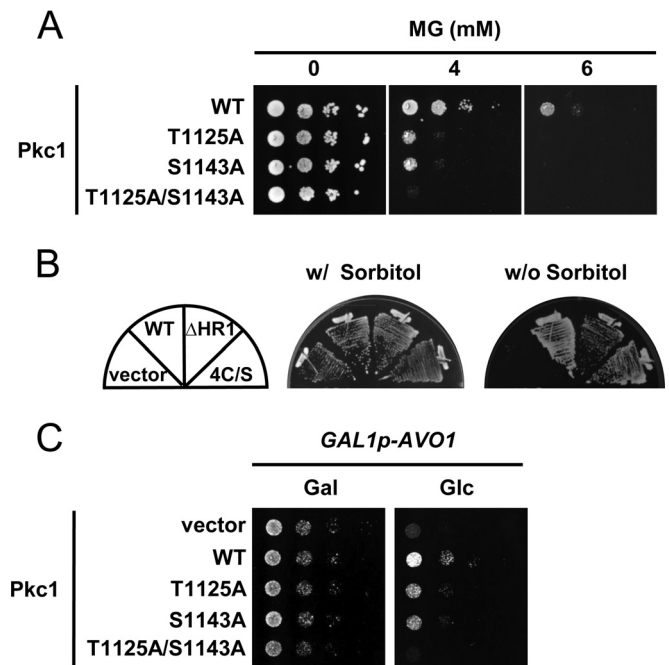
Since Thr<sup>1125</sup> and Ser<sup>1143</sup> in Pkc1 were the direct phosphorylation sites of TORC2 (Fig. 3), we investigated whether these amino acids are necessary for the MG-induced phosphorylation of Mpk1. As shown in Fig. 5B, Mpk1 was slightly phosphorylated in *PKC1<sup>S1143A</sup>* cells but not in *PKC1<sup>T1125A</sup>* or *PKC1<sup>T1125A/S1143A</sup>* cells following treatment with MG. These results indicate that Thr<sup>1125</sup> and Ser<sup>1143</sup> in Pkc1 are involved in the MG-induced activation of the Mpk1 MAP kinase cascade.

We next determined whether MG enhances the phosphorylation of Pkc1. As shown in Fig. 5C, the phosphorylation levels of Thr<sup>1125</sup> in the TM of Pkc1 remained unchanged following treatment with MG. Meanwhile, the phosphorylation levels of Ser<sup>1143</sup> in HM were increased by MG treatment (Fig. 5D). To examine whether MG directly affects the activity of TORC2 to increase the phosphorylation level of Pkc1 at Ser<sup>1143</sup>, we added MG in a mixture of the *in vitro* kinase assay. However, the phosphorylation level of Pkc1<sup>Ser1143</sup> was substantially unchanged in the presence of MG (see Fig. S2 in the supplemental material). Next, to verify that the increase in the phosphorylation level of Pkc1 at Ser<sup>1143</sup> in MG-treated cells is dependent on TORC2, cells carrying the *AVO1* gene driven by the *GAL1* promoter were shifted from the galactose medium to the glucose medium in order to reduce the activity of TORC2 (see Fig. S5 in the supplemental material). These cells were then treated with MG. As shown in Fig. 5E, the phosphorylation levels of Ser<sup>1143</sup> in TORC2-impaired cells were not increased by treatment with MG. These results imply that MG may have facilitated TORC2 signaling, thereby increasing the phosphorylation level of Pkc1 at Ser<sup>1143</sup>, which transmitted the signal downstream to the Mpk1 MAP kinase cascade.

