Phosphatidylinositol 3,5-bisphosphate is involved in methylglyoxal-induced activation of the Mpk1 mitogen-activated protein kinase cascade in Saccharomyces cerevisiae

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Running title: Role of PtdIns(3,5)P2 in MG-induced Mpk1 phosphorylation

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ABSTRACT

Methylglyoxal (MG) is a natural metabolite derived from glycolysis, and this 2-oxoaldehyde has been implicated in some diseases including diabetes. However, the physiological significance of MG for cellular functions is yet to be fully elucidated. We previously reported that MG activates the Mpk1 (MAPK) cascade in the yeast Saccharomyces cerevisiae. To gain further insights into the cellular functions and responses to MG, we herein screened yeast-deletion mutant collections for susceptibility to MG. We found that mutants defective in the synthesis of phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2) are more susceptible to MG. PtdIns(3,5)P2 levels increased following MG treatment, and vacuolar morphology concomitantly changed to a single swollen shape. MG activated the Pkc1–Mpk1 MAPK cascade in which a small GTPase Rho1 plays a crucial role, and the MG-induced phosphorylation of Mpk1 was impaired in mutants defective in the PtdIns(3,5)P2 biosynthetic pathway. Of note, heat shock-induced stress also provoked Mpk1 phosphorylation in a Rho1-dependent manner; however, PtdIns(3,5)P2 was dispensable for the heat shock-stimulated activation of this signaling pathway. Our results suggest that PtdIns(3,5)P2 is specifically involved in the MG-induced activation of the Mpk1 MAPK cascade and in the cellular adaptation to MG-induced stress.

INTRODUCTION

Catabolic processes are necessary in all heterotrophs for life functions. Although numerous intermediates and their derivatives are produced during the metabolism of organic compounds to produce energy, some metabolic intermediates affect cellular functions (1-3). Methylglyoxal (MG) is one of the metabolites derived from glycolysis, and is mainly formed from the reaction of triosephosphate isomerase (4,5). Since MG is a highly reactive carbonyl compound, it produces advanced glycation end products (AGEs) with more than 20000-fold greater efficiency than glucose (4,5). Therefore, MG is involved in some diseases such as diabetes and its complications, cancers, Alzheimer’s disease, and autism (6-14), suggesting that MG plays a role as a functional metabolic intermediate under pathological conditions. However, the molecular mechanisms by which MG causes such diseases currently remain unclear.

We have been attempting to reveal the physiological significance of MG using Saccharomyces cerevisiae and Schizosaccharomyces pombe as model organisms (4). We previously reported that MG causes acute
cellular responses in yeast. For example, the exogenous addition of MG activated yeast AP-1-like transcription factors (15,16) and the signaling of the protein kinase C (Pkc1)-Mpk1 mitogen-activated protein (MAP) kinase (MAPK) cascade (17). MG was also found to inhibit glucose uptake through the attenuation of hexose transporter activities as well as the enhancement of their endocytosis (18). In the present study, we found that mutants defective in the synthesis of phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2) showed greater susceptibility to MG.

The phosphorylated phosphatidylinositol (PtdIns) derivatives are crucial for various cellular processes including signal transduction, membrane traffic, and cytoskeleton organization (19,20). Four species of phosphoinositides have been identified in the budding yeast S. cerevisiae (PtdIns3P, PtdIns4P, PtdIns(3,5)P2, and PtdIns(4,5)P2) (20). PtdIns(3,5)P2 is synthesized from PtdIns3P by PtdIns3P 5-kinase (Fab1) in S. cerevisiae (19,20). In yeast, PtdIns(3,5)P2 is probably involved in the regulation of vacuolar morphology, i.e. the deletion of Fab1 was found to enhance vacuolar fusion and increase vacuolar size (21,22). PtdIns(3,5)P2 is also necessary for the stability of vacuolar H+ ATPase (V-ATPase) (23) and vacuolar acidification (21-24). In contrast, hyperosmotic stress, which transiently increases PtdIns(3,5)P2 levels, induces the fragmentation of vacuoles (25). Therefore, PtdIns(3,5)P2 plays a role in vacuolar functions and morphology.

In the present study, we show that MG causes alterations in vacuolar morphology and increases PtdIns(3,5)P2 levels in a Fab1-dependent manner. Mutants defective in the biosynthetic pathway of PtdIns(3,5)P2 (vps34Δ, fab1Δ, and vac7Δvac14Δ) showed sensitivity to MG. The Fig4 protein mostly functions as a phosphatidylinositol phosphatase; however, it is also involved in the Fab1 complex (Fab1-Vac7-Vac14) through an interaction with Vac14 (25,26). The steady state level of PtdIns(3,5)P2 in fig4Δ cells was similar to that in wild type cells; however, MG-induced increases in PtdIns(3,5)P2 levels did not occur in this mutant. We found that the MG-induced activation of the Pck1-Mpk1 MAPK cascade was impaired in mutants defective in the synthetic pathway of PtdIns(3,5)P2 (vps34Δ, fab1Δ, and vac7Δvac14Δ). In contrast, the heat shock-induced activation of this cascade occurred in these mutants. Notably, the deletion of FIG4 did not affect the MG-induced phosphorylation of Mpk1 or tolerance to MG, indicating that an increase in PtdIns(3,5)P2 levels is not necessarily required for the MG-induced activation of the Mpk1 MAPK cascade.

