Methylglyoxal as a Signal Initiator for Activation of the Stress-activated Protein Kinase Cascade in the Fission Yeast Schizosaccharomyces pombe*

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Yoshifumi Takatsume, Shingo Izawa, and Yoshiharu Inoue

From the Laboratory of Molecular Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Methylglyoxal (MG) is a typical 2-oxoaldehyde derived from glycolysis. We have recently found that MG activates transcription factors such as Yap1 and Msn2, and triggers a Hog1 mitogen-activated protein kinase cascade in Saccharomyces cerevisiae. Regarding the activation of Hog1 by MG, we found that Sin1, an osmosensor possessing histidine kinase activity, functions as a sensor of MG (Maeta, K., Izawa, S., and Inoue, Y. (2005) J. Biol. Chem. 280, 253–260). To gain further insight into the role of MG as a signal initiator, here we analyze the response of Schizosaccharomyces pombe to extracellular MG. Spc1, a stress-activated protein kinase (SAPK), was phosphorylated following the treatment with MG. No phosphorylation was observed in a wis1Δ mutant. The His-to-Asp phosphorelay system consisting of three histidine kinases (Phk1, Phk2, and Phk3), a phosphorelay protein (Spy1), and a response regulator (Mcs4) exists upstream of the Spc1-SAPK pathway. The phosphorylation of Spc1 following MG treatment was observed in phk1Δphk2Δphk3Δ and spy1Δ cells, but not in mcs4Δ cells. These results suggest that S. pombe has an alternative module(s) that directs the MG signal to the SAPK pathway via Mcs4. Additionally, we found that the transcription factor Pap1 is concentrated in the nucleus in response to MG, independent of the Spc1-SAPK pathway.

Methylglyoxal (MG,2 CH₃COCHO) is a typical 2-oxoaldehyde derived from glycolysis, a ubiquitous energy-generating system present in all types of organism; nevertheless, MG inhibits the growth of cells (1). For example, MG induces apoptosis in some mammalian cells by provoking production of reactive oxygen species (2). Actually, cells exhibiting MG-induced apoptosis, such as Jurkat cells and HL60 cells, are sensitive to reactive oxygen species (3, 4). On the other hand, MG induces necrosis in the budding yeast Saccharomyces cerevisiae (5). In this case, MG does not enhance production of reactive oxygen species, and therefore, mutants lacking antioxidant enzymes (for example sod1Δ, sod2Δ, and gpx3Δ) do not show increased sensitivity to MG (5).

Because the major source of MG is glycolysis, and an overaccumulation of MG adversely affects cellular function, cells of all types possess a ubiquitous enzymatic pathway, the glyoxalase system, for detoxification of MG. The glyoxalase system consists of glyoxalase I and glyoxalase II. The former catalyzes the conversion of MG to S-D-lactoylglutathione in the presence of glutathione. The latter hydrolyzes the resultant glutathione thiolester to L-lactic acid and glutathione (see Fig. 1). Previously, we have reported that the disruption of GLO1, the structural gene for glyoxalase I, enhances susceptibility to MG in S. cerevisiae (6). A mutant defective in glyoxalase II was also sensitive to MG (7). On the other hand, S. cerevisiae has another enzymatic route by which to convert MG to lactic acid, i.e. MG is reduced to L-lactaldehyde by the action of an NADPH-dependent methylglyoxal reductase, and L-lactaldehyde is further oxidized to L-lactic acid by an NAD⁺-dependent lactaldehyde dehydrogenase (8, 9). Chen et al. (10) have reported that GRE2 encodes methylglyoxal reductase, although, as far as we could determine, gre2Δ cells were not sensitive to MG. Aguilera and Prieto (11) have reported that GRE3 encodes aldose reductase that is involved in the metabolism of MG; however, a gre3Δ mutant did not exhibit an MG-sensitive phenotype. Consequently, the glyoxalase system is the major pathway to detoxify MG in S. cerevisiae (12). We have confirmed that a glyoxalase I-deficient mutant of the fission yeast Schizosaccharomyces pombe was also sensitive to MG (13).

