Methylglyoxal, a Metabolite Derived from Glycolysis, Functions as a Signal Initiator of the High Osmolarity Glycerol-Mitogen-activated Protein Kinase Cascade and Calineurin/Crz1-mediated Pathway in Saccharomyces cerevisiae*

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Methylglyoxal (MG) is a typical 2-oxoaldehyde derived from glycolysis, although it inhibits the growth of cells in all types of organism. Hence, it has been questioned why such a toxic metabolite is synthesized via the ubiquitous energy-generating pathway. We have previously reported that expression of GLO1, coding for the major enzyme detoxifying MG, was induced by osmotic stress in a high osmolarity glycerol (HOG)-mitogen-activated protein (MAP) kinase-dependent manner in Saccharomyces cerevisiae. Here we show that MG activates the HOG-MAP kinase cascade. Two osmosensors, Sln1 and Sho1, have been identified to function upstream of the HOG-MAP kinase cascade, and we reveal that MG initiates the signal transduction to this MAP kinase cascade through the Sln1 branch. We also demonstrate that MG activates the Msn2 transcription factor. Moreover, MG activated the uptake of Ca\(^{2+}\) in yeast cells, thereby stimulating the calcineurin/Crz1-mediated Ca\(^{2+}\) signaling pathway. We propose that MG functions as a signal initiator in yeast.

Glycolysis is a ubiquitous energy-generating pathway in organisms. Triosephosphate isomerase is one of the enzymes involved in this anaerobic oxidation pathway for glucose. During the triosephosphate isomerase reaction, methylglyoxal (MG, CH\(_2\)C(O)COH) is synthesized by the β-elimination reaction from an enediol phosphate intermediate (1, 2). Although MG is synthesized during normal metabolism of glucose, this aldehyde inhibits the growth of cells from microorganisms to higher eukaryotes, eventually killing them, and therefore, the raison d'être of MG has been of considerable interest.

By the systematic biochemical analysis of the metabolic fate of MG using the budding yeast Saccharomyces cerevisiae as a model organism, we revealed that MG is metabolized to lactic acid by two different routes (for reviews, see Refs. 3 and 4). One route is a glyoxalase system in which glyoxalase I and glyoxalase II are involved. In this route, MG is condensed with glutathione to give S-d-lactoylglutathione by the action of glyoxalase I, and the glutathione thiolester is then hydrolyzed to lactic acid and glutathione by glyoxalase II. The other route is a reduction/oxidation system consisting of methylglyoxal reductase and lactaldehyde dehydrogenase. MG is reduced to lactaldehyde by NADPH-dependent methylglyoxal reductase, and lactaldehyde is further oxidized to lactic acid by NAD\(^+\)-dependent lactaldehyde dehydrogenase. To evaluate the physiological importance of these enzymatic routes in vivo, we cloned the structural gene for glyoxalase I (GLO1) and demonstrated that a glo1Δ mutant showed hypersensitivity to MG (5). Similarly, Bito et al. (6) reported that a mutant defective in glyoxalase II (glo2Δ) was also hypersensitive to MG. On the other hand, Chen et al. (7) recently reported that GRE2 is one of the genes encoding NADPH-dependent methylglyoxal reductase activity, although a gre2Δ mutant did not exhibit sensitivity to MG as far as we discerned.2 Alternatively, the GRE3 gene encodes an aldose reductase, and the corresponding gene product is supposed to be involved in MG metabolism, although the disruption of GRE3 did not enhance susceptibility to MG (8). These results indicate that the glyoxalase system is the most important pathway for MG detoxification in S. cerevisiae. This was also the case in the fission yeast (9).

