

Running title: Methylglyoxal activates Gcn2 without affecting TORC1 function

Methylglyoxal activates Gcn2 to phosphorylate eIF2 α independently of the TOR pathway in *Saccharomyces cerevisiae*

Wataru Nomura, Kazuhiro Maeta*, Keiko Kita, Shingo Izawa[§], and Yoshiharu Inoue[¶]

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

* Present address: Chemical Genetics Laboratory, RIKEN, Wako, Saitama 351-0198, Japan.

[§] Present address: Laboratory of Microbial Engineering, Division of Applied Biology, Kyoto Institute of Technology, Kyoto 606-8585, Japan.

[¶] Corresponding author: Laboratory of Molecular Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan. Phone: +81 774 38-3773; Fax: +81 774-38-3789; e-mail: y_inoue@kais.kyoto-u.ac.jp.

Abstract

Methylglyoxal is a ubiquitous 2-oxoaldehyde derived from glycolysis. Previously, we have reported that methylglyoxal attenuates the rate of overall protein synthesis in *S. cerevisiae* through phosphorylation of the α subunit of translation initiation factor 2 (eIF2 α) in a Gcn2-dependent manner. Phosphorylation of eIF2 α impedes the formation of a translation initiation complex, and subsequently, overall protein synthesis is reduced. Uncharged tRNA plays an important role in the activation of Gcn2, although we found that MG treatment did not elevate the levels of uncharged tRNA. Rapamycin, a potent inhibitor of TOR kinase, is known to induce phosphorylation of eIF2 α without affecting the levels of uncharged tRNA. We determined the correlation between methylglyoxal and TOR kinase activity, and found that phosphorylation of eIF2 α by methylglyoxal occurred independently of the TOR pathway.

Key words:

Methylglyoxal, TORC1, Gcn2, eIF2 α , *S. cerevisiae*, rapamycin

Introduction

Methylglyoxal (MG) is a ubiquitous 2-oxoaldehyde (CH_3COCHO), which is synthesized during glycolysis. Although a natural metabolite, since MG is highly reactive due to its two carbonyl groups, its overaccumulation has several adverse effects on cellular functions (Inoue and Kimura 1995). We have been trying to reveal the physiological significance of MG using *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as models (Maeta et al. 2004, Maeta et al. 2005, Takatsume et al. 2006). Regarding the adverse effects of MG, we have previously reported that MG attenuates the rate of overall protein synthesis in yeast (Nomura et al. 2008). We found that MG treatment activated the protein kinase Gcn2 to phosphorylate the α subunit of translation initiation factor 2 (eIF2 α). Phosphorylation of eIF2 α is known to cause the inhibition of translation initiation (Hinnebusch 2005).

Basically, amino acid starvation attenuates the rate of overall protein synthesis in all types of organisms. Amino acid starvation inactivates the target of rapamycin (TOR) pathway. *S. cerevisiae* has two TOR proteins, Tor1 and Tor2, both serine/threonine protein kinases. Tor1 and Tor2 are involved in many important biological events, such as transcription, protein synthesis including ribosome biogenesis, protein degradation, cell growth, and cell cycle control (Wullschleger et al. 2006). The activity of TOR kinase is upregulated in the presence of amino acids, and downregulated under nutrient-starved conditions. For example, TORC1 is involved in the regulatory mechanism of nitrogen catabolite repression (NCR) through regulation of the phosphorylation state of Gln3 transcription factor (Beck and Hall 1999, Bertram et al. 2000). Gln3 is substantially phosphorylated and present in the cytoplasm when sufficient amino acids are available. However, when cells are starved of amino acids, Gln3 is dephosphorylated by Sit4 protein phosphatase and translocated into the nucleus, thereby activating the transcription of NCR-sensitive genes (Beck and Hall 1999, Bertram et al. 2000). Sit4 is responsible for dephosphorylation

of Gln3 under amino acid-starved conditions, of which the activity is regulated by TORC1 (Beck and Hall 1999, Bertram et al. 2000)) (see Fig. 6). Rapamycin, a macrocyclic lactone, is a potent inhibitor of Tor1 and Tor2 when they are present in the TOR complex 1 (TORC1) (Stan et al. 1994, Loewith et al. 2002). Therefore, Gln3 is dephosphorylated following treatment with rapamycin via activation of Sit4 (see Fig. 6). Phosphorylation state of Gln3 is also regulated by Snf1 protein kinase when cells are starved of glucose (Bertram et al. 2002, Cox et al. 2002) (see Fig. 6). Meanwhile, level of Gln3 protein is regulated by Npr1 protein kinase, and Rsp5 and its associated proteins Bul1 and Bul2 (Crespo et al. 2004). The activity of Npr1 correlates with phosphorylation/dephosphorylation state, which is regulated by TORC1 through Sit4 (see Fig. 6).