Aberrant metabolism of MG has been implicated in diseases such as colon cancer, diabetes, Alzheimer disease, and autism (14–17). For example, in type 2 diabetes, the basal activity of the mitogen-activated protein (MAP) kinase family is increased, and the up-regulation of p38, one of the MAP kinases, is associated with a loss of expression of the GLUT4 glucose transporter that results in a lowering of glucose uptake, which may exacerbate hyperglycemia (18). Therefore, it is implied that there is a correlation between high MG levels and increased basal activation of p38 in type 2 diabetes. However, it remains to be clarified whether a high MG level is a consequence of diabetes, or diabetes induces aberrant MG metabolism, which leads to an increase in the MG level. We have recently found that extracellular MG activates Hog1-MAP kinase, a homologue of p38 MAP kinase in S. cerevisiae (19). Later, Aguilera et al. (20) reported similar results. Therefore, MG seems to have the capability to activate p38 MAP kinase.

We have also found that MG activates Yap1, a crucial transcription factor in the response to oxidative stress in S. cerevisiae (21). An intramolecular disulfide bond is formed between Cys residues of Yap1 following treatment with H₂O₂, with the aid of Gpx3 and Ybp1 (22, 23). By contrast, neither an intramolecular nor intermolecular disulfide bond is formed upon the activation of Yap1 by MG, although MG seems to modify Cys residues of Yap1 directly and reversibly (21). Therefore, Yap1 is constitutively activated in a glyoxalase I-deficient (glbΔ) mutant because the steady state level of MG in glbΔ cells is high (21). Besides oxidative stress response, Yap1 is involved in diverse aspects of cellular stress response, and consequently, the glbΔ mutant exhibits a pleiotropic phenotype in terms of stress response, because of the con-
Methylglyoxal Activates SAPK in the Fission Yeast

Strain—S. pombe PR109 (h− leu1-32 uralD18) and mutants derived from this strain, IM554 (wls1Δ::ura4+), KS1366 (spe1Δ::ura4+), and TP108-3c (pap1Δ::ura4+) (24, 25), were donated by Dr. K. Shiozaki. A glo1Δ mutant in the background was described previously (13). AV8 (h+ ade6-M210 leu1-32 uralD18 sty1-HA::kan) (26) was a gift from Dr. E. Hidalgo. JY879 (h+ ade6-M210 leu1 uralD18) and mutants derived from this strain, PNN5 (phk1Δ::ura4− phk2Δ::ura4+ phk3Δ::ura4+), PNN6 (spe1Δ::ura4+), and PNN7 (mcs4Δ::kan1), were donated by Dr. H. Aiba (27).

Media—Basically, S. pombe cells were cultured in YES medium (3% glucose, 0.5% yeast extract, 100 µg/ml each of L-His, L-Leu, L-Lys, ade- nine, and uracil) at 28 °C unless otherwise stated. For determination of the intracellular MG level and cellular localization of EGFP-Pap1 cells were cultured in EMM medium (28).

Spot Assay—S. pombe cells were cultured in YES medium until the A595 reached 0.1 and were diluted with a sterilized 0.85% NaCl solution serially (1/10, 1/100, and 1/1000). Then, 3 µl of each cell suspension was spotted onto YES agar plates containing various concentrations of MG, and cells were cultured at 28 °C.

Growth Experiment—Cells were cultured in 200-ml flasks containing 50 ml of YES medium at 28 °C with reciprocal shaking. When the A595 reached 1.0, 10 mM MG was added and cultivation was continued, and the A595 of the culture was monitored periodically.

Western Blotting—Cells were cultured in YES medium at 28 °C until the A595 reached 0.8, and 10 mM MG or 0.3 mM H2O2 was added to the culture. After 10 min, cell extracts were prepared according to the method of Bell et al. (29). To detect phosphorylated Spc1, anti-diphospho-p38 monoclonal antibody (Sigma) was used (30). The Spc1 protein level was detected using anti-Hog1 antibody (Santa Cruz Biotechnology). The HA-tagged protein and Cdc2 (for loading control, Ref. 31) were detected using anti-HA antibody (New England Labs) and anti-Cdc2 (PSTAIRE) antibody (Santa Cruz Biotechnology), respectively.