To obtain a clue as to the cellular function of MG and its metabolic pathway, we have attempted to find the conditions that alter the intracellular MG level and/or MG-metabolizing enzyme activity in S. cerevisiae. We found that the expression of GLO1 is specifically induced by osmotic stress in a high osmolarity glycerol (HOG)-mitogen-activated protein (MAP) kinase-dependent manner (10). The GLO1 promoter contains the characteristic cis-acting element, STRE (stress response element). A gene possessing the STRE usually responds to a wide variety of stress stimuli such as oxidative stress, heat shock stress, and osmotic stress (11). Man2 binds to this element, and this C\(_2\)H\(_2\)-type zinc finger transcription factor was concentrated into the nucleus when cells were exposed to these stress stimuli (12). However, interestingly, GLO1 did not respond to any form of stress but osmotic stress, even if the GLO1 promoter contained two sets of STRE (10). Under a highly osmotic stress, S. cerevisiae cells produce glycerol as a compatible osmolyte from glucose (13). It has been reported that the expression of HXT1 and GLK1 coding for hexose transporter and glucokinase, respectively, was elevated in cells exposed to high osmotic stress (14). These expression profiles suggest that the influx of glucose for glycolysis is facilitated to efficiently produce glycerol. Indeed, we revealed

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1 The abbreviations used are: MG, methylglyoxal; MAP, mitogen-activated protein; HOG, high osmolarity glycerol; GFP, green fluorescent protein; NLS, nuclear localization signal; NES, nuclear export signal.

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that consumption of glucose was enhanced in cells exposed to osmotic stress, and subsequently the cellular MG level increased (10), although other stress stimuli such as oxidative stress did not increase the MG level. Therefore, the physiological significance of the induction of GLO1 under high osmotic stress is most likely the control of the intracellular MG level (4, 10).

In this study, to gain further insight into the roles of MG, we examined the cellular responses of yeast cells to this aldehyde. We found that Hog1 is phosphorylated and concentrated into the nucleus of cells treated with a low dose of MG. Msn2 was also translocated into the nucleus with MG treatment. Moreover, we found that MG treatment enhanced the uptake of Ca$^{2+}$ in the medium by cells allowing activation of calcineurin. Consequently, Crz1, speculated to be the only transcription factor that functions under the control of calcineurin (15), was concentrated into the nucleus. Taken together, we hypothesize that MG may function as a signal initiator in yeast.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—S. cerevisiae strains used in this study have the genetic background of YPH250 (MATa trp1-Δ1 his3Δ200 leu2-3,112 ade2-101 ura3-5. mid1Δ: KanMX4, ccb1Δ: KanMX4, and yec1Δ: KanMX4 mutants have the genetic background of BY4741 (MATa his3Δ1 leu2-3,112 met15Δ1 ura3Δ1) (Invitrogen). To construct a plasmid to disrupt HOG1 with LEU2, the plasmid pUHOG1 (10) was digested with Styl and used to delete an ~500-bp fragment within the open reading frame of HOG1, and the region was replaced with LEU2. The resultant plasmid (pUHOG1Leu2) was digested with BamH I and SpH I, and the hog1Δ: LEU2 cassette was used to disrupt the HOG1 gene.

The alleles of ssh1Δ: URA3 and sholΔ: HIS3 with a TM151 background, which was donated by Dr. T. Maeda (16), were amplified by PCR with the following primers: ssh1Δ: URA3, 5'-CGTGGTTGATCCGAATTCGACCTGCTATGGACGGGC-3’ and 5’-GTCGACGAGGAGAAGAAACGCTACTGCGTAAG-3’; sholΔ: HIS3, 5'-CGTAAAAGCTTTGACCGCTACCGACCATG-3’ and 5’-CCACACATGCTCCTCAGGTAGAGCATGACG-3’. The SK1 and SHO1 loci of S. cerevisiae YPH250 were disrupted using these DNA fragments.

The double mutant gpd1Δ: TRP1 gpd2Δ: URA3 with the W303-1A background was donated by Dr. S. Hohmann. Each allele was amplified by PCR using the following primers: gpd1Δ: TRP1, 5’-ACCCACACCACAAATACGTAACAGGGGGCGC-3' and 5’-GCGAAGGACCGTAATGCGGTCATTTTAGCC-3'; gpd2Δ: URA3, 5’-CGCAGCGCTATTTCGACCGCTACCGACCATG-3’ and 5’-GGCATAACAGGTCTTGGCACAAGTGACCGCA-3'. The GPD1 and GPD2 loci of YPH250 were disrupted using these DNA fragments.