**The phosphorylation status of Pkc1 at Thr<sup>1125</sup> affects its protein levels.** Although Thr<sup>1125</sup> in the TM of Pkc1 did not respond to MG (Fig. 5C), the basal phosphorylation level of this site appeared to be necessary for transmitting the signal to the Mpk1 MAP kinase cascade (Fig. 5B). However, it currently remains unclear why the phosphorylation of Mpk1 did not occur in Pkc1<sup>T1125A</sup> cells following treatment with MG, in spite of Ser<sup>1143</sup> being intact. Previous studies reported that the phosphorylation of Thr<sup>450</sup> in the TM of Akt secured its stability in mammalian cells (35, 50). To verify whether the replacement of Thr<sup>1125</sup> in TM by Ala affects the stability of Pkc1, whereby the MG-triggered signal is not adequately transmitted to the Mpk1 MAP kinase cascade, we determined Pkc1 protein levels. We raised anti-Pkc1 antibodies to monitor Pkc1 protein levels. Although some nonspecific bands appeared in the blots, the Pkc1 antibodies raised were able to detect Pkc1 proteins in cell extracts by Western blotting (see Fig. S6 in the supplemental material). As shown in Fig. 5F, the steady-state levels of the Pkc1<sup>T1125A</sup> protein were markedly lower than those of the Pkc1<sup>WT</sup> protein. This may be one of the reasons why the MG-induced phosphorylation of Mpk1 did not occur in Pkc1<sup>T1125A</sup> cells (Fig. 5B).

**Role of phosphorylation of Pkc1 at Thr<sup>1125</sup> and Ser<sup>1143</sup> under conditions of MG stress.** To explore its physiological relevance to the TORC2-dependent phosphorylation of Pkc1, we investigated the effects of the substitution of the phosphorylation sites of Pkc1 on growth and sensitivity to MG. The phosphorylation sites of Pkc1 were found to be irrelevant to the growth of yeast cells. The *pkc1Δ* mutant is unable to grow in the absence of an osmoprotectant, such as sorbitol, because cell wall biogenesis is defective (51, 52). However, we found that the Pkc1<sup>T1125A/S1143A</sup> mutant was able to grow in the medium without sorbitol (Fig. 6A). Pkc1 mutants defective in the HR1 and C1 domains, in which the phosphorylation levels of Thr<sup>1125</sup> and Ser<sup>1143</sup> were reduced (Fig. 4B), were also able to grow without sorbitol (Fig. 6B). These results suggest that the phosphorylation of Thr<sup>1125</sup> and Ser<sup>1143</sup> in Pkc1 is dispensable for the growth of yeast cells under normal osmotic conditions. However, the replacement of Thr<sup>1125</sup> and/or Ser<sup>1143</sup> by Ala enhanced the susceptibility of cells to MG (Fig. 6A).