Pap1 Mobility Assay—Cells carrying EGFP-Pap1 were cultured in EMM medium until the A595 reached 0.8, and 15 mM MG, 20 mM diethylmaleimide (DEM), or 15 mM MG + 20 mM DEM was added to the culture. Cells were incubated for 30 min at 28 °C. To remove chemicals, cells were collected by centrifugation, washed twice with EMM medium, and incubated in the fresh EMM medium for 60 min. The nuclear localization of EGFP-Pap1 was monitored using a fluorescence microscope as described below. After incubation, cells (~1.6 × 107 cells) were collected by centrifugation, washed with 20% trichloroacetic acid solution, and disrupted with glass beads in 20% trichloroacetic acid solution (200 µl). Cell homogenates were transferred to another tube, the glass beads were washed twice with 5% trichloroacetic acid (200 µl), and the resultant suspension was mixed. The mixture was centrifuged at 4 °C, 14,000 rpm for 10 min, and the pellets were washed with acetone. The dried materials were suspended in the buffer (40 µl) containing 1% SDS, 100 mM Tris-HCl buffer (pH 8), 1 mM EDTA and 10 unit/ml calf intestinal alkaline phosphatase (New England Biolabs), and the mixture was incubated at 37 °C for 1 h (32). After incubation, 10 µl of 5X sample buffer (non-reducing) was added, and the mixture was kept at 65 °C for 10 min, and the resultant sample was subjected to non-reducing SDS-PAGE. EGFP-Pap1 was detected by Western blotting using anti-EGFP monoclonal antibody (Santa Cruz Biotechnology).

Fluorescence Microscopy—Cells expressing EGFP-Pap1 (pR41FPap1) (33) were cultured in EMM medium at 28 °C until the A595 reached 0.2,
Methylglyoxal Activates SAPK in the Fission Yeast

Effect of MG on growth of SAPK-deficient mutants. A, cells (WT, PR109; wis1Δ, JM544; spc1Δ, KS1366; and glo1Δ in PR109 background) cultured in YES medium until \( A_{595} = 0.1 \) were diluted serially, and aliquots of cell suspension were spotted onto YES agar plates containing 10 mM MG. Cells were cultured at 28 °C for 4 days. B, methylglyoxal was added to the culture in which cells were grown logarithmically \( (A_{595} = 1.0) \), and cultivation was continued at 28 °C. \( A_{595} \) was monitored periodically. Symbols used are: circle, WT (PR109); square, wis1Δ (JM544); triangle, spc1Δ (KS1366). An arrow indicates when MG was added.

and 10 mM MG or 0.3 mM H\(_2\)O\(_2\) was added. Cells were incubated at 28 °C for 10 min, and the localization of EGFP-Pap1 was observed using a fluorescence microscope (Olympus BX51).

Measurement of Intracellular MG Content—The extraction and measurement of cellular MG content were done as described previously (21).

Measurement of Intracellular Oxidation Levels—The cellular oxidation level was measured using an oxidant-specific fluorescent probe, 2′,7′-dichlorofluorescein diacetate, as described previously (5).

RESULTS

Importance of Spc1-SAPK for Growth in the Presence of MG—Recently, we found that the expression of \( GLO1 \) is enhanced in a Hog1-dependent fashion when cells are exposed to MG extracellularly, and therefore, hog1Δ cells were sensitive to MG (19). To determine the significance of the Spc1-SAPK pathway in the MG-evoked response of \( S. pombe \), the susceptibility to MG of mutants defective in the SAPK pathway was investigated. As shown in Fig. 2A, spc1Δ cells were sensitive to MG, although not as sensitive as glo1Δ cells. Wis1 is a MAP kinase kinase that phosphorylates Spc1 (Fig. 1), and therefore, a wis1Δ mutant was also sensitive to MG.

Next, we treated the logarithmically growing \( S. pombe \) cells with MG (Fig. 2B). Cell growth in the wild-type strain was retarded by the addition of 10 mM MG, whereas that in spc1Δ and wis1Δ cells was severely repressed, although viability did not drop at this concentration. At much higher concentrations of MG, cells died rapidly (data not shown). These results suggest that the Spc1-SAPK pathway plays some role in cells challenged with moderate concentrations of MG.