RESULTS
Screening of MG-sensitive mutants.
To explore the physiological significance of MG in the biological system, we screened non-essential gene knockout collections of S. cerevisiae BY4741 for sensitivity to MG. Each mutant was cultured in YPD liquid medium in 96-well microtiter plates for 2 days and then replica plated onto YPD agar plates containing various concentrations of MG. Among approximately 4800 mutants, 204 mutants showed poor or no growth on YPD agar plates containing 30 mM MG. The cells of the candidates (204 mutants) (Table S1) from master plates (0 mM MG) were picked up and then streaked on YPD agar plates containing 30 mM MG to verify their sensitivity to MG, and the mutants that clearly showed sensitivity to MG were selected (62 mutants) (Table S1). The resultant candidates were cultured in YPD medium until the log phase of growth, serially diluted, and spotted on YPD agar plates containing various concentrations of MG. Thirty-one mutants showed severe sensitivity to MG (Table 1). GLO1 and GLO2 code for glyoxalase I and glyoxalase II, respectively, which catalyze the detoxification of MG to lactic acid (4), and mutants defective in these genes were involved in this screening, indicating that our screening system worked appropriately. The activities of glyoxalases I and II in these mutants, except for glo1Δ and glo2Δ, were not significantly different from those in wild type cells (data not shown).

The responsible genes of these 31 mutants obtained by our screening method were classified into 7 categories (Table 1), among which several mutants defective in lipid metabolism were involved. Notably, VPS34 and VPS15, which encode PtdIns 3-kinase and its regulatory protein (Ser/Thr kinase), respectively, were involved. The phosphatidylinositol species in S. cerevisiae identified to date were PtdIns3P, PtdIns4P, PtdIns(3,5)P2, and PtdIns(4,5)P2 (Fig. 1A). Since vps34Δ and vps15Δ mutants showed greater susceptibility to MG, we investigated whether a
mutant defective in \textit{FAB1}, encoding PtdIns3P 5-kinase that synthesizes PtdIns(3,5)P$_2$, also exhibits MG sensitivity on YPD agar plates. A \textit{fab1}$\Delta$ mutant with the BY4741 background did not show strong susceptibility to 20 mM MG on YPD agar plates (Fig. 1B); however, \textit{fab1}$\Delta$ cells showed severe growth defects on SD agar plates containing 8 mM MG (Fig. 1C). Since the catalytic activity of Fab1 is regulated by the activator proteins Vac7 and Vac14 (22,27-29), we assessed the effects of a deficiency in these genes on growth in SD medium containing MG. \textit{FAB1}, \textit{VAC7}, and \textit{VAC14} were disrupted in YPH250, and the susceptibilities of resultant mutants to MG were evaluated. As shown in Fig. 1D, a \textit{fab1}$\Delta$ mutant (YPH250) showed severe susceptibility to MG (no growth on SD agar plates containing 4 mM MG), and a \textit{vac7}$\Delta$\textit{vac14}$\Delta$ mutant was unable to grow on SD agar plates containing 6 mM MG, in which medium wild type cells showed poor growth. These results suggest that PtdIns(3,5)P$_2$ is involved in tolerance or adaptation to MG-induced stress. In the present study, we focused on the physiological role of PtdIns(3,5)P$_2$ with respect to MG-induced stress responses.

\textbf{MG changes vacuolar morphology.}

PtdIns(3,5)P$_2$ is enriched in the vacuolar membrane and is involved in the control of vacuolar morphology and functions (22,30-34). Previous studies reported that mutants defective in the production of PtdIns(3,5)P$_2$, i.e. \textit{vps34}$\Delta$, \textit{vps15}$\Delta$, \textit{fab1}$\Delta$, \textit{vac7}$\Delta$, and \textit{vac14}$\Delta$, have a single grossly enlarged vacuole (21,22,35,36). Vacuolar morphology dynamically changes in response to alterations in environmental conditions around the cell, e.g. vacuoles are fragmented and shrunken under hyperosmotic conditions, but swollen under hypo-osmotic conditions (37). We previously reported that MG enhances the internalization of hexose transporters (Hxts) from the plasma membrane as well as the degradation of Hxts in vacuoles through the endocytotic pathway (18). In the process of observing the endocytosis of Hxts to vacuoles, we noted that MG changed vacuolar morphology. Therefore, we examined the effects of MG on vacuolar morphology.

Wild type cells at the logarithmic growth phase predominantly have two to four fragmented vacuoles. As shown in Fig. 2A, vacuolar morphology changed from a fragmented form to a single swollen shape following the treatment with 10 mM MG for 60 min. To evaluate this phenomenon quantitatively, we measured the number of vacuoles per cell and the size (diameter) of vacuoles. As shown in Fig. 2A, the number of vacuoles per cell decreased, while vacuolar size inversely increased following the treatment with MG. In addition, vacuolar morphology reverted to the fragmented form when MG was removed from the medium (Fig. 2B). The HOPS (homotypic vacuole fusion and vacuole protein sorting) complex and SNAREs (soluble \textit{N-ethylmaleimide-sensitive factor attachment protein receptor}) were previously shown to be needed for homotypic vacuolar fusion (38). Previous studies reported that the deletion mutant of \textit{Vps41} (one of the subunits of HOPS) or \textit{Vam3} (a vacuolar \textit{t-SNARE}) has highly fragmented vacuoles (36,39). As shown in Fig. 2C, MG-induced vacuolar defragmentation did not occur in these mutants, suggesting that MG induces the fusion of vacuoles.