The growth defect in TORC2-knockdown cells was partially

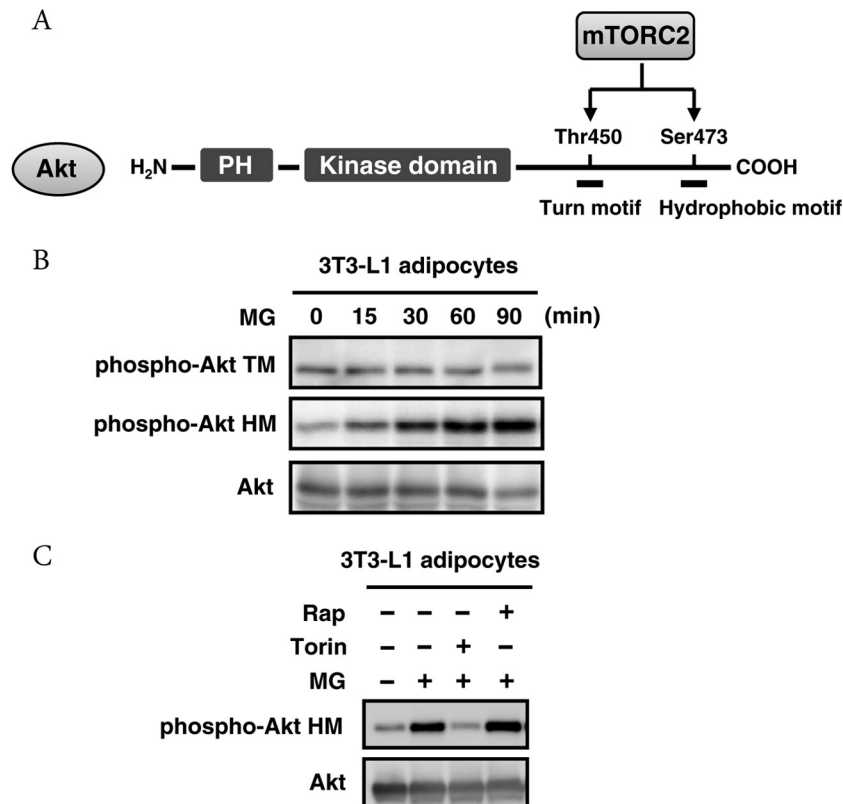


**FIG 6** Physiological significance of the phosphorylation states of Pkc1 at Thr<sup>1125</sup> and Ser<sup>1143</sup> on the growth of yeast cells. (A) *pkc1Δ* (DL376) cells carrying various *PKC1* alleles (WT, *PKC1*<sup>WT</sup>; T1125A, *PKC1*<sup>T1125A</sup>; S1143A, *PKC1*<sup>S1143A</sup>; T1125A/S1143A, *PKC1*<sup>T1125A/S1143A</sup>) with YCp50 (a *CEN*-type plasmid) were cultured in SD medium until the log phase of growth. Cells were then serially diluted (1:10) with a 0.85% NaCl solution, and 4  $\mu$ l of each cell suspension was spotted onto SD agar plates containing MG. Cells were grown at 28°C for 3 days. (B) *pkc1Δ* cells (DL376) carrying various *PKC1* alleles (WT, *PKC1*<sup>WT</sup>;  $\Delta$ HR1, *PKC1* <sup>$\Delta$ HR1</sup>; 4C/S, *PKC1*<sup>4C/S</sup>) with pFL39 (a *CEN*-type plasmid) were streaked onto SD agar plates with or without 1 M sorbitol and cultured at 28°C for 3 days. (C) Cells with *GAL1* promoter-driven *AVO1* (RL25-1C) carrying various *PKC1* alleles (WT, *PKC1*<sup>WT</sup>; T1125A, *PKC1*<sup>T1125A</sup>; S1143A, *PKC1*<sup>S1143A</sup>; T1125A/S1143A, *PKC1*<sup>T1125A/S1143A</sup>) with YEp352 (a 2- $\mu$ -type plasmid) were cultured in SC-Gal medium until the log phase of growth. Cells were then serially diluted (1:10) with 0.85% NaCl solution, and 4  $\mu$ l of each cell suspension was spotted onto SC-Gal (Gal) or SD (Glc) agar plates. Cells were cultured at 28°C for 3 days.

suppressed by multiple copies of *PKC1*<sup>WT</sup>, *PKC1*<sup>T1125A</sup>, or *PKC1*<sup>S1143A</sup> without sorbitol in the medium (Fig. 6C). Since the addition of sorbitol to a medium was previously shown to suppress the growth defect in TORC2-knockdown cells (22), suppression of the growth defect in TORC2-impaired cells by multiple copies of *PKC1* may be attributed to the recovery of cell wall integrity. However, the growth defect in TORC2-knockdown cells was not suppressed by multiple copies of *PKC1*<sup>T1125A/S1143A</sup>. To explore the physiological relationship between TORC2 and Pkc1, we examined the organization of actin. As shown in Fig. S7 in the supplemental material, the proportion of cells with depolarized actin increased when TORC2 was impaired (16.9% of the wild-type cells had depolarized actin, and 59.8% of the TORC2-impaired cells had depolarized actin); however, it reverted to nearly wild-type levels (29.3%) when *PKC1* was expressed in a multicopy vector. However, multiple copies of *PKC1*<sup>T1125A/S1143A</sup> were not able to recover the polarization of actin. This may have been because of a failure in the growth recovery of TORC2-knockdown cells carrying multiple copies of *PKC1*<sup>T1125A/S1143A</sup>.