Spc1 Is Phosphorylated following MG Treatment—MG induces the phosphorylation of Hog1 in \( S. cerevisiae \) (19). To determine whether Spc1 is phosphorylated in cells treated with MG, the status of Spc1 was monitored using anti-diphospho-p38 antibody. It has been reported that Spc1 is phosphorylated in response to various environmental stimuli (34). We reconfirmed that Spc1 was phosphorylated in cells exposed to \( H_2O_2 \), osmotic stress, and heat shock stress (Fig. 3A), and found that Spc1 was phosphorylated following the treatment with MG as well. The protein levels of Spc1 were determined using commercially available anti-Hog1 antibody (Fig. 3A). Because no immunoreactive band was detected in cells lacking Spc1 (Fig. 3A), use of the anti-Hog1 antibody to detect Spc1 protein in \( S. pombe \) seems appropriate. We verified the validity of this approach using an \( S. pombe \) strain expressing HA-tagged Spc1 (Fig. 3B). Phosphorylation of Spc1-HA was observed in cells treated with \( H_2O_2 \) or MG, and the amount of Spc1-HA protein in each cell was estimated to be equal using anti-HA antibody, a result that was reproduced using anti-Hog1 antibody (Fig. 3B). Therefore, we used a commercially available anti-Hog1 antibody to determine the Spc1 protein level.

Because Spc1 is phosphorylated by Wis1 MAP kinase kinase, we determined the status of Spc1 in the wis1Δ mutant following the treatment with MG. As shown in Fig. 3C, Spc1 was not phosphorylated in wis1Δ cells even after 60 min of MG treatment, indicating that MG activates the SAPK pathway, leading to phosphorylation of Spc1.

Mcv4 Functions as the Upstream Modulator for Activation of SAPK by MG—The His-to-Asp phosphorelay system has been identified upstream of the Spc1-SAPK pathway (Fig. 1). Buck et al. (35) have

FIGURE 2. Effect of MG on growth of SAPK-deficient mutants. A, cells (WT, PR109; wis1Δ, JM544; spc1Δ, KS1366; and glo1Δ in PR109 background) cultured in YES medium until \( A_{595} = 0.1 \) were diluted serially, and aliquots of cell suspension were spotted onto YES agar plates containing 10 mM MG. Cells were cultured at 28 °C for 4 days. B, methylglyoxal was added to the culture in which cells were grown logarithmically \( (A_{595} = 1.0) \), and cultivation was continued at 28 °C. \( A_{595} \) was monitored periodically. Symbols used are: circle, WT (PR109); square, wis1Δ (JM544); triangle, spc1Δ (KS1366). An arrow indicates when MG was added.

FIGURE 3. Spc1-SAPK pathway is activated following MG treatment. A, cells (WT, PR109; spc1Δ, KS1366) cultured in YES medium until \( A_{595} = 0.8 \) at 28 °C, then 0.3 mM \( H_2O_2 \), 1 M sorbitol (osmotic stress), or 10 mM MG was added to the culture, and the cells were cultured for another 1 h. For heat shock stress, the flask containing the cell culture was transferred to a water bath incubator preheated at 42 °C, and cultivation was continued for 1 h. Western blotting was carried out as described in the text. Anti-Hog1 antibody was used to detect Spc1 protein. The Cdc2 protein level was monitored as a loading control (32). B, cells carrying the Spc1-HA allele (AV8) were cultured as described above, and 10 mM MG or 0.3 mM \( H_2O_2 \) was added to the culture. After 1 h, cells were collected and Western blotting was carried out. C, cells (WT, PR109; wis1Δ, JM544) were cultured as described above and treated with 10 mM MG. Cells were collected at the prescribed time as indicated in the figure, and Western blotting was carried out.
reported that Mak1, Mak2, and Mak3 (also referred to as Phk3, Phk1, and Phk2, respectively (27)) are sensors of peroxides, which possess histidine kinase activity. Spy1 (also known as Mpr1) is a phosphorelay protein (also referred to as histidine-containing phosphotransfer (HPT) factor) that converts phosphoric acid from the phosphorylated His residue of the sensor kinase to the Asp residue of the Mcs4 response regulator (Fig. 1) (36). Another response regulator, Prr1, has been found in *S. pombe* (37), although Prr1 is not linked to the Spc1-SAPK pathway regarding the response to oxidative stress (37, 38). To address whether this His-to-Asp phosphorelay system (Phk1/Phk2/Phk3-Spy1-Mcs4) functions as a modulator to direct the extracellular MG signal to the Spc1-SAPK pathway, we determined the phosphorylation status of Spc1 in mutants lacking these proteins. As shown in Fig. 4, MG-induced phosphorylation did not occur in *mcs4*Δ cells, suggesting that the MG signal is sent to Mcs4 for activation of the SAPK pathway. Because Spy1 negatively regulates Mcs4, disruption of *spy1*Δ enhanced the basal phosphorylation of Spc1. Intriguingly, further phosphorylation of Spc1 was observed in *spy1*Δ cells following the treatment with MG, suggesting that Spy1 is dispensable for the MG-evoked response regarding Spc1 phosphorylation. Similarly, the basal Spc1 phosphorylation level was increased in *phk1Δ/ phk3Δ* cells, because these histidine kinases negatively function as downstream modulators under normal conditions. Although the maximal level of phosphorylation was slightly lower than that attained in wild-type cells, Spc1 was further phosphorylated in *phk1Δ/ phk2Δ phk3Δ* cells following the treatment with MG.