\textbf{MG increases PtdIns(3,5)P$_2$ levels.}

PtdIns(3,5)P$_2$ cellular levels increased under hyperosmotic stressed conditions, in which cell vacuoles shrank and fragmented; therefore, an increase in PtdIns(3,5)P$_2$ levels is considered to be related to the fragmentation of vacuoles (25,40). Since we found that the defragmentation of vacuoles occurred in the presence of MG, we expected PtdIns(3,5)P$_2$ levels to decrease under these conditions. However, contrary to our expectations, PtdIns(3,5)P$_2$ cellular levels increased by approximately 3-fold after the treatment with MG for 60 min (Table 2). PtdIns3P and PtdIns4P levels remained unchanged, while PtdIns(4,5)P$_2$ levels slightly (~15%) decreased following the treatment with MG (Table 2). We then examined the time course of changes in vacuolar morphology and PtdIns(3,5)P$_2$ levels in the presence of MG. As shown in Fig. 3A, the number of vacuoles per cell decreased, and vacuolar size increased with increasing levels of PtdIns(3,5)P$_2$ following the treatment with MG.

Previous studies reported that Atg18 binds to PtdIns(3,5)P$_2$, and Atg18-GFP is detected in the limited area of the vacuolar membrane as well as punctate structures (41-43). PtdIns(3,5)P$_2$ is necessary for recruiting Atg18 to the vacuolar membrane (41-43). As shown in Fig. 3B, MG enhanced the recruitment of Atg18 to the vacuolar membrane in wild type cells. The punctate structures of Atg18-GFP in wild type
cells were rarely observed in our system, and the number of punctate structures did not increase in the presence of MG. Vac7 and Vac14 form a complex with Fab1 (22,27-29), and this complex is crucial for the synthesis of PtdIns(3,5)P2; therefore, PtdIns(3,5)P2 rarely exists or is hardly detectable in these mutants (22,27,28). We were unable to detect PtdIns(3,5)P2 in these mutants in the presence or absence of MG by our analytical system (data not shown); therefore, the recruitment of Atg18-GFP to the vacuolar membrane did not appear to occur in vac14Δ cells. These results suggest that MG increases PtdIns(3,5)P2 levels in the vacuolar membrane.

**MG does not affect vacuolar acidification.**

A correlation between PtdIns(3,5)P2 levels and the regulation of vacuolar acidification has been reported (21-24). The pH of vacuoles is kept low (~6.0) by the actions of V-ATPases, whereas the acidification of vacuoles is not achieved in mutants defective in the synthesis of PtdIns(3,5)P2, such as fab1Δ, vac7Δ, and vac14Δ (21-24). Therefore, PtdIns(3,5)P2 appears to play a crucial role in the acidification of vacuoles. A recent study reported that the loss of vacuolar acidity by the pharmacological inhibition of V-ATPase activity induces vacuolar fusion (44). These findings imply that MG-induced vacuolar defragmentation is caused by an impairment in vacuolar acidification, and an increase in PtdIns(3,5)P2 levels is one of the adaptation mechanisms that enhance V-ATPase activity. To investigate this possibility, we assessed vacuolar acidity using quinacrine, a fluorescent weak base to assess vacuolar acidification (28,45). Consistent with previous findings (21-24), vacuoles in fab1Δ, vac7Δ, and vac14Δ cells were not acidified (Fig. 4). The fluorescence intensity of vacuoles derived from quinacrine in wild type cells was not decreased by the MG treatment (Fig. 4), indicating that MG does not affect vacuolar acidification.

**PtdIns(3,5)P2 is necessary for the MG-induced activation of the Mpk1 MAPK cascade.**

We previously reported that MG activated Pkc1–Mpk1 signaling, and mutants defective in the Mpk1 MAPK cascade exhibited susceptibility to MG (17,46). Since mutants defective in the PtdIns(3,5)P2 biosynthetic pathway were also sensitive to MG (Fig. 1), PtdIns(3,5)P2 may be involved in the MG-induced activation of Pkc1–Mpk1 signaling. In order to verify this possibility, we introduced the constitutively active allele of PKC1 (PKC1<sup>R398F</sup>) into fab1Δ cells, and susceptibility to MG was investigated. As shown in Fig. 5A, PKC1 (PKC1<sup>R398F</sup>) suppressed the susceptibility of fab1Δ cells to MG. This result supports our hypothesis that a relationship exists between PtdIns(3,5)P2 levels and the activation of the Pkc1–Mpk1 MAPK cascade. We then monitored the phosphorylation status of Mpk1 as an index of the activation of Pkc1–Mpk1 signaling in mutants defective in the synthesis of PtdIns(3,5)P2. In wild type cells, the phosphorylation levels of Mpk1 were elevated following the treatment with MG (Fig. 5A) (17,46). Interestingly, the MG-induced phosphorylation of Mpk1 in vps34Δ, fab1Δ, and vac7Δvac14Δ cells was significantly weaker than that in wild type cells (Fig. 5B). Heat shock stress is also known to activate the Pkc1–Mpk1 MAPK cascade (47). However, the heat shock-induced phosphorylation of Mpk1 in vps34Δ, fab1Δ, and vac7Δvac14Δ cells was not markedly impaired (Fig. 5C). These results indicate that the biosynthetic pathway of PtdIns(3,5)P2 plays a role in the MG-induced activation of the Pkc1–Mpk1 MAPK cascade.