**MG activates mTORC2 signaling in mammalian cells.** We demonstrated that MG functions as a signal initiator for TORC2





**FIG 7** Effects of MG on mTORC2 signaling. (A) Diagram of Akt. PH, the pleckstrin homology domain. (B) Mouse 3T3-L1 adipocytes were treated with 5 mM MG. After being incubated for the prescribed time, the phosphorylation of Thr<sup>450</sup> in the turn motif (p-Akt TM) and Ser<sup>473</sup> in the hydrophobic motif (p-Akt HM) of Akt was determined using the respective specific antibodies. (C) Mouse 3T3-L1 adipocytes were preincubated with 250 nM torin 1 or 20 nM rapamycin (Rap) for 60 min before treatment with 5 mM MG for 60 min.

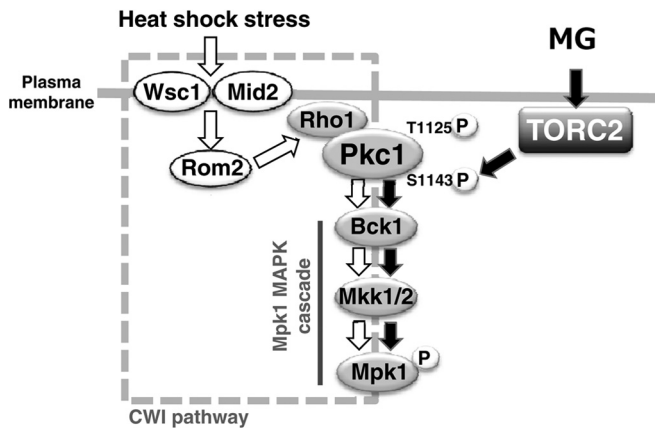
signaling using the budding yeast *S. cerevisiae* as a model organism. To determine whether this is also the case in higher eukaryotes, we measured the phosphorylation levels of Akt, a substrate of mammalian TORC2 (mTORC2) (Fig. 7A), using mouse 3T3-L1 adipocytes. The phosphorylation level of Ser<sup>473</sup> in the HM of Akt increased in adipocytes following treatment with MG (Fig. 7B). Meanwhile, Thr<sup>450</sup> in the TM of Akt was constitutively phosphorylated, and its level remained unchanged with MG treatment (Fig. 7B). To verify that the MG-induced phosphorylation of Akt<sup>Ser473</sup> was dependent on mTORC2, we used torin 1 to inhibit mTOR. Rapamycin specifically inhibits TORC1, while torin 1 acts on both mTORC1 and mTORC2 (53). The MG-induced phosphorylation of Ser<sup>473</sup> was completely inhibited in the presence of torin 1; however, rapamycin did not inhibit the MG-induced phosphorylation of Ser<sup>473</sup> (Fig. 7C). In mouse adipocytes, the phosphorylation of Akt at Ser<sup>473</sup> was previously shown to depend on mTORC2 (54). Therefore, these results suggest that MG may function as a signal initiator of mTORC2 signaling in mammalian cells, similar to yeast cells.

## DISCUSSION

In the present study, we demonstrated that MG activates the Pkc1-Mpk1 MAP kinase cascade in *S. cerevisiae*. The activation of this kinase cascade appeared to be necessary for yeast cells to combat MG-induced stress. An important feature of our results is that TORC2 was involved in activating the Pkc1-Mpk1 signaling pathway triggered by MG. We have demonstrated herein for the first