Disruption of PBS2 and WHI2 was carried out using pJB4D (17) and pHY38 (18), respectively, which were donated by Dr. F. Estruch and Y. Kikuchi.

Mutants glo1Δ: URA3, glo1Δ: HIS3, msn2Δ: HIS3, msn4Δ: URA3, crz1Δ: LEU2, and ccb1Δ: HIS3 with the YPH250 background were described previously (5, 10, 19).

**Adaptation Test**—Cells were cultured in the SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids) supplemented with appropriate amino acids and bases at 28 °C until $A_{610}$ = 0.5, and 8 mM MG was added. After incubation for 3 h, 50 mM MG was added, and a portion of the cell suspension was recovered periodically.

**Spot Assay**—Cells cultured in the SD minimal medium to $A_{610}$ = 0.5 were diluted serially (1:10) with the same solution to spread on YPD agar plates. After incubation at 28 °C for 2 days, the colonies were counted. For control experiments, cells cultured in SD medium to $A_{610}$ = 0.5 were directly challenged with 50 mM MG.

**Spot Assay**—The cells were cultured in YPD medium (2% glucose, 1% yeast extract, 2% peptone) until $A_{610}$ = 0.1, diluted serially (1:10) with the sterilized 0.85% NaCl solution, and spotted (5 μl) onto YPD agar.

**Fig. 1.** Effect of MG on viability of yeast. A, wild-type cells were cultured in SD medium until $A_{610}$ = 0.5, and 50 mM MG was added (open squares). For induction of adaptation, the cells were pretreated with 8 mM MG for 0.5 h (closed diamonds), 1 h (closed circles), 2 h (closed triangles), and 3 h (open squares) and then challenged with 50 mM MG. The data are for three independent experiments (averages ± S.D.). B, wild-type cells were cultured in SD medium until $A_{610}$ = 0.5, and 8 mM MG was added. The data are for three independent experiments (averages ± S.D.).

**Fig. 2.** MG activates HOG-MAP kinase cascade. A, cells were spotted onto YPD agar plates with or without 20 mM MG and cultured at 28 °C for 3 days. B, hog1Δ cells expressing Hog1-GFP were cultured in the SD minimal medium until $A_{610}$ = 0.5, and 8 mM MG was added. The localization of Hog1-GFP was monitored at the prescribed time. C, Northern blotting analysis of cells treated with 8 mM MG was carried out as described in the text. In each lane, 20 μg of RNA was loaded. D, phosphorylation of Hog1 and its protein level of cells treated with 8 mM MG were detected by Western blotting as described in the text. WT, wild type.
FIG. 3. MG activates HOG-MAP kinase cascade in a Sln1 branch-dependent manner. A, cells were spotted onto YPD agar plates with or without 20 mM MG and cultured at 28 °C for 3 days. B, cells of each mutant were treated with 8 mM MG for 15 min, and Hog1 phosphorylation was monitored by Western blotting. C, cells of each strain carrying Hog1-GFP were treated with 8 mM MG for 30 min, the localization of Hog1-GFP was observed. All of the strains used have a hog1Δ background. WT, wild type.

plates containing various concentrations of MG.