**Increases in PtdIns(3,5)P2 levels are not necessarily required for the MG-induced phosphorylation of Mpk1.**

Intercellular levels of PtdIns(3,5)P2 are regulated by PtdIns kinases as well as PtdIns phosphatases. The Fig4 protein mostly functions as a PtdIns(3,5)P2 5-phosphatase; however, it is also a member of the Fab1 complex via binding to Vac14 (25,48). Therefore, Fig4 is necessary for the biosynthetic process of PtdIns(3,5)P2 under hyperosmotic stressed conditions (48). As shown in Fig. 6A, steady state levels of PtdIns(3,5)P2 in wild type and fig4Δ cells were almost similar; however, the MG-induced increase in PtdIns(3,5)P2 levels did not occur in fig4Δ cells. Accordingly, the recruitment of Atg18-GFP to vacuolar membranes was not observed in fig4Δ cells following the treatment with MG (Fig. 6B). Meanwhile, the punctate structures of Atg18-GFP were more clearly observed in fig4Δ cells in the presence of MG; however, the reason for this currently remains unclear. These results indicate that the MG-induced increase in PtdIns(3,5)P2 levels occurs in vacuolar membranes in a Fig4-dependent manner.

To investigate whether the MG-induced increase in PtdIns(3,5)P2 levels in vacuolar
membranes is crucial for the MG-induced activation of Pkc1–Mpk1 MAPK signaling, we examined the phosphorylation levels of Mpk1 following the treatment with MG in \( \text{fig4} \Delta \) cells. The MG-induced phosphorylation of Mpk1 occurred even in \( \text{fig4} \Delta \) cells (Fig. 6C). In addition, the deletion of \( \text{FIG4} \) did not enhance susceptibility to MG (Fig. 6D). Taking the results of Fig. 5 into account, the presence of a similar amount of PtdIns(3,5)\( \_ \)P\(_2\) with the steady state level in wild type cells is sufficient for activation of the Pkc1–Mpk1 MAPK cascade in \( \text{fig4} \Delta \) cells, and further increases in PtdIns(3,5)\( \_ \)P\(_2\) levels are not necessarily required for this response cued by extracellular MG. Therefore, the presence of a similar amount of PtdIns(3,5)\( \_ \)P\(_2\) in \( \text{fig4} \Delta \) cells to that in wild type cells appears to be sufficient for adaptation to MG stress (Fig. 6D).

**DISCUSSION**

In the present study, we performed a comprehensive screening of mutants showing MG sensitivity using the non-essential gene knockout collections of \( S.\ cer<sup>erb</sup>vi<sub>siae</sub> \) BY4741. We eventually identified 31 mutants, the responsible genes of which were classified into 7 categories. For example, deficiencies in several transcriptional regulators (\( \text{NGG1, HF11, SW13, TAF14, and ERT1} \)) were found to exhibit sensitivity to MG. We previously reported that the yeast AP-1-like transcription factor Yap1 is activated through the reversible modification of C-terminal Cys residues within Yap1 with MG (15). Yap1 regulates the expression of \( \text{GSH1} \) encoding the rate-limiting step enzyme of glutathione biosynthesis, and glutathione is necessary for the detoxification of MG (4); therefore, a \( \text{yap1} \Delta \) mutant showed increased sensitivity to MG in SD medium (15). However, since YPD medium contained large amounts of glutathione, neither the \( \text{yap1} \Delta \) nor \( \text{gsh1} \Delta \) mutant was isolated in the 1st screening by our method. The genome-wide DNA microarray analysis revealed that the expression profile of yeast genes markedly changed following the treatment with MG (15). Therefore, the mutants defective in some transcriptional regulators were isolated as MG-sensitive mutants.

Some mutants categorized in protein quality control also showed MG sensitivity. MG is capable of binding with the amino, guanidino, and sulfhydryl groups of the amino acid residues of proteins to cause protein carbonylation, which eventually leads to the production of advanced glycation end products (AGES). Since this step is irreversible, MG-modified proteins must be removed from cells in order to avoid adverse effects on biological systems. For example, the accumulation of MG as well as MG-derived AGEs has been implicated in type 2 diabetes and its complications. Therefore, some mutants defective in protein quality control, including E3 ubiquitin ligase (\( \text{HEL2} \)), may be isolated as MG-sensitive mutants. Besides protein carbonylation, MG is known to exert genotoxicity, thereby causing DNA damage (5,49). Hence, some mutants defective in DNA repair (\( \text{RAD1, RAD50, and RAD51} \)) conceivably exhibit MG sensitivity.

We previously reported that MG induces the endocytosis of Hxts from the cytoplasmic membranes in \( S.\ cer<sup>erb</sup>vi<sub>siae</sub> \) (18). Some mutants defective in membrane traffic, such as \( \text{CHC1 and CLC1} \) (encoding the clathrin heavy chain and light chain, and crucial for the formation of endocytic vesicles) (50,51), \( \text{PEP12} \) (involved in the endocytotic process) (52), and \( \text{PEP3} \) (encoding the COEVET component, and functioning in the vesicular docking/fusion process) (53). Although the physiological significance of MG-induced endocytosis of Hxts in yeast cells remains unclear, it may provide a model of hyperglycemia in diabetic patients whose blood MG levels are higher than those of healthy individuals (18). A deficiency in the membrane traffic process, including endocytosis, appears to have serious effects on cellular functions in the presence of MG.