time that Pkc1 is a substrate of TORC2; i.e., TORC2 phosphorylated Thr<sup>1125</sup> in the TM and Ser<sup>1143</sup> in the HM of Pkc1. Genetic studies previously reported a relationship between Tor2 and the Pkc1-Mpk1 pathway; i.e., *PKC1* and *MPK1* have been isolated as multicopy suppressors that rescue growth defects in the *tor2-21<sup>ts</sup>* mutant at nonpermissive temperatures (22, 23, 43). Since the *tor2-21<sup>ts</sup>* mutant was defective in TORC2 only at nonpermissive temperatures (22), TORC2 appeared to communicate with the Pkc1-Mpk1 pathway. However, the heat shock-induced activation of Mpk1 was previously shown to be controlled independently of Tor2 (23, 55). We demonstrated that TORC2-Pkc1 signaling is involved in the MG-induced activation of the Mpk1 MAP kinase cascade (a schematic model is shown in Fig. 8). However, since the MG-induced phosphorylation of Mpk1 occurred in *wsc1Δ mid2Δ* cells as well as in mutants defective in other sensor proteins (*Wsc2* or *Mtl1*), MG appeared to trigger TORC2-Pkc1 signaling via routes different from the known pathway for the heat shock-induced activation of the Pkc1-Mpk1 MAP kinase cascade (Fig. 8).

We have previously demonstrated that MG treatment triggers other cellular responses, including the high-osmolarity glycerol (HOG) pathway and the Ca<sup>2+</sup>/calcineurin signaling pathway (10, 12). The cytoplasmic membrane proteins Sln1 and Sho1 acted as MG sensors in the activation of the HOG pathway, although the phosphorylation of Mpk1 following treatment with MG occurred in mutants defective in both branches (data not shown). On the other hand, it was reported that both Mpk1 and calcineurin are



**FIG 8** Schematic model for the integration of TORC2 into the Pkc1-Mpk1 MAPK cascade in *S. cerevisiae*. Details are described in the text. Briefly, heat shock stress is sensed by the stress sensor proteins Wsc1 and Mid2 on the cytoplasmic membrane, and the heat shock signal is transmitted to Rom2 (guanine-nucleotide exchange factor for Rho1), which in turn activates Rho1. GTP-bound Rho1 interacts with Pkc1, and Pkc1 activates the Mpk1 MAP kinase cascade (the so-called cell wall integrity [CWI] pathway). TORC2 phosphorylates Pkc1 at Thr<sup>1125</sup> in the turn motif and at Ser<sup>1143</sup> in the hydrophobic motif. The phosphorylation of these amino acid residues is necessary for transmitting the MG signal downstream to the Mpk1 MAP kinase cascade. The phosphorylation level of Ser<sup>1143</sup> in Pkc1 increases following treatment with MG.

involved in the regulatory mechanism of G<sub>2</sub>/M delay by a high dose of Ca<sup>2+</sup> (56). However, the MG-induced phosphorylation of Mpk1 occurred in the presence of the Ca<sup>2+</sup> chelator EGTA (data not shown) under conditions in which we have previously shown that the uptake of Ca<sup>2+</sup> into the cell was inhibited and activation of Ca<sup>2+</sup>/calcineurin signaling subsequently did not occur (12). Collectively, the MG-triggered stimulation of TORC2-Pkc1 signaling does not seem to cross talk with other MG-induced cellular responses.