GFP-tagged Proteins—To construct Hog1-GFP, the open reading frame of HOGL with its promoter (487-bp upstream region) (20) was cloned by PCR using primers Hog1SacF, 5′-AGTTAATTGAGCTCGAT and Hog1BamR, 5′-AAACACGGATCCTGTTTGAACCTGATTGAGCTCGAT-3′, and Hog1BamR, 5′-AAACACGGATCCTGTTTGAACCTGATTGAGCTCGAT-3′, and Hog1BamR, 5′-AAACACGGATCCTGTTTGAACCTGATTGAGCTCGAT-3′, were cultured in the SD minimal medium until
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Measurement of Intracellular Ca2+ with Fura 2-AM—The uptake of Ca2+ in the medium by yeast cells was measured using the Ca2+ specific fluorescence probe Fura 2-AM as described by Iida et al. (25) with some modifications. Briefly, the cells were cultured in SD medium in the presence of 2 μM Fura 2-AM (Dojindo Laboratories) dissolved in Me2SO under darkness until A610 = 0.5 at 28 °C, and then 8 mM MG was added. After incubation for 15 min, the cell extracts were prepared as described by Bell et al. (24). Phosphorylation of Hog1 was monitored using an anti-diphosphorylated p38 monoclonal antibody (Sigma). Protein levels of Hog1 were measured using anti-Hog1 antibody (Santa Cruz Biotechnology).

Measurement of Intracellular Oxidation Levels—Intracellular oxidation levels were measured using the oxidant-sensitive probe 2′,7′-dichlorofluorescin diacetate (Molecular Probes) as described previously (28).

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Measurement of cAMP and glycerol—Intracellular cAMP and glycerol were extracted from cells with distilled water by heating cell suspension at 100 °C for 2 min. After removal of cell debris by centrifugation, the amount of cAMP and glycerol in the resultant supernatants was deter-
RESULTS

MG Induces Stress Response—Consistent with the high reactivity of the aldehyde group in nature, MG, a typical 2-oxoaldehyde, has been reported to react with DNA, RNA and protein to make adducts (for reviews, see Refs. 3 and 29). MG consequently disrupts cellular functions. To explore the effect of MG on cell growth in the budding yeast S. cerevisiae, we monitored the viability of yeast cells after exposure to high concentrations of MG. As shown in Fig. 1A, 50 μM MG killed S. cerevisiae cells, and cell viability declined rapidly. On the other hand, when MG was added at low concentrations (8 μM) to the culture in which yeast cells were growing logarithmically, cell growth was temporarily suspended, but cell viability did not drop, and the cell growth resumed after 3 h (Fig. 1B). Du et al. (30) reported that MG depletes intracellular glutathione causing the production of reactive oxygen species, and subsequently Ask1 is activated in Jurkat cells to induce apoptosis. To determine whether MG causes the production of reactive oxygen species in S. cerevisiae, we measured intracellular oxidation levels using an oxidant-specific fluorescent probe in cells treated with 8 μM MG. We found that the cellular oxidation level did not increase (data not shown) and thus concluded that a low dose of MG per se induces some kind of stress response, and the cells exposed to 8 μM MG may acquire tolerance to this aldehyde during the period of temporal growth arrest.

To address this possibility, cells pretreated with 8 μM MG were exposed to 50 μM MG. As shown in Fig. 1A, the pretreatment of yeast cells with a low dose of MG conferred resistance to MG. Interestingly, the longer the period of pretreatment, the greater the extent of the rise in the rate of survival. These results imply that there is some regulatory system(s) to transfer the MG signal to the nucleus to induce MG-responsive gene expression resulting in the acquisition of MG resistance.

MG Activates HOG-MAP Kinase Cascade—We have previously reported that GLO1 expression is specifically induced by osmotic stress in a HOG-MAP kinase-dependent manner (10). To gain further insights into the correlation between MG metabolism and HOG-MAP kinase, we determined the phenotype of a hog1Δ mutant in terms of its susceptibility to MG. The hog1Δ mutant showed increased sensitivity to MG (Fig. 2A). Hog1 is activated through phosphorylation by Pbs2 MAP kinase, and consequently, a pbs2Δ mutant also exhibited susceptibility to MG (Fig. 2A). In addition, the hog1Δ mutant was impaired in the acquisition of tolerance to MG after pretreatment with a low dose of MG (data not shown).

Hog1 is translocated into the nucleus under high osmotic stress (12, 20) and functions as a component of the transcription machinery (31). We monitored the intracellular localization of Hog1 using GFP-tagged protein and found that Hog1 rapidly translocated into the nucleus after treatment with 8 μM MG (Fig. 2B). Subsequently, expression of the genes under the control of Hog1, such as GLO1 (10) and CTT1 (11), was induced following MG treatment (Fig. 2C).