In the present study, we focused on the mutants categorized in lipid metabolism. Besides PtdIns3P mutants, another interesting result was that mutants defective in \( \text{CSG2} \) and \( \text{ISCI} \), gene products involved in sphingolipid biosynthesis, showed MG sensitivity. We recently reported that MG activated the target of rapamycin complex 2 (TORC2) signaling in \( S.\ cer<sup>erb</sup>vi<sub>siae</sub> \) as well as mammalian cells (17). TORC2 is involved in the regulation of sphingolipid biosynthesis in yeast (54). Mutants defective in the sphingolipid biosynthetic process are involved in MG-sensitive mutants, suggesting a correlation between MG and the TORC2 signaling pathway.

Vacuoles are dynamic organelles and their morphology is altered by environmental changes as well as some stress conditions, including hypo-osmotic stress, heat stress, and ethanol stress, conditions under which vacuoles are
swollen (37,55). The metabolism of PtdIns(3,5)\(P_2\) is involved in vacuolar morphology. PtdIns(3,5)\(P_2\) levels are lower in yeast cells than other phosphoinositides, although hyperosmotic stress has been shown to cause an acute elevation in PtdIns(3,5)\(P_2\) levels within 15 min (40). The elevation induced in PtdIns(3,5)\(P_2\) levels by MG was slower than that induced by hyperosmotic stress (Fig. 3A). Therefore, our results suggest that MG activates the machinery involved in the biosynthesis of PtdIns(3,5)\(P_2\) in a different manner by which osmotic stress elevates PtdIns(3,5)\(P_2\) levels. Hyperosmotic stress causes vacuolar fragmentation accompanied by a decrease in the volume of vacuoles. Since mutants defective in the synthetic pathway of PtdIns(3,5)\(P_2\) have a single enlarged vacuole, PtdIns(3,5)\(P_2\) appears to be linked with vacuolar morphology and size. We found that MG altered vacuolar morphology from a fragmented form to a single swollen shape through the induction of vacuolar fusion (Fig. 2), despite MG increasing PtdIns(3,5)\(P_2\) levels (Fig. 3A). A previous study reported that heat shock stress, which also alters vacuolar morphology from a fragmented form to a single swollen shape, increased PtdIns(3,5)\(P_2\) levels (56). Consequently, increase in PtdIns(3,5)\(P_2\) levels seems not necessarily correlate with vacuolar fragmentation.

Fragmented vacuoles become a large single vacuole in the stationary phase of growth (37). We previously reported that intracellular levels of MG increase with cell growth and reach a maximum during the transition period from the logarithmic phase to the stationary phase, which is referred to as the diauxic shift (15). Although the mechanisms by which MG promotes vacuolar fusion currently remain unclear (Fig. 2), the MG-induced defragmentation of vacuoles may be involved in entry into the diauxic shift.

A certain level of PtdIns(3,5)\(P_2\) appears to be necessary for activation of the Pkc1–Mpk1 MAPK cascade following the treatment with MG (Figs. 5 and 6); however, the heat shock-induced activation of this signaling pathway did not depend on PtdIns(3,5)\(P_2\), despite heat shock also increasing PtdIns(3,5)\(P_2\) levels (56) (Fig. 5). We recently reported that MG activated TORC2-Pkc1 signaling, which led to the phosphorylation of Mpk1 (17). Therefore, PtdIns(3,5)\(P_2\) may be involved in the regulation of TORC2 signaling. The small GTPase Rho1 is a regulator of the cell wall integrity pathway, in which the Pkc1–Mpk1 MAPK cascade is involved (47). Rho1 is also necessary for the MG-induced activation of TORC2–Pkc1 signaling (17). GFP-tagged Rho1 has been reported to localize at the plasma membranes and endomembrane including vacuolar membranes, in which PtdIns(3,5)\(P_2\) is abundant (57). A recent study reported that the C terminus of Rho1 has the ability to bind to some anionic lipids including PtdIns(3,5)\(P_2\) in vitro (58). These findings imply that PtdIns(3,5)\(P_2\) is involved in the MG-induced activation of the Pkc1–Mpk1 MAPK cascade via Rho1. Further studies are needed in order to elucidate the molecular mechanisms underlying the connection between PtdIns(3,5)\(P_2\) and the Pkc1–Mpk1 MAPK cascade.

**EXPERIMENTAL PROCEDURES**

**Media**

The media used were YPD (2% glucose, 1% yeast extract, and 2% peptone) and SD (2% glucose and 0.67% yeast nitrogen base without amino acids) with appropriate amino acids and bases being added to SD media where necessary.

**Strains**

The yeast strains used had the YPH250 background (MATa trp1-1Δ1 his3-Δ200 leu2-Δ1 lys2-801 ade2-101 ura3-52) unless otherwise stated. Gene deletions of FAB1 and VAC14 were constructed by PCR-based methods with his5' selective markers (59). Deletion constructs were amplified by PCR from BY4741-based deletion mutants (Invitrogen), and the corresponding loci of YPH250 were disrupted using PCR products. The VAC7 gene in YPH250 was disrupted by gene replacement methods using the *Candida glabrata* LEU2 gene (CgLEU2) as a selectable marker (60) with the following primers: 5'-ACAGAAGAAAGATAGAAAGCTCAGTGA GAGACAGAAACAGTACGAAGTTATTAGG TCTAG-3' and 5'-GGTTGCCTTTGCTGCTGCTGCGTGC TGGAGGAGTCAGACGAAGTTATTAGG TCTAG-3' and 5'-GGTTGCCTTTGCTGCTGCTGCGTGC TGGAGGAGTCAGACGAAGTTATTAGG TCTAG-3' and the plasmid BYP1419 as a template.