Gcn2 is the sole protein kinase responsible for the phosphorylation of eIF2 α that is activated under conditions of amino acid starvation in *S. cerevisiae* (Wek et al. 1995). The level of uncharged (deacylated) tRNA increases when cells are starved of amino acids. Uncharged tRNA plays an important role in the activation of Gcn2, although in the present study we showed that MG treatment did not cause an increase in the levels of uncharged tRNA. Previously, Kubota et al. (2003) have reported that rapamycin activates Gcn2 without affecting the levels of uncharged tRNA. However, here we show that MG activates Gcn2 without affecting TOR kinase activity.

Materials and methods

Strains

S. cerevisiae YPH250 (*MATa trp1- Δ 1 his3- Δ 200 leu2- Δ 1 lys2-801 ade2-101 ura3-52*) was used as a wild type strain. *S. cerevisiae* TB123a (*MATa leu2-3,112 ura3-52 trp1 his4 rme1 HMLa GLN3-¹³myc-kanMX*) (Beck and Hall 1999) was donated by Dr. M. Hall. The *SNF1* gene in TB123a was disrupted by a gene

replacement method using the *Candida glabrata* *LEU2* gene (*CgLEU2*) as a selectable marker (Sakumoto et al. 1999) with the following primers: 5'-AGCAGTAACAACAACACAAACACAGCACCTGCCAATGCAATACGAAGTTATTAGGTCTAG-3' and 5'-TCGTTACAAGAGGAGATATTTTAGAGGCAGCTGGCGAACCACGAAGTTATATTAAGGGTT-3'; and *CgLEU2* as a template. *S. cerevisiae* W303H (*MATa ade2-1 trp1-1 leu2-3 ura3-1 can1-100*) and its isogenic mutants (WH1, *gcn1Δ::kanMX*; WH2, *gcn2Δ::kanMX*; and WH3, *TOR1-1*) (Kubota et al. 2003) were donated by Dr. T. Ito.

Plasmids

A plasmid carrying *SNF1-3HA* (McCartney Schmidt 2001), *HA-NPR1* (Schmidt et al. 1998), and *PHO80Δ60* (pTN9; Noda et al. 1995) were donated by Dr. M. Hall, Dr. M. Schmidt, and Dr. Y. Ohsumi, respectively. A plasmid carrying *CgLEU2* (p3008) was obtained from the National Bio-Resource Project (NBRP) of the MEXT, Japan.

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 as necessary. When the A_{610} of the culture reached 0.3-0.5, 10 mM MG or 200 ng/ml rapamycin was added, and cells were incubated at 28°C with reciprocal shaking for the prescribed time.

Induction of autophagy

Cells (YPH250) were cultured in SD medium until the A_{610} reached 0.5, and 1 mM phenylmethylsulfonyl fluoride (PMSF) was added together with 10 mM MG or 200 ng/ml rapamycin. After 3 h, autophagic bodies were observed using differential interference contrast microscopy (Olympus, BX60). Alkaline phosphatase (ALP) activity was measured as described (Noda and Ohsumi 1998).

Western blotting

Cells were cultured in SD medium until the A_{610} reached 0.3, and treated with chemicals for the prescribed time. To detect phospho-Ser⁵¹ of eIF2 α , Gln3-¹³myc, and HA-Npr1, cell extracts were prepared as described by Kubota et al. (2001), Beck and Hall (1999), and Schmidt et al. (1998), respectively. To detect phospho-Thr²¹⁰ of Snf1 and HA-tagged Snf1, cells were disrupted with glass beads in 50 mM Tris-HCl buffer (pH 7.5) containing 120 mM NaCl, 2 mM EDTA, 2 mM PMSF, 10 mM NaF, and 10 mM sodium pyrophosphate. Each cell extract was subjected to SDS-PAGE, separated proteins were transferred to PVDF membranes (Millipore), and proteins of interest were detected using appropriate antibodies (anti-phospho Ser⁵¹ eIF2 α antibody (BIOSOURCE), anti eIF2 α antibody (BIOSOURCE), anti-c-Myc monoclonal antibody (MC045, Nacalai tesque), anti-HA monoclonal antibody (6E2, Cell Signaling), and anti-phospho Thr²¹⁰ Snf1 antibody (McCartney and Schmidt 2001) that was donated by Dr. M. Schmidt, and with respective secondary antibodies conjugated with alkaline phosphatase. Immunoreactive bands were visualized using a BCIP-NBT solution kit for alkaline phosphatase (Nacalai tesque).