Phosphorylation is necessary for the translocation of Hog1 into the nucleus (32). Both Thr174 and Tyr176 of Hog1 are phosphorylated by Pbs2. We determined whether Hog1 is phosphorylated using anti-diphospho-p38 monoclonal antibody. As shown in Fig. 2D, Hog1 is phosphorylated upon MG treatment. The timing of the phosphorylation coincided well with the nuclear localization of Hog1 following MG treatment (Fig. 2D). Hog1 was redistributed in the cytoplasm after 60 min of MG treatment (Fig. 2B), and concomitantly, Hog1 phosphorylation reverted to the initial level (Fig. 2D).

MG Activates an Msn2 Transcription Factor—In addition to Hog1, the Msn2 and Msn4 transcription factors were also necessary for the osmotic stress response of GLO1 (10). We then determined the susceptibility of the msn2Δ and msn4Δ mutants to MG to evaluate the contribution of these transcription factors to MG metabolism. We found that msn2Δ exhibited increased sensitivity to MG, whereas the sensitivity of msn4Δ was more moderate (Fig. 4A). We determined whether msn2Δ cells could acquire resistance to MG stress if pretreated with a low dose of MG and found that the msn2Δ mutant was impaired in its adaptation to MG stress (data not shown).
Msn2 is concentrated in the nucleus when cells are exposed to several forms of environmental stress such as oxidative stress, heat shock stress, and osmotic stress. The intracellular localization of Msn2 was monitored in cells treated with 8 mM MG. As shown in Fig. 4B, Msn2-GFP was concentrated in the nucleus following the treatment with MG.

It has been reported that cAMP negatively regulates Msn2 through the activation of protein kinase A (12, 35, 36). We measured cAMP levels in cells treated with 8 mM MG but found no distinct difference between the MG-treated cells and untreated cells (untreated, 55.4 ± 6.0 pmol/10^9 cells; treated, 51.5 ± 0.06 pmol/10^9 cells).

Kikuchi and co-workers (18) have reported that Whi2 protein phosphatase is involved in regulating the activity of Msn2 by controlling its phosphorylation. They reported that the transcriptional activity of Msn2 for the STRE-lacZ reporter gene decreased in a whi2Δ mutant; nevertheless, Msn2 was accumulated in the nucleus of whi2Δ cells treated with H_2O_2 or NaCl (Ref. 18; also see Fig. 4B). As shown in Fig. 4B, Msn2 was concentrated in the nucleus of whi2Δ cells after treatment with 8 mM MG, although the whi2Δ mutant showed sensitivity to MG (Fig. 4C). The additive effect in terms of susceptibility to MG of msn2Δwhi2Δ cells was not observed (data not shown). These results imply that the nuclear localization of Msn2 following MG treatment is regulated irrespective of Whi2; however, both Msn2 and Whi2 seem to be involved in the same detoxification pathway of MG in yeast.

MG Activates Calcineurin/Crz1-mediated Ca^{2+} Signaling Pathway—We have demonstrated that MG activates the HOG-MAP kinase cascade in a Sln1-dependent manner. Therefore, the factor(s) that interacts with this pathway is expected to be involved in MG metabolism and/or MG stress response either directly or indirectly. Miyakawa and co-workers (37) recently reported that Hog1 activity is antagonistically regulated by calcineurin to control the cell cycle of yeast. Calcineurin plays a crucial role in Ca^{2+} signaling in eukaryotes from yeasts to mammals (38). Crz1 is supposed to be the sole transcription factor that functions under the control of calcineurin in yeast (15). Crz1 is phosphorylated and predominantly present in the cytoplasm under normal conditions, although calcineurin in an active form dephosphorylates Crz1 to allow this zinc finger transcription factor to translocate into the nucleus (39). To explore the correlation between MG metabolism and the calcineurin/Crz1 pathway, we monitored the localization of GFP-Crz1 in cells treated with MG. As shown in Fig. 5A, Crz1 was concentrated in the nucleus with MG treatment, and consequently, the expression of the Crz1 target gene, FKS2 (40), was enhanced with MG treatment (Fig. 5C). However, the nuclear
localization of Crz1 following MG treatment was not observed in the calcineurin-deficient (cnb1Δ) mutant or when FK506, a potent inhibitor of calcineurin, was present (Fig. 5B).