In order to disrupt *FIG4*, the *fig4α*::LEU2 allele in SEY6210 (61) was amplified by PCR with the following primers: 5'-GCCGAGCACTATTTGAATGTAAAGTGT GTG-3' and 5'-TGGTGAATCCTCATCTACTTCATGACTG AT-3', and the resultant PCR product was introduced into YPH250.
In order to disrupt *VPS34*, pKTY38, a derivative of pPHY38 (35), was digested with HindIII and KpnI, and the resultant HindIII-KpnI fragment was introduced into YPH250.

**Plasmids**

In order to create pRS306-ATG18-GFP, the C-terminal region of *ATG18* was amplified with the following primers: ATG18-GFP-F-XbaI (5'-GGATCTAGAGGATCCAAACAGCAGTA AC GG-3') and ATG18-GFP-R-XhoI (5'-TCCCTCGAGCATCCCTCAAGATGGAAT ACT-3'). The PCR product was digested with XbaI and XhoI, and the resultant fragment was introduced into the XbaI and XhoI sites of pHXT1-GFP (18). pRS306-ATG18-GFP was digested with ClaI, and linearized DNA was introduced at the *ATG18* locus.

A plasmid carrying *PKC1*<sup>R398P</sup> (YCp50-PKC1<sup>R398P</sup>) was donated by Dr. M.N. Hall (62).

**Treatment with chemicals**

Cells were cultured in SD medium (2% glucose, 0.67% yeast nitrogen base without amino acids) supplemented with appropriate amino acids and bases where necessary. When the *A<sub>610</sub>* of the culture reached 0.3-0.5, 5 or 10 mM MG was added, and cells were incubated at 28°C with reciprocal shaking. We verified that cell growth was temporally arrested in the presence of 10 mM MG, while cell viability was maintained.

**Spot assay**

Cells were cultured in SD medium until the early log phase of growth, and then diluted to *A<sub>610</sub> = 0.1* with the sterilized 0.85% NaCl solution. Cells were diluted serially (1:10) with sterilized 0.85% NaCl solution, and spotted (4 µl) onto YPD or SD agar plates containing various concentrations of MG.

**Fluorescence microscopy**

The fluorescence microscope BX51 (OLYMPUS) equipped with a digital camera DP70 (OLYMPUS) was used.

**Western blotting**

Procedures for the detection of Mpk1 were described previously (17). Anti-phospho-p44/42 MAP kinase (#9101; Cell Signaling) and anti-Mpk1 (sc6803; Santa Cruz Biotechnology) were used as the primary antibody. Immunoreactive bands were visualized with a BCIP-NBT solution kit (Nacalai tesque). The intensity of the immunoreactive bands was quantified using ImageJ.

**Vacuolar staining**

Yeast vacuoles were visualized in vivo by labeling with FM4-64 (Molecular Probes, Inc., Eugene, OR, U.S.A.) (63). Quinacrine (Sigma) staining for the evaluation of vacuolar acidification was performed as previously reported (28,45).

**Analysis of phosphatidylinositols**

Cells were cultured in SD medium containing myo-[2-<sup>3</sup>H]inositol for 20 h. When *A<sub>610</sub>* reached 0.8-1.0, 10 mM MG was added and cultivation was continued for another 60 min. Phosphatidylinositols were extracted from cells, and deacylated with methylamine as described by Hama et al. (64). Phosphatidylinositols (PtdIns3P, PtdIns4P, PtdIns(3,5)P<sub>2</sub>, and PtdIns(4,5)P<sub>2</sub>) were separated with HPLC, and radioactive counts were measured (64).

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article.

**AUTHOR CONTRIBUTIONS**

W.N., K.M., and Y.I. designed experiments and W.N. and K.M. performed experiments. W.N. and Y.I. analyzed data and wrote the manuscript.
REFERENCES


trafficking. Methods 20, 465-473

**FOOTNOTES**

**Abbreviations:** MG, methylglyoxal; MAPK, mitogen-activated protein kinase; PtdIns, phosphatidylinositol; V-ATPase, vacuolar H\(^+\) ATPase; Hxt, hexose transporter; TORC2, target of rapamycin complex 2.
### Table 1. List of relevant genes involved in susceptibility to MG

<table>
<thead>
<tr>
<th>Functional categories</th>
<th>Relevant genes</th>
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<tbody>
<tr>
<td>Cell organization</td>
<td>SAC6, WHI2, BUD25</td>
</tr>
<tr>
<td>Gene expression</td>
<td>NGG1, HFI1, SWI3, TAF14, ERT1</td>
</tr>
<tr>
<td>Protein quality control</td>
<td>SSZ1, HEL2, VMS1, ATG17</td>
</tr>
<tr>
<td>Sugar or amino acid metabolism</td>
<td>GLO1, GLO2, GLY1, SHP1</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>VPS34, VPS15, CSG2, ISCI, KCSI, ERG2, ERG3</td>
</tr>
<tr>
<td>DNA repair</td>
<td>RAD50, RAD51, RAD1</td>
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<td>Membrane traffic</td>
<td>PEP3, PEP12, CHC1, CLC1</td>
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<td>Unknown</td>
<td>YEL045c</td>
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Wild type cells were treated with 10 mM MG for 60 min. The level of each phosphatidylinositol is shown as a percentage of total phosphatidylinositol levels (average ± standard deviation, n≥3).