Northern blotting

To detect charged and uncharged tRNA^[Met], total RNA was prepared essentially as described by Kubota et al. (2003). Cells were suspended in AE buffer (50 mM sodium acetate (pH 5.3), 10 mM EDTA), and disrupted with glass beads by FastPrep for 45 sec at 4°C. Total RNA was extracted with AE-saturated phenol, and precipitated with ethanol. It was then electrophoresed on a 10% acrylamide gel containing 8 M urea in 100 mM sodium acetate buffer (pH 5.0) at 4°C and 95 volts for 7 h. To deacylate tRNA, the final preparation of total RNA was incubated in 100 mM Tris-HCl (pH 8.0) at 75°C for 5 min as described (Kubota et al. 2003). Preparation of the specific probe for tRNA^[Met] was done as reported (Sarkar 1999).

Results

MG does not affect the levels of uncharged tRNA

Gcn2 is the sole protein kinase responsible for the phosphorylation of eIF2 α that is activated under conditions of amino acid starvation in *S. cerevisiae* (Wek et al. 1995). We have previously demonstrated that the protein kinase responsible for the phosphorylation of eIF2 α in cells treated with MG is Gcn2 (Nomura et al. 2008). Regarding the activation of Gcn2, several pathways have been proposed (Hinnebusch 2005). Importantly, Gcn1 and uncharged tRNA play crucial roles in the activation of Gcn2. The rate of overall protein synthesis is reduced under conditions of amino acid starvation, which leads to the accumulation of uncharged tRNAs (Hinnebusch 2005). Gcn1 forms a complex with Gcn20 (Vazquez de Aldana et al. 1995), and the resultant complex, a positive regulator of Gcn2, binds the N-terminal segment of Gcn2. Therefore, phosphorylation of eIF2 α did not occur in *gcn1* Δ cells following treatment with MG (Nomura et al. 2008). On the other hand, uncharged tRNA binds the histidyl-tRNA synthetase (HisRS)-related domain of Gcn2. Since Gcn1/Gcn20 does not affect the expression of *GCN2* or kinase activity of Gcn2 *in vitro*, this complex is speculated to mediate the activation of Gcn2 by uncharged tRNA

in yeasts starved of amino acids (Vazquez de Aldana et al. 1995, Marton et al. 1993). It has been reported that Gcn2 binds different uncharged tRNAs with the same affinity (Dong et al. 2000). We determined the levels of uncharged methionyl-tRNA (tRNA^[Met]). As shown in Fig 1A, the levels of uncharged tRNA^[Met] did not increase following the treatment of yeast cells with MG. Since the uptake of amino acids from the medium was not impaired by treatment with MG (Nomura et al. 2008), we speculated that there might be another cause, other than amino acid starvation, of the activation of Gcn2 by MG treatment.

Role of Gcn2 in phosphorylation of eIF2 α following treatment with MG

Several amino acid residues in Gcn2 have been assigned to its activity (Hinnebusch 2005). Ser⁵⁷⁷ is critical to the activity of Gcn2 (Garcia-Barrio et al. 2000). Ser⁵⁷⁷ of Gcn2 is phosphorylated by an as yet unidentified protein kinase when sufficient amino acids exist (Cherkasova and Hinnebusch 2003), and phospho-Ser⁵⁷⁷ is dephosphorylated by some protein phosphatase(s) whose activity is under the control of the TOR pathway (Hinnebusch 2005). Rapamycin induces phosphorylation of eIF2 α (Kubota et al. 2003; also see Fig. 2A), which is presumably a consequence of dephosphorylation of Ser⁵⁷⁷ of Gcn2. Garcia-Barrio et al. (2000) have reported that eIF2 α was constitutively phosphorylated in a *gcn2*^{S577A} mutant, which mimics a dephosphorylated (i. e. an active) form of Gcn2. Consequently, as shown in Fig. 2B, the basal phosphorylation level of eIF2 α was high in *gcn2*^{S577A} cells, and the level of phosphorylated eIF2 α was not further increased following treatment with rapamycin. This was also the case in MG-treated *gcn2*^{S577A} cells (Fig. 2B). In addition, Kubota et al. (2003) have reported that rapamycin treatment did not increase the levels of uncharged tRNA^[His]. Here we verified that the levels of uncharged tRNA^[Met] were not increased following treatment with rapamycin (Fig. 1B). Furthermore, this was also the case in MG-treated cells (Figs. 1A and 1B). These results imply that MG inactivates TOR kinase in