The phosphorylation levels of Ser<sup>1143</sup> in the HM of Pkc1 were increased following treatment with MG, whereas those of Thr<sup>1125</sup> in the TM were not (Fig. 5C and D). Nevertheless, the MG-induced phosphorylation of Mpk1 did not occur in the Pkc1<sup>T1125A</sup> mutant (Fig. 5B). In mammalian cells, the phosphorylation of Akt at Thr<sup>450</sup> in the TM secured the stability of the Akt protein *per se* (35, 50). We showed that Pkc1 protein levels were markedly decreased when Thr<sup>1125</sup> in the TM of Pkc1 was changed to Ala (Fig. 5F). Therefore, the phosphorylation of this site appears to affect the stability of Pkc1 in *S. cerevisiae* cells. In addition, intriguingly, we noticed that the phosphorylation level of Ser<sup>1143</sup> in Pkc1<sup>T1125A</sup> cells was significantly lower than that in Pkc1<sup>WT</sup> cells (Fig. 3E). This may be another reason why the phosphorylation of Mpk1 did not occur in Pkc1<sup>T1125A</sup> cells following treatment with MG. Since not only the Pkc1 protein levels but also the phosphorylation levels of Ser<sup>1143</sup> were lower in Pkc1<sup>T1125A</sup> cells, the MG-triggered signal was not adequately transmitted to the Mpk1 MAP kinase cascade in Pkc1<sup>T1125A</sup> cells. Therefore, the maintenance of a certain level of phosphorylation at both Thr<sup>1125</sup> and Ser<sup>1143</sup> appeared to secure the transmittance of the signal downstream to the Mpk1 MAP kinase cascade. To the best of our knowledge, this is the first study to demonstrate that the phosphorylation status of TM affected the phosphorylation levels of HM in AGC kinases.

The heat shock-induced activation of Mpk1 occurs through

the activation of Rho1 (38, 39) (Fig. 8). We showed that the basal phosphorylation levels of Pkc1 were decreased by a deficiency in the Pkc1 domains (HR1 and C1 domains) needed for the physical interaction with Rho1 (37, 47–49) (Fig. 4B). Previous studies reported that TORC2 was predominantly located in punctate structures adjacent to the cytoplasmic membrane in *S. cerevisiae* cells (57, 58). Since TORC2 exists beneath the cytoplasmic membrane, its substrate (Pkc1) also must be present near the cytoplasmic membrane, in which the enzyme (TORC2) exists. Rho1 is associated with the plasma membrane (38, 59); therefore, the physical interaction between Rho1 and Pkc1 may guarantee contact between TORC2 and Pkc1. In addition to the physical interaction with Rho1, the C1 domain of Pkc1 has been suggested to bind to lipids (49, 55). A previous study using multiple copies of green fluorescent protein-labeled Pkc1 showed that Pkc1 localized to the bud cortex or bud neck (60). In addition, the C1 domain of Pkc1 was shown to be sufficient for its peripheral localization in the cell (60); therefore, the C1 domain of Pkc1 may contribute to TORC2-Pkc1 signaling not only by interacting with Rho1 but also by binding to lipids in the plasma membrane, thereby allowing Pkc1 to interact with TORC2 beneath the cytoplasmic membrane. Overexpression of the C1 domain may compete with endogenous Pkc1 to interfere with the interaction with Rho1, thereby inhibiting the MG-induced phosphorylation of Mpk1 (Fig. 4D). A possible explanation for these results is that both the HR1 and C1 domains are necessary for Pkc1 to be present near the cytoplasmic membrane through a physical interaction with Rho1, whereby TORC2 phosphorylates Pkc1.

The physiological significance of the MG-induced activation of TORC2-Pkc1 signaling in yeast cells is the prevention of physiological disorders caused by MG. We demonstrated here that MG also facilitates mTORC2 signaling in mammalian cells. Although the mechanism by which MG activates TORC2 currently remains unknown, a study is now being conducted to elucidate the mechanism underlying the sensing of MG and facilitation of TORC2 signaling using yeast as a model organism and also to determine the common physiological significance of MG-induced TORC2 signaling in eukaryotes.

## ACKNOWLEDGMENTS

We thank T. Kawada, N. Takahashi, T. Goto, Y. Kim, S. Izawa, and K. Maeta for their technical support and helpful discussions. We are grateful to M. N. Hall, D. E. Levin, T. Miyakawa, J. Thorne, Y. Ohya, J. J. Heinisch, M. Y. Chen, M. S. Cyert, J. R. Broach, K. Irie, and the National Bio-Resource Project (NBRP) of MEXT, Japan, for providing the plasmids and yeast strains.

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