Next, we determined the susceptibility of mutants lacking these His-to-Asp phosphorelay proteins to MG (Fig. 4B). Spc1 phosphorylation was not observed in *mcs4*Δ cells, and consequently, the *mcs4*Δ mutant exhibited an increased sensitivity to MG. Meanwhile, the susceptibility to MG of the *spy1*Δ and *phk1Δ/ phk2Δ phk3Δ* mutants was comparable to that of the wild-type strain. Taken together, although Phk1, Phk2, and Phk3 partially function as the sensor kinase in MG stress response, these sensor kinases are likely not linked to Spy1 regarding the response, and consequently, another pathway may exist to direct the extracellular MG signal to Mcs4.

**MG Activates Pap1 in an Spc1-independent Fashion—**Pap1 is a homologue of Yap1, and its activity is regulated at the nucleocytoplasmic localization step as is that of Yap1 (33, 39). We have recently reported that Yap1 was concentrated in the nucleus upon the exposure of *S. cerevisiae* cells to MG (21). In MG-induced Yap1 activation, three Cys residues in the C terminus play crucial roles, and these residues are well conserved in Pap1 (33). We determined whether Pap1 is also concentrated in the nucleus following the treatment with MG. Quinn et al. (40) have reported that the nuclear localization of Pap1 in response to H$_2$O$_2$ is partially dependent upon Spc1, i.e. at lower concentrations of H$_2$O$_2$ (~0.3 mM), Pap1 is accumulated in the nucleus irrespective of Spc1; however, at higher concentrations (>1 mM), the nuclear localization of Pap1 is dependent upon Spc1. As shown in Fig. 5, EGFP-tagged Pap1 was concentrated in the nucleus after 10 min of 0.3 mM H$_2$O$_2$ treatment even in *spc1*Δ cells. Similarly, Pap1 was accumulated in the nucleus in *mcs4*Δ cells following treatment with 0.3 mM H$_2$O$_2$. On the other hand, at 1 mM H$_2$O$_2$, Pap1 was not able to concentrate in the nuclei of *spc1*Δ and *mcs4*Δ cells. By contrast, Pap1 was accumulated in the nucleus after 10 min following treatment with 20 mM MG in the presence or absence of Spc1 or Mcs4. These

**FIGURE 4.** MG stress signal converges to Mcs4. A, cells (WT, YB79; phk1Δphk2Δphk3Δ, PNN5; spy1Δ, PNN6; and mcs4Δ, PNN7) were cultured in YES medium until *A$_{600}$* = 0.8 at 28 °C, and 0.3 mM H$_2$O$_2$ or 10 mM MG was added to the culture. After 1 h, cells were collected, and Western blotting was performed. The density of each band was measured using densitometry, and the Spc1 phosphorylation level was shown as the ratio of the density of phosphorylated Spc1 to Spc1 protein. The Spc1 phosphorylation level in H$_2$O$_2$-treated wild-type cells was taken as 100. B, cells cultured in YES medium until *A$_{600}$* = 0.1 were diluted serially, and aliquots of cell suspension were spotted onto YES agar plates containing various concentrations of MG. Cells were cultured at 28 °C for 4 days.