The increase in the intracellular Ca\(^{2+}\) level is necessary for activation of calcineurin. To activate calcineurin, a high concentration of CaCl\(_2\) (200–500 mM) is usually loaded into yeast cells (37); however, we did not supply Ca\(^{2+}\) to the culture medium in our experimental conditions. To determine whether Ca\(^{2+}\) in the medium is involved in MG-dependent activation of calcineurin, we added EGTA, a Ca\(^{2+}\) chelator, to the culture medium before MG treatment. As shown in Fig. 5A, preloading of EGTA blocked the nuclear localization of Crz1 following MG treatment. This result implies that MG-treated cells actively take up Ca\(^{2+}\) in the medium to activate the calcineurin/Crz1-mediated pathway. To address this possibility, the changes in intracellular Ca\(^{2+}\) levels were evaluated using the Ca\(^{2+}\)-specific fluorescent probe Fura 2-AM. As shown in Fig. 5D, MG enhanced the uptake of Ca\(^{2+}\), thereby activating calcineurin to allow the nuclear localization of Crz1.

**Nuclear Export of Msn2 and Crz1 Is Normal with MG Treatment**—We have demonstrated that MG activates Hog1, Msn2, and calcineurin resulting in the nuclear localization of Crz1. The nuclear export of Hog1 is regulated by Crm1/Xpo1 (20). Recently, we revealed that exogenously added MG did not impair the function of Crm1/Xpo1 (27). Therefore, the nuclear localization of Hog1 following the treatment with MG seems to occur irrespective of the blockade of the nuclear export.

The nuclear export of Msn2 and Crz1 is performed by Msn5 (22, 41). If the function of Msn5 was damaged by MG, these transcription factors would be accumulated in the nucleus because of a blockade of the nuclear export. To address this possibility, we monitored the localization of a reporter protein that contains three GFP moieties followed by the nuclear localization signal (NLS) of SV40 large T antigen (NLSSV40) and the nuclear export signal (NES) for Msn5, which is derived from Crz1 (amino acid residue, 186–279) (22). This reporter protein (3xGFP-NLSSV40-NESCrz1 (186–279)) is imported into the nucleus because of the NLSSV40, although it is immediately exported from the nucleus by Msn5, and thus this reporter protein is basically cytoplasmic-resident (22). If Msn5 is impaired by MG, this reporter protein is expected to accumulate in the nucleus. As shown in Fig. 6, 3xGFP-NLSSV40-NESCrz1 (186–279) is distributed in the cytoplasm when cells were treated with MG, suggesting that Msn5 functions normally.

Next, to exclude the possibility that NLSSV40 was impaired by the MG treatment and so 3xGFP-NLSSV40-NESCrz1 (186–279) was not translocated into the nucleus, we determined the nucleocytoplasmic localization of another reporter protein consisting of NLSSV40 and mutant NES of human protein kinase inhibitor (PKI) a (NESPKI-P12) followed by two moieties of GFP (Fig. 6). The human PKI a contains Crm1/Xpo1-recognizable NES, and the NESPKI-P12 mutant contains a single substitution of leucine to alanine in the NESPKI motif (42), rendering the NLSSV40-NESPKI-P12-2xGFP mutant unable to interact with Crm1/Xpo1. Therefore, this reporter protein is translocated into the nucleus because of NLSSV40 and is constitutively concentrated in the nucleus under normal conditions (21). We found that NLSSV40-NESPKI-P12-2xGFP was located in the nucleus following the treatment with MG (Fig. 6), indicating that the function of NLSSV40 was not impaired with MG treatment. Collectively, Msn5 functions normally in the presence of MG, and therefore, the nuclear accumulation of Hog1, Msn2, and Crz1 following the treatment with MG was not attained by the blockade of the nuclear export. This result also suggests that the activation of Hog1, Msn2, and Crz1 by MG is a result of the positive response of yeast cells and not a consequence of cellular damage by MG.