Table 2. Levels of phosphatidylinositols

<table>
<thead>
<tr>
<th>Phosphatidylinositols</th>
<th>w/o MG</th>
<th>w/ MG</th>
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<tbody>
<tr>
<td>PtdIns3P</td>
<td>1.00 ± 0.11</td>
<td>0.97 ± 0.06</td>
</tr>
<tr>
<td>PtdIns4P</td>
<td>1.64 ± 0.07</td>
<td>1.51 ± 0.07</td>
</tr>
<tr>
<td>PtdIns(3,5)P$_2$</td>
<td>0.020 ± 0.004</td>
<td>0.065 ± 0.001</td>
</tr>
<tr>
<td>PtdIns(4,5)P$_2$</td>
<td>0.68 ± 0.04</td>
<td>0.57 ± 0.02</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

FIGURE 1. Involvement of the PtdIns(3,5)P_2 biosynthesis pathway in tolerance to MG. A, Outline of the phosphoinositide synthesis pathway in S. cerevisiae. B, Wild type (BY4741), glo1Δ, vps15Δ, vps34Δ, and fab1Δ cells, which are BY4741 background strains, were cultured in YPD medium until A_610 = 0.3, 4 µl of each cell suspension was spotted onto YPD agar plates with (20 mM) or without MG, and cells were then incubated at 28°C for 3 days. C, Wild type (BY4741) and fab1Δ (BY4741 background) cells were cultured in SD medium until A_610 = 0.3, and cell suspensions were spotted onto SD agar plates with (8 mM) or without MG, as described in B. D, Wild type (YPH250), fab1Δ, and vac7Δvac14Δ cells were cultured in SD medium until A_610 = 0.3, and cell suspensions were spotted onto SD agar plates with or without MG, as described in B.

FIGURE 2. Effects of MG on vacuolar morphology. A, Cells (YPH250) were cultured in SD medium until A_610 = 0.3-0.5, and treated with 10 mM MG for the period indicated. The vacuolar membrane was stained with FM4-64. The number of vacuoles per cell and vacuole size were measured for more than 50 cells, and mean values ± standard deviation were shown below each picture. Bar, 5 µm. B, Wild type (YPH250) cells were cultured in SD medium until A_610 = 0.3-0.5, and treated with 10 mM MG for 60 min. Cells were washed with 0.85% NaCl solution, suspended in fresh SD medium with or without 10 mM MG for 30-60 min, and the vacuolar membrane was then stained with FM4-64. The number of vacuoles per cell and vacuole size were measured for more than 50 cells, and mean values (± standard deviation) were shown below each picture. Bar, 5 µm. C, Wild type (YPH250), vps41Δ, and vam3Δ cells were cultured in SD medium until A_610 = 0.3-0.5, and treated with 10 mM MG for 90 min. The vacuolar membrane was stained with FM4-64.

FIGURE 3. Effects of MG on PtdIns(3,5)P_2 levels. A, Wild type (YPH250) cells were cultured in SD medium until A_610 = 0.3-0.5, and treated with 10 mM MG. The time course of the number of vacuoles per cell (open circles), vacuole size (closed circles) (left panel), and PtdIns(3,5)P_2 levels (right panel) were assessed. B, Wild type (YPH250) and vac14Δ cells carrying ATG18-GFP were cultured in SD medium until A_610 = 0.3-0.5, and treated with 10 mM MG. After 90 min, Atg18-GFP and the vacuole (FM4-64) were observed using a fluorescence microscope.

FIGURE 4. Vacuolar acidification is not affected by a treatment with MG. Wild type (YPH250), fab1Δ, and vac7Δvac14Δ cells were cultured in SD medium until A_610 = 0.4, and then treated with 10 mM MG for 60 min. Cells were labeled with 200 µM quinacrine to assess vacuolar acidification. Quinacrine fluorescence images were superimposed on low-light bright field images to reveal cell outlines.

FIGURE 5. Involvement of PtdIns(3,5)P_2 in the MG-induced activation of the Mpk1 MAPK cascade. A, Wild type (YPH250) and fab1Δ cells carrying either an empty plasmid (vector, YCp50) or YCp50-PKCI^{R198F} were cultured in SD medium until A_610 = 0.3, 4 µl of each cell suspension was spotted onto SD agar plates with (4 mM) or without MG, and cells were then incubated at 28°C for 3 days. B, Wild type (YPH250), vps34Δ, fab1Δ, and vac7Δvac14Δ cells were cultured in SD medium until A_610 = 0.3, and 10 mM MG was added. The levels of phosphorylation of Mpk1 (p-Mpk1) and the Mpk1 protein (Mpk1) were assessed after incubations for the prescribed times. The intensity of immunoreactive bands was quantified by image analysis. The ratio of p-Mpk1/Mpk1 at 0 min in each strain was relatively taken as 1. Data are for three independent experiments (average plus standard deviation, n≥3). C, Wild type (YPH250), vps34Δ, fab1Δ, and vac7Δvac14Δ cells were cultured in SD medium at 28°C until A_610 = 0.3, and were then shifted to 38°C. The levels of phosphorylation of Mpk1 (p-Mpk1) and the Mpk1 protein (Mpk1) were assessed after incubations for the prescribed times. The intensity of immunoreactive bands was quantified as described in B.