**FIGURE 5.** Effect of MG on activation of Pap1. Cells (spc1Δ and mcs4Δ having the PR109 and YB79 background, respectively) carrying pR41FPap1 (EGFP-Pap1) were cultured in EMM medium until *A$_{600}$* = 0.2 at 28 °C, and chemicals at the concentrations indicated in the figure were added to the culture. After 10 min, the localization of EGFP-Pap1 was determined using a fluorescence microscope.

**Methylglyoxal Activates SAPK in the Fission Yeast**
Methylglyoxal Activates SAPK in the Fission Yeast

FIGURE 6. Reversible activation of Pap1 by MG. A and B, cells (PR109) carrying EGFP-Pap1 were cultured in EMM medium until A650 = 0.8 at 28 °C, and 15 mM MG, 20 mM DEM, or both were added to the culture. After 30 min, chemicals were removed, and cells were suspended in the fresh EMM medium without chemicals. Localization of Pap1 was monitored using a fluorescence microscope (A), and the mobility of EGFP-Pap1 in cells treated with each chemical for 60 min or in cells after removal of chemical for 60 min was determined in non-reducing SDS-PAGE (B).

results suggest that the nucleocytoplasmic localization of Pap1 is regulated irrespective of the Spc1-SAPK pathway and its upstream modulators.

Reversible Activation of Pap1 with MG—Previously, we have reported that the MG-induced Yap1 nuclear accumulation is reversible (21). To assess whether this is also the case in Pap1, S. pombe cells carrying EGFP-Pap1 were treated with MG for 30 min, washed with the medium, and then suspended in the fresh medium without MG. As shown in Fig. 6A, Pap1 was redistributed in the cytoplasm if MG was removed from the medium. On the other hand, although DEM, a Cys-alkylating agent, is also able to cause the nuclear accumulation of Pap1 (33), Cys-modification with DEM is irreversible; and therefore, the nuclear export of Pap1 was blocked even if DEM was removed from the medium (Fig. 6A). In Yap1 activation with MG, Cys residues are the targets of MG (21). If Cys residues of Pap1 are irreversibly modified with MG, Pap1 redistributed in the cytoplasm after removal of MG from the medium will not respond to DEM any further; however, Pap1 was accumulated in the nucleus again following DEM treatment (data not shown).

To further verify the reversibility of Pap1 activation following MG treatment, the migration ratio of Pap1 in non-reducing SDS-PAGE was determined. As shown in Fig. 6B, the migration speed of Pap1 prepared from cells treated with DEM was slightly retarded compared with that prepared from untreated cells. The migration ratio of Pap1 prepared from MG-treated cells was the same as that from untreated cells.

As we have mentioned above, we found that Cys residues of Pap1 are free after treatment with MG in terms of the response to DEM. However, MG can make the irreversible adducts with Arg and Lys residues of protein. If MG modifies such amino acid residues of Pap1, Pap1 prepared from cells treated with both MG and DEM will migrate more slowly compared with that prepared from cells treated with DEM alone. As shown in Fig. 6B, Pap1 from MG+DEM-treated cells exhibited the same migration ratio as that from the DEM-treated cells. Taken together, MG-induced Pap1 nuclear accumulation is reversible.

Effect of Glyoxalase I-deficiency on MG-induced Activation of Spc1 and Pap1—We have previously reported that Yap1 is constitutively concentrated in the nucleus of glo1Δ cells of S. cerevisiae (21). To determine whether this is also the case for Pap1 in S. pombe, we introduced EGFP-Pap1 into a glo1Δ mutant of S. pombe. As shown in Fig. 7A, Pap1 was found in the cytoplasm of glo1Δ cells under non-stressed conditions. However, Pap1 nuclear localization in glo1Δ cells was attained faster and at lower concentrations of MG when compared with that in wild-type cells.