**DISCUSSION**

In this study, we have demonstrated that MG activates Hog1 and Msn2, both of which are involved in the metabolism of MG through the regulation of GLO1 expression (10). In addition, MG also activated calcineurin, leading to the nuclear localization of Crz1. Recently, we found another example of the MG-dependent activation of a yeast transcription factor, i.e. MG-activated Yap1 (27). In the activation of Yap1 by MG, an increase in the intracellular Mg level was necessary, and the threshold for the activation was approximately ~5–6 mmol/10\(^7\) cells. The cellular Mg level increased slowly after exposure of yeast cells to 8 mM MG (untreated, 2.14 ± 0.33 nmol/10\(^7\) cells; treated with 8 mM MG for 30 min, 3.94 ± 0.17 nmol/10\(^7\) cells; for 1 h, 7.48 ± 0.44 nmol/10\(^7\) cells), and therefore, the nuclear localization of Yap1 following MG treatment was attained after 60 min. On the other hand, the nuclear localization of Hog1, Msn2, and Crz1 was accomplished rapidly (within 10 min). These observations lead us to consider two possibilities, i.e. threshold for the activation of these factors is lower than that for the activation of Yap1, or there is some sensory system for external MG on the cell surface to convey signals to these factors. To address the former possibility, we determined the localization of Hog1-GFP, Msn2-GFP, and GPP-Crz1 in g1oΔ cells, in which the Mg level (5.25 ± 1.17 nmol/10\(^7\) cells) was high enough to cause the constitutive nuclear localization of Yap1 (27). None of these factors were found to have concentrated in the nucleus of g1oΔ cells (data not shown). In addition, the intracellular Mg level did not increase after treatment with 8 mM MG for 10 min (2.15 ± 0.35 nmol/10\(^7\) cells). These results suggest that the nuclear accumulation of Hog1, Msn2, and Crz1 is attained irrespective of the intracellular Mg level.

O’Rourke and Herskowitz (43) reported that under low level osmotic stress only the Snl1 branch can activate Hog1. MG activates Hog1 in a Snl1 branch-dependent manner (Fig. 3), and Msn2, which is concentrated in the nucleus under conditions of osmotic stress, also accumulated in the nucleus following the treatment with MG (Fig. 4). Therefore, one might assume that 8 mM MG caused osmotic stress. S. cerevisiae
produces glycerol as a compatible osmolyte under osmotic stress (13). However, the intracellular glycerol content did not increase after MG treatment for up to 3 h (before treatment, $0.30 \pm 0.07$ mg/10^9 cells; after 3 h, untreated, $0.31 \pm 0.03$ mg/10^9 cells; 8 mM MG treated, $0.29 \pm 0.05$ mg/10^9 cells; 0.5 mM NaCl treated, $11.7 \pm 0.88$ mg/10^9 cells). In addition, gpd1Δgpd2Δ, a mutant that exhibits hypersensitivity to osmotic stress caused by a defect in glycerol production (44), did not show susceptibility to MG (data not shown). Taken together, we conclude that 8 mM MG does not cause osmotic stress, and therefore, Hog1 activation following MG treatment is not due to changes in osmolarity.