FIGURE 6. An increase in PtdIns(3,5)P_2 is not necessary for the MG-induced activation of the Mpk1 MAPK cascade. A, Wild type (YPH250) and gsg4Δ cells were labeled with myo-[2-^3H]inositol, and treated with MG for 60 min. The extraction of phosphatidylinositol and measurement of PtdIns(3,5)P_2 levels were described in the EXPERIMENTAL PROCEDURES. PtdIns(3,5)P_2 levels are
shown as a percentage of total phosphatidylinositol levels. Data are for three independent experiments (average ± standard deviation, n≥3). B, Wild type (YPH250) and fig4Δ cells carrying ATG18-GFP were cultured in SD medium until $A_{610} = 0.3-0.5$, and treated with 10 mM MG. After 90 min, Atg18-GFP and the vacuole (FM4-64) were observed using a fluorescence microscope. C, Wild type (YPH250) and fig4Δ cells were cultured in SD medium until $A_{610} = 0.3$, and 10 mM MG was added. The levels of phosphorylation of Mpk1 (p-Mpk1) and the Mpk1 protein (Mpk1) were assessed after incubations for the prescribed times. Quantification of the ratio of p-Mpk1/Mpk1 was described in FIGURE 5B, and data are for three independent experiments (average plus standard deviation, n≥3). D, Wild type (YPH250) and fig4Δ cells were cultured in SD medium until $A_{610} = 0.3$, 4 µl of each cell suspension was spotted onto SD agar plates with (4 mM) or without MG, and cells were then incubated at 28°C for 3 days.
**FIGURE 1**

**A**

```
Vps34/Vps15 → PtdIns3P → PtdIns(3,5)P2

PtdIns

Pik1,
Stt4, or Lsb6

PtdIns4P → PtdIns(4,5)P2

Vac7 Vac14

Fab1

Wild-type

glo1Δ
vps15Δ
vps34Δ
fab1Δ
```

**B**

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<td></td>
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<td></td>
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</tr>
<tr>
<td>glo1Δ</td>
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<tr>
<td>vps15Δ</td>
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<td>fab1Δ</td>
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**C**

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<td>WT (BY4741)</td>
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**D**

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Wild-type

fab1Δ
vac7Δvac14Δ
**FIGURE 2**

**A**

<table>
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<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
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<tr>
<td>- MG</td>
<td>1.96 ± 0.99</td>
<td>1.80 ± 0.82</td>
<td>1.92 ± 0.85</td>
<td>1.96 ± 0.88</td>
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<tr>
<td>+ MG</td>
<td>1.67 ± 0.76</td>
<td>1.43 ± 0.77</td>
<td>1.22 ± 0.59</td>
<td>1.05 ± 0.22</td>
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Mean number of vacuoles per cell

Mean vacuole size (µm)

**B**

<table>
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<tr>
<th>Time (min)</th>
<th>0</th>
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<th>60</th>
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<tr>
<td>- MG</td>
<td>1.97 (± 0.97)</td>
<td>1.42 (± 0.62)</td>
<td>2.34 (± 0.88)</td>
</tr>
<tr>
<td>+ MG</td>
<td>1.65 (± 0.73)</td>
<td>1.03 (± 0.17)</td>
<td>1.13 (± 0.35)</td>
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Mean number of vacuoles per cell

Mean vacuole size (µm)

**C**

<table>
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<tr>
<th></th>
<th>Wild-type</th>
<th>vps41Δ</th>
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<tr>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>FM4-64</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BF</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</table>
FIGURE 3

A

Mean vacuole size (µm)

Mean number of vacuoles per cell

Time (min)

0
0.5
1.0
1.5
2.0
2.5
3.0

0
0.5
1.0
1.5
2.0
2.5
3.0

0
15
30
45
60
75
90

B

Wild-type  vac14Δ

MG  -  +  -  +

Atg18-GFP

FM4-64

BF

Percentage of total PtdIns

Time (min)

0
0.02
0.04
0.06

0
15
30
45
60

+ MG  - MG
FIGURE 4
FIGURE 5

A

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<tr>
<td>PKC1&lt;sup&gt;R398P&lt;/sup&gt;</td>
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<td>fab1Δ</td>
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B

<table>
<thead>
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<tr>
<td>vac7Δ vac14Δ</td>
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C

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</table>

p-Mpk1/Mpk1 ratio

Time (min)
FIGURE 6

A

B

C

D

WT

fig4Δ

MG

p-Mpk1

Mpk1

p-Mpk1

Mpk1

Figure 6: Various panels showing experiments and results.

A) Graph showing PtdIns(3,5)P₂ levels in Wild-type and fig4Δ strains with or without MG treatment.

B) Images comparing Atg18-GFP and FM4-64 staining in Wild-type and fig4Δ strains with MG treatment.

C) Western blot analysis of p-Mpk1 and Mpk1 levels in WT and fig4Δ strains under MG treatment.

D) Images showing Wild-type and fig4Δ strains under different MG concentrations.