Next, we determined the timing of Spc1 phosphorylation in wild-type and glo1Δ cells. In contrast to the nuclear localization of Pap1, the phosphorylation of Spc1 occurred at the same time following the treatment with MG in both wild-type and glo1Δ cells (Fig. 7B). Interestingly, the maximal level of phosphorylated Spc1 in the glo1Δ mutant was slightly higher than that in the wild type. Furthermore, Spc1 phosphorylation continued longer in glo1Δ cells.

DISCUSSION

In the present study, we have demonstrated that MG activates the Spc1-SAPK pathway. Spc1 phosphorylation following MG treatment was absolutely dependent upon Wis1 MAP kinase kinase (Fig. 3C). Because Wis1 is the only kinase identified to date that is able to phosphorylate Spc1, the MG signal enters this signaling cascade upstream of Wis1.

Spc1 phosphorylation also occurs in response to oxidative stress, heat shock stress, high osmotic stress, and UV radiation (Ref. 34, also see Fig. 3A). Mcs4 is a response regulator that constitutes the His-to-Asp phosphorelay system along with the Spy1 phosphorelay protein and histidine kinases (Phk1, Phk2, and Phk3) upstream of the Spc1-SAPK pathway; and therefore, Spc1 phosphorylation was not observed in mutants carrying the mcs4Δ (D412N) allele, which cannot receive phosphoric acid from Spy1 following H2O2 treatment (35). However, intriguingly, it has been reported that Spc1 is phosphorylated in such mutant cells following treatment with paraquat (methyl viologen) and 4-nitroquinoline oxide (35), chemicals by which the superoxide anion radical, but not H2O2, is produced. We have demonstrated in this study that Spc1 phosphorylation did not occur in mcs4Δ cells after treatment with MG (Fig. 4A). Therefore, we initially suspected that MG treatment induces production of H2O2 in S. pombe cells, which in turn activates the Spc1-SAPK pathway. Indeed, it has been reported that MG treatment provokes H2O2 production in some types of mammalian cells (3). Furthermore, Phk1/Mak2, Phk2/Mak3, and Phk3/Mak1 were originally cloned as peroxide sensors functioning upstream of the Spc1-SAPK pathway (35). We then measured the intracellular oxidation level; however, it was substantially the same before and after the treatment with MG (data not shown). We have previously reported that MG treatment does not induce H2O2 production in S. cerevisiae (5, 21). Taken together, MG itself activates the Spc1-SAPK pathway in an Mcs4-dependent fashion.

We have previously reported that MG activates Hog1, a p38 MAP kinase homologue in S. cerevisiae (19), a counterpart of Spc1 in S. pombe. The phosphorylation of Hog1 is strictly dependent upon Pbs2 MAP kinase kinase, and therefore, was not observed following MG treatment in pbs2Δ cells (19). There are at least two ways by which
highly osmotic stress signals are transmitted to Pbs2; i.e. via Ssk2 and Ssk22, redundant MAP kinase kinase kinases, as well as via Ste11, which independently phosphorylates Pbs2 in response to osmotic stress. Both Ssk2 and Ssk22 lie downstream of the Sin1 branch (Sin1-Ypd1-Ssk1; His-to-Asp phosphorelay system), where Sin1 is a histidine kinase, Ypd1 is a phosphorelay protein, and Ssk1 is a response regulator (Fig. 1). Meanwhile, it has been reported that Ste11 is activated by two different osmosensors, Sho1 and Msb2, which operate in parallel (41). Because Hog1 phosphorylation following MG treatment was not observed in cells defective in the Sin1 branch (19), it is likely that in both S. cerevisiae and S. pombe, the His→Asp phosphorelay system is involved in sensing and transmitting the MG signal to the p38 MAP kinase family.