Reiser et al. (45) reported that the changes in turgor pressure of cells caused by treatment with nystatin or zymolyase activate Hog1 through the Sln1 branch but not Sho1 branch. Of the two osmosensors, MG specifically stimulated the Sln1 branch (Fig. 3). To determine the correlation between MG treatment and turgor pressure, we examined cellular volume by measuring the colony-forming unit (cell number/A_{610}) of cells following the treatment with MG. As shown in Fig. 1, 8 mM MG temporarily halted the increase in cell number (but not killed cells) for 3 h, although the absorbance of the culture at 610 nm (A_{610}) continued to increase during this period; hence, the colony-forming unit value decreased ~35%. This observation implies that MG arrests the budding process at a certain point, but cells continue to proliferate, and consequently, cellular volume increases during the first 3 h of MG treatment. This suggests that turgor pressure tends to increase on treatment with 8 mM MG; however, Reiser et al. (45) reported that a decrease in turgor pressure caused by nystatin treatment inactivated the Sln1 histidine kinase activity, leading to the activation of the HOG-MAP kinase cascade. Therefore, the activation of Hog1 through the Sln1 branch following MG treatment may not be due to the decrease in the turgor pressure of cells. Nevertheless, cellular volume seemed to increase with MG treatment, which may elevate turgor pressure.

Regarding Hog1 activation through the Sln1 branch following MG treatment, another possibility exists. Singh (46) has previously reported that the Sln1 branch mediates oxidative stress elicited by H_2O_2 and diamide, although the mechanism involved has not yet been clarified. Recently, we found that MG potentially modifies the cysteine residues of a protein reversibly (27). A single cysteine residue is present within the external region of Sln1; hence, we cannot rule out the possibility that MG and some oxidants directly irritate Sln1 through such a residue.

We found that MG transiently activates the uptake of Ca^{2+} from the medium (Fig. 5D). It has been reported that yeast cells take up Ca^{2+} in the medium through Mid1 and Cch1, both of which belong to a family of stretch-activated channels (47), in response to the changes in tension of the cytoplasmic membrane under hyperosmotic stress (48). We have concluded that MG treatment itself does not seem to cause hyperosmotic stress. Rather, our data suggest that MG mimics hypotonic stress. Batiza et al. (49) reported that hypotonic stress activates unidentified stretch-activated Ca^{2+} channel(s) in yeast, leading to a transient increase in the Ca^{2+} level in the cytoplasm; however, such an increase in cytoplasmic Ca^{2+} seems to be the consequence of mobilization from an intracellular Ca^{2+} pool. We monitored the localization of GFP-Crz1 in mid1Δ or cch1Δ cells following treatment with MG. GFP-Crz1 was concentrated in the nucleus of such mutant cells after treatment with MG (data not shown). Furthermore, the nuclear localization of GFP-Crz1 was also observed in cells defective in YVC1 (data not shown), the gene product of which is a transient receptor potential-like channel and releases vacuolar Ca^{2+} in response to hyperosmotic stress (50). Because we have demonstrated that the EGTA treatment blocked the transient increase in the Ca^{2+} level of cells treated with MG (Fig. 5D), we concluded that yeast cells take extracellular Ca^{2+} in response to MG treatment through some Ca^{2+} channel(s) yet to be identified.

Collectively, MG seems to cause the cell wall stress and block a certain step in the budding process, but cells continue to proliferate, and subsequently, MG influences the integrity of the cell wall and/or cell membrane, leading to the increase in turgor pressure. Consequently, MG causes activation of Hog1, and concomitantly, activation of calcineurin through enhancement of the influx of Ca^{2+} (Fig. 7).

Several reports have demonstrated a strong correlation between the aberrant metabolism of MG and pathological processes of some diseases, such as cancer and diabetes mellitus.
and its complications (for reviews, see Refs. 29 and 51). For example, cells in the tissues of diabetic patients are constantly exposed to high concentrations of extracellular MG, because MG levels are higher in the red blood cells and plasma of such patients than those of healthy individuals (52, 53); however, it has long been questioned whether this is a cause or consequence of diabetes. Carlson et al. (54) have recently reported that the basal activity of the MAP kinase family, including p38, 10. Inoue, Y., Tsujimoto, Y., and Kimura, A. (1998) J. Biol. Chem. 273, 2255–22558.

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Methylglyoxal, a Metabolite Derived from Glycolysis, Functions as a Signal Initiator of the High Osmolarity Glycerol-Mitogen-activated Protein Kinase Cascade and Calcineurin/Crz1-mediated Pathway in *Saccharomyces cerevisiae*

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