some way, which alters the phosphorylation state of Ser⁵⁷⁷ of Gcn2, thereby phosphorylating eIF2 α . However, eIF2 α phosphorylation was further increased in the cells of *TOR1-1* mutant (Kubota et al. 2003), which is resistant to rapamycin, following treatment with MG (Fig. 2B); suggesting that an alternative pathway to activate Gcn2 exists. Hence, the effect of MG on the TOR pathway is controversial, and thus, in the next paragraph, we discuss the correlation between MG and the TOR pathway.

Effect of MG on TORC1 activity

To address whether MG affects the phosphorylation of eIF2 α by impairing the function of TORC1 or independently of the TOR pathway, we determined the effect of MG on TOR kinase activity by analyzing several readouts of this signaling pathway.

First, we determined the status of the Gln3 transcription factor, the phosphorylation of which is regulated in the TOR pathway (Beck and Hall 1999). Gln3 is involved in nitrogen catabolite repression (NCR) (Kathleen et al. 2000). When cells are starved of nitrogen, Gln3 is dephosphorylated; meanwhile, when a sufficient amount of a good nitrogen source such as glutamate is present, Gln3 is predominantly in the phosphorylated form (Beck and Hall 1999, Kathleen et al. 2000). As shown in Fig. 3A, Gln3 is dephosphorylated when TORC1 is deactivated by rapamycin. In contrast, Gln3 was hyperphosphorylated following treatment with MG (Figs. 3A and 3B). Sit4, a type 2A-related protein phosphatase, is responsible for dephosphorylation of Gln3 in the TOR pathway (Beck and Hall 1999, Tate et al. 2006). Since MG induced hyperphosphorylation of Gln3, we assumed that it might impair the activity of Sit4. However, as shown in Fig. 3C, overproduction of Sit4 did not reduce the rate of Gln3 phosphorylation following treatment with MG.

Npr1 is a negative regulator of the nuclear localization of Gln3, the activity for which is also

regulated *via* phosphorylation/dephosphorylation in a TORC1-dependent manner (Schmidt et al. 1998); we therefore determined the status of Npr1. As shown in Fig. 3D, MG induced further phosphorylation of Npr1 as opposed to that brought about by rapamycin.

Next, we determined the effect of MG on autophagy, the bulk protein degradation system, the process of which is also regulated in TOR pathway (Noda and Ohsumi 1998, Kamada et al. 2000). When cells were treated with rapamycin, small vesicular structures that are referred to as autophagic bodies appeared in the vacuole, which can be viewed by differential interference contrast microscopy (Fig. 4A). However, in vacuole of cells treated by MG, such vesicular structures were not observed (Fig. 4A). To quantify the induction of autophagy, processing of Pho8, a vacuolar alkaline phosphatase, was monitored using Pho8 Δ 60. This truncated mutant of Pho8 substantially resides in the cytoplasm in an inactive form under normal conditions; whereas, upon nutrient starvation or rapamycin treatment, the conditions of which induce autophagy, Pho8 Δ 60 is delivered to the vacuole and turned to be an active form through processing by the vacuolar proteinase (Noda et al. 1995). As shown in Fig. 4B, the alkaline phosphatase activity was increased in cells treated with rapamycin, however, it was substantially unchanged in cells treated with MG. It should be noted that newly synthesized protein is not necessary for the processing of Pho8 Δ 60 in this biochemical assay. Taken together, MG does not seem to impair the function of TORC1.