In S. cerevisiae, disruption of SLN1 is lethal due to hyperactivation of Hog1 MAP kinase (42). By contrast in S. pombe, the phk1Δphk2Δphk3Δ triple mutant is viable, and its rate of growth was comparable to that of the wild type. Although the basal phosphorylation level of Spc1 was slightly increased in phk1Δphk2Δphk3Δ cells, further phosphorylation occurred following MG treatment. Similarly, Spy1 was dispensable for MG-induced Spc1 phosphorylation (Fig. 4A). Some reports have indicated the possibility that several stress signals enter the Spc1-SAPK pathway from Ws1 or Ws4 (35, 36, 43, 44); however, importantly, the MG signal converges on the Msc4 response regulator. Hence, our data strongly suggest that there is another pathway to transmit the MG signal to Msc4 in parallel with the Phk1/Phk2/Phk3-Spy1 His-to-Asp phosphorelay system. Nevertheless, no homologue of Phk1, Phk2, or Phk3 was found in the S. pombe data base with a BLAST search. Furthermore, we could not find a Spy1 homologue in the data base. Therefore, different mechanism(s) to activate Msc4 may exist in terms of the response to MG (Fig. 1).

The next question is whether the phosphorylation of Spc1 following MG treatment occurs through the perception of a rise in the MG level in the cell, or of the existence of MG outside of the cell. To help answer this question, we determined the nuclear localization of the transcription factor Pap1, a Yap1 homologue in S. pombe. We have recently found that MG activates Yap1 in S. cerevisiae (21). We revealed that an increase in the intracellular MG level is a direct trigger of Yap1 activation (21). In the present study, we have demonstrated that Pap1 is also activated by MG reversibly. The steady state level of MG in glo1Δ cells of S. pombe was substantially the same as that in wild-type cells (data not shown), and the constitutive nuclear localization of Pap1 was not observed in glo1Δ cells (Fig. 7A). Nevertheless, because glo1Δ cells are impaired in the detoxification of MG compared with wild-type cells, the intracellular MG level increases faster in glo1Δ cells than wild-type cells after treatment with 10 mM MG (increase in cellular MG level after 10 min: wild-type, 3 nmol/g-cell; glo1Δ, 20 nmol/g-cell). Subsequently, Pap1 nuclear localization in glo1Δ cells was attained at lower concentrations of MG than that attained in wild-type cell (Fig. 7A). The nuclear localization of Pap1 in response to H2O2 is regulated through the formation of disulfide bonds between Cys residues within Pap1 (45), and the Cys-rich domain, which is crucial for the Yap1 nuclear localization in response to H2O2, is well conserved in Pap1 also (33). However, as we described above, MG treatment does not induce H2O2 production in S. pombe cells. Collectively, the intracellular MG level seems to be the factor determining the nuclear localization of Pap1 in response to MG, as was observed in Yap1 (21). The involvement of Cys residues of Pap1 in MG-induced nuclear localization was recently reported by Zuin et al. (46).

Then, does the phosphorylation of Spc1 correspond to the rise in the level of MG in the cell? To answer this question, we measured the time course of Spc1 phosphorylation in wild-type and glo1Δ cells after treatment with 5 mM MG. The phosphorylation began after 5 min and reached a maximum after 10 min in both wild-type and glo1Δ cells (Fig. 7, B and C). However, the intracellular MG level in wild-type and glo1Δ cells changed little under such conditions, suggesting that Spc1 phosphorylation occurs irrespective of intracellular MG content. Hence, the existence of MG outside of the cell is likely to be the trigger for activation of the Spc1-SAPK pathway, a feature that is conserved in MG-induced activation of Hog1 in S. cerevisiae (19). Intriguingly, Spc1 phosphorylation continued longer in glo1Δ cells than in wild-type cells (Fig. 7, B and C). This suggests that the loss of glo1Δ does not influence the
activation of Spc1, although the impairment of MG detoxification seems to affect the deactivation of the Spc1-SAPK pathway, e.g. MG may inactivate some phosphatases that dephosphorylate Spc1, such as Pyp1. Taken together, it is conceivable that there is some machinery that perceives the extracellular concentration of MG, leading to activation of the Spc1-SAPK pathway through Mcs4 in S. pombe.

Cells in the tissues of diabetic patients are constantly exposed to high concentrations of extracellular MG, because MG levels in the red blood cells and plasma of diabetic patients are higher than those of healthy individuals (47, 48). It has been reported that MG activates p38 MAP kinase in human endothelial cells (49). Therefore, the identification of an MG sensor would provide important information about the molecular basis of diabetes and other metabolic diseases in which MG is involved. We are currently searching for such a sensor in both S. cerevisiae and S. pombe cells.

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Methylglyoxal Activates SAPK in the Fission Yeast