Snf1 is not involved in the MG-induced phosphorylation of Gln3

Besides amino acid starvation, Gcn2 is activated by several forms of environmental stress such as glucose-limited conditions (Yang et al. 2000). In addition to NCR, phosphorylation of Gln3 is also regulated by the protein kinase Snf1 under glucose-limited conditions (Bertram et al. 2002). Snf1 is inactive when glucose is sufficient in the medium, but is activated through phosphorylation of Thr²¹⁰

when glucose is limited (McCartney and Schmidt 2001). Gln3 is one of the targets of Snf1 kinase, and therefore, Gln3 is phosphorylated by Snf1 under glucose-starved conditions, a process which occurs independently of the TOR pathway (Bertram et al. 2002). As shown in Fig. 3A, we confirmed that Gln3 was hyperphosphorylated under conditions of glucose starvation.

To address whether the activation of Snf1 is involved in the MG-induced phosphorylation of Gln3, we determined the phosphorylation state of Thr²¹⁰ of Snf1. As shown in Fig. 5A, Thr²¹⁰ was not phosphorylated following treatment with MG. Glucose starvation induced phosphorylation of Thr²¹⁰ but rapamycin did not (Fig. 5A). This suggests that MG-induced phosphorylation of Gln3 occurs independently of Snf1. To verify this, phosphorylation of Gln3 was determined in an *snf1Δ* mutant. As shown in Fig. 5B, highly phosphorylated Gln3 was observed in cells treated with MG in the presence or absence of Snf1, although this was not the case for glucose starvation, i. e. hyperphosphorylation was not observed under glucose-starved conditions in *snf1Δ* cells.

Collectively, we concluded that phosphorylation of Gln3 following treatment with MG occurs independently of the inactivation of TORC1 as well as activation of Snf1. Consequently, phosphorylation of eIF2 α after treatment with MG is supposed to occur independently of the inactivation of TORC1. An unknown protein kinase or protein phosphatase may be involved in the regulation of Gln3's phosphorylation in MG-treated cells. Recently, Tate and Cooper (2007) reported that environmental stress, including temperature, osmotic stress, and oxidative stress, induced phosphorylation of Gln3 even under nitrogen-starved or TOR-inactivating conditions, although the regulatory mechanism behind this has yet to be determined. MG may cause a similar response in terms of the phosphorylation of Gln3.

Discussion

Previously, we have demonstrated that MG blocks the initiation of protein synthesis in vivo (Nomura et al. 2008). TORC1 is involved in the cap-dependent translation initiation via eIF4G (Cameroni et al. 2006), although since MG did not affect the function of TORC1 but enhanced the phosphorylation of eIF2 α , we speculated that phosphorylation of eIF2 α is a part of the MG-induced attenuation of overall protein synthesis in *S. cerevisiae*. eIF2 plays a crucial role in translation initiation, because the GTP-bound form of eIF2 delivers charged initiator methionyl-tRNA to the 40S ribosomal subunit to form the 43S preinitiation complex. Phosphorylation of Ser⁵¹ changes eIF2 α into a competitive inhibitor of the guanine nucleotide exchanging factor of eIF2. Since Gcn2 is the sole protein kinase responsible for eIF2 α 's phosphorylation in *S. cerevisiae*, Gcn2 also plays important roles in the regulation of translation initiation. Amino acid starvation enhances the levels of uncharged tRNAs in cells, which leads to activation of Gcn2 through the binding of uncharged tRNA to the HisRS-like domain of Gcn2, thereby phosphorylating eIF2 α . We have previously reported that MG treatment did not provoke deprivation of free amino acids in the cells (Nomura et al. 2008), and subsequently, here we showed that the levels of uncharged tRNA^[Met] did not increase (Fig. 1).

Activation of Gcn2 by the Gcn1/Gcn20 complex is thought to be mediated by uncharged tRNA. Hinnebusch (2005) has proposed two alternative models for the activation of Gcn2 by uncharged tRNA; i. e. (i) Gcn1/Gcn20 accelerates binding of uncharged tRNA to the A-site of the ribosome, or (ii) Gcn1/Gcn20 facilitates the transfer of uncharged tRNA from the A-site to the HisRS-like domain of Gcn2. Since Gcn1 directly binds to Gcn2, the latter is more likely (Hinnebusch 2005). We have demonstrated in this study that MG-induced phosphorylation of eIF2 α did not occur in *gcn1* Δ cells. On the other hand, Kubota et al. (2003) have reported that rapamycin treatment induced phosphorylation of eIF2 α even though the levels of uncharged tRNA did not increase. Additionally, they have shown that eIF2 α

phosphorylation was not induced in *gcn1Δ* cells following treatment with rapamycin for 120 min. However, as shown in Fig. 2A, we found that eIF2α was phosphorylated in *gcn1Δ* cells after treatment with rapamycin for 30 min. In our experimental conditions, levels of phosphorylated eIF2α were evaluated after 30 min of treatment with MG as well as rapamycin. We confirmed that eIF2α was phosphorylated in a *gcn1Δ* strain (WH1) that was used by Kubota et al. (2003) following treatment with rapamycin for 30 min (data not shown). Kubota et al. (2003) proposed that dephosphorylation of Ser⁵⁷⁷ of Gcn2 through inactivation of TORC1 with rapamycin enhanced the affinity of Gcn2 for the uncharged tRNA in a Gcn1-dependent manner. The protein kinase domain of Gcn2 is divided into two segments (N-lobe and C-lobe), and phospho-Ser⁵⁷⁷ in the N-lobe inhibits the binding of ATP to the ATP-binding site in the C-lobe, and consequently, Gcn2 cannot phosphorylate eIF2α (Hinnebusch 2005). Meanwhile, dephosphorylation of Ser⁵⁷⁷ of Gcn2 in rapamycin-treated cells may induce the conformational change of Gcn2 and enhance the affinity for uncharged tRNA, thereby phosphorylating eIF2α under normal levels of uncharged tRNA (Kubota et al. 2003). On the other hand, we have demonstrated that eIF2α is phosphorylated in *gcn1Δ* cells at the early stage of treatment with rapamycin (Fig. 2A), which may suggest that the conformational change of Gcn2 via dephosphorylation of Ser⁵⁷⁷ temporarily decreases the dependency of Gcn2 on Gcn1 for binding the uncharged tRNA to activate its eIF2α kinase activity. However, the phosphorylation of eIF2α did not occur following treatment with MG for 30 min in *gcn1Δ* cells (Fig. 2A). The activity of Sit4, a protein phosphatase considered responsible for dephosphorylation of Ser⁵⁷⁷ of Gcn2 (Beck and Hall 1999, Bertram et al. 2000), is regulated by the TOR pathway. To address whether MG affects the activity of Sit4 through inhibiting TORC1's functions, we determined the phosphorylation state of Gln3 and Npr1, both are readouts of the TORC1 pathway through Sit4 (Fig. 6). These proteins were dephosphorylated following treatment with rapamycin, because repression of the

activity of Sit4 protein phosphatase is derepressed through inactivation of TORC1. Conversely, we have shown that both Gln3 and Npr1 were hyperphosphorylated following treatment with MG, and thus concluded that MG does not, at least, inhibit TORC1's function. Therefore, MG seems to activate Gcn2 without affecting the phosphorylation status of Ser⁵⁷⁷ of Gcn2. On the other hand, Tyr¹¹¹⁹ and Arg¹¹²⁰ in the HisRS-like domain seem crucial for binding uncharged tRNA as well as conformational stabilization of the protein kinase domain of Gcn2, because Y1119A and R1120L mutations abolish the kinase activity of Gcn2 (Hinnebusch 2005). Two mutations, R794G and F842L, which can suppress these mutations have been identified in the protein kinase domain of Gcn2 (Qui et al. 2002). However, these mutations suppress the Y1119A and R1120L mutations without affecting the interaction between Gcn2 and Gcn1 (Qui et al. 2002). Since MG-induced phosphorylation of eIF2 α was strictly dependent upon Gcn1 (Nomura et al 2008, also see Fig. 2A), MG seems likely to affect the function of Gcn1 to activate Gcn2, thereby phosphorylating eIF2 α , but not affecting the activity of Gcn2 directly (Fig. 6).

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Figure legends

Fig. 1. Effect of MG on accumulation of uncharged tRNA^[Met]. (A) Cells (YPH250) were cultured in SD medium until $A_{610} = 0.3$, and treated with various concentrations of MG for 60 min. After treatment, total RNA was prepared as described in Materials and Methods, followed by Northern blotting to detect charged (aminoacylated) and uncharged (deacylated) tRNA^[Met]. Deacylation of tRNA was achieved by incubating at alkaline pH at high temperature. (B) Cells (YPH250) were cultured in SD medium until $A_{610} = 0.3$, and treated with 10 mM MG or 200 ng/ml rapamycin (Rapa) for the period indicated in the figure.

Fig. 2. Effect of MG on phosphorylation of eIF2 α . (A) Cells of the wild type (YPH250) and isogenic mutants *gcn2* Δ and *gcn1* Δ were cultured in SD medium until $A_{610} = 0.3$, and 10 mM MG (M) or 200 ng/ml rapamycin (R) was added. C represents the control experiments (not treated with chemicals). After 30 min of incubation, the phosphorylation status and protein levels of eIF2 α were determined. (B) Cells of the wild type (W303H) and isogenic mutants *gcn2* Δ (WH2) and *TOR1-1* (WH3) were treated with 10 mM MG (M) or 200 ng/ml rapamycin (R) for 30 min, and the phosphorylation status of eIF2 α was determined. C represents the control experiments (not treated with chemicals).

Fig. 3. Effect of MG on phosphorylation of Gln3 and Npr1. (A) Cells (TB123a) were cultured in SD medium until $A_{610} = 0.3$, 10 mM MG or 200 ng/ml rapamycin (Rapa) was added, and the cells were incubated for 30 min (Rapa) or 60 min (MG). For glucose starvation experiments (-Glc), cells at $A_{610} = 0.3$ were collected by centrifugation, suspended in SD medium without glucose, and then incubated for 30 min. After each stress treatment, the phosphorylation status of Gln3 was determined with anti-Myc

monoclonal antibody. (B) Cells (TB123a) were cultured in SD medium until $A_{610} = 0.3$, and 10 mM MG was added. At the prescribed time as indicated in the figure, cells were collected, and the phosphorylation status of Gln3 was determined. (C) Cells (TB123a) carrying pRS426 (vector) or pRS426-*SIT4* (2 μ *SIT4*) were treated with MG or rapamycin (Rapa) as described in (A), and the phosphorylation status of Gln3 was determined. (D) Cells (YPH250) carrying a plasmid expressing *HA-NPR1* were cultured in SD medium until $A_{610} = 0.3$, and treated with MG or rapamycin (Rap) as described in (A). The phosphorylation status of Npr1 was determined using anti-HA monoclonal antibody.

Fig. 4. Effect of MG on induction of autophagy. (A) Cells (YPH250) were cultured in SD medium until $A_{610} = 0.5$, and treated with 10 mM MG or 200 ng/ml rapamycin (Rapa) in the presence of 1 mM PMSF. After 3 h, cells were observed using a differential interference contrast microscope. (B) Cells (YPH250) expressing *PHO80 Δ 60* were cultured in SD medium until $A_{610} = 0.5$, and 10 mM MG or 200 ng/ml rapamycin (Rapa) was added. After 3 h, cell extracts were prepared and alkaline phosphatase (ALP) activity was measured (Noda and Ohsumi 1998). Data are a summary of three independent experiments (average $\pm$ standard deviation).

Fig. 5. Snf1 is not involved in the MG-induced phosphorylation of Gln3. (A) *snf1 Δ* cells of YPH250 carrying a plasmid expressing HA-tagged Snf1 were cultured in SD medium. When the A_{610} reached 0.3, 10 mM MG or 200 ng/ml rapamycin (Rapa) was added, and the cells were incubated for 30 min. For glucose starvation experiments (-Glc), cells at $A_{610} = 0.3$ were collected by centrifugation, and suspended in SD medium without glucose, and then incubated for 60 min. After each stress treatment, the phosphorylation status of Snf1 was determined with anti-phospho Thr²¹⁰ Snf1 antibody (α -PT210). The

amount of HA-tagged Snf1 was monitored using anti-HA monoclonal antibody (α -HA). (B) Cells of TB123a with the wild-type *SNF1* or *snf1* Δ allele were cultured in SD medium until $A_{610} = 0.3$, and treated with 10 mM MG (60 min), 200 ng/ml rapamycin (Rapa) (30 min), or glucose starvation stress (-Glc) (30 min). The phosphorylation status of Gln3 was determined.

Fig. 6. A proposed model of regulation of eIF2 α by MG. Detailed description of each regulatory step was given in the text. P represents the phosphorylation status of each protein. Inhibitory effect of MG on translation initiation in *S. cerevisiae* is a consequence of eIF2 α phosphorylation. Gcn2 is responsible for phosphorylation of eIF2 α following treatment with MG, although protein kinase(s) or protein phosphatase(s) that may influence the phosphorylation states of Gln3 and Npr1 have yet to be determined.

Fig. 1 Nomura et al.

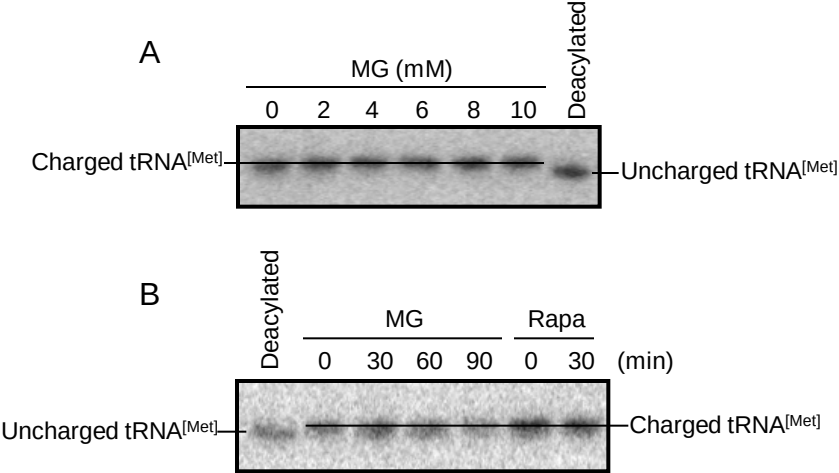


Fig. 2 Nomura et al.

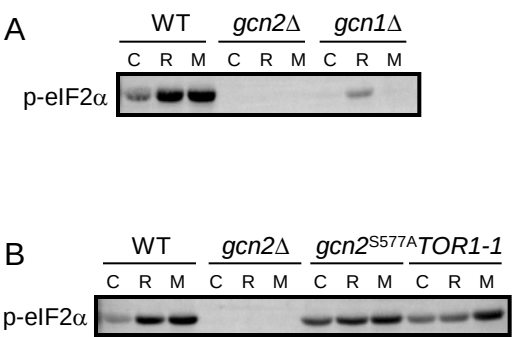


Fig. 3 Nomura et al.

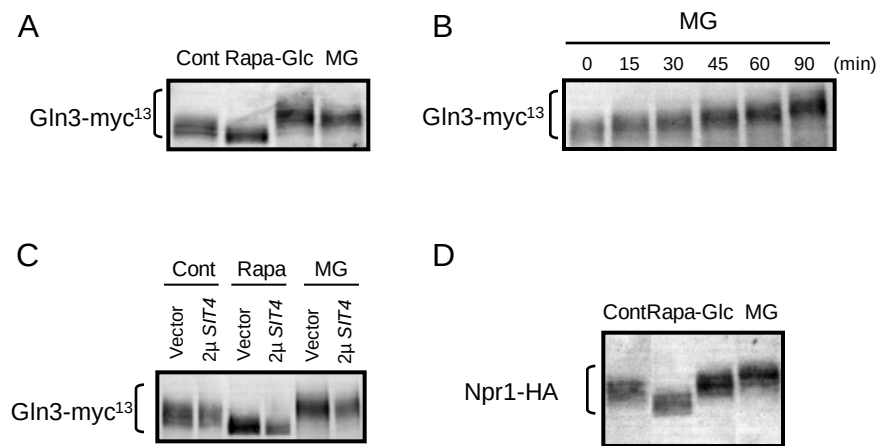


Fig. 4 Nomura et al.

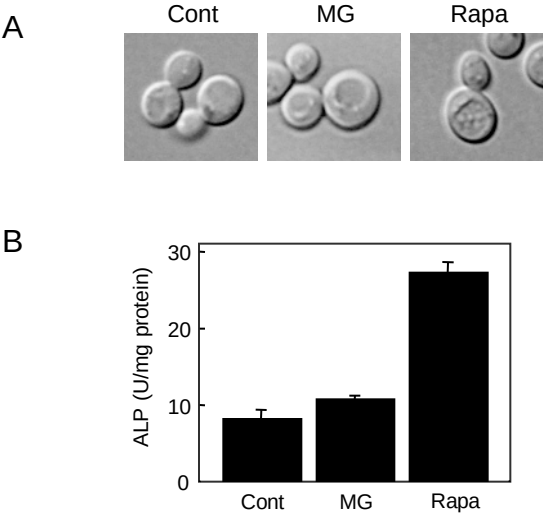


Fig. 5 Nomura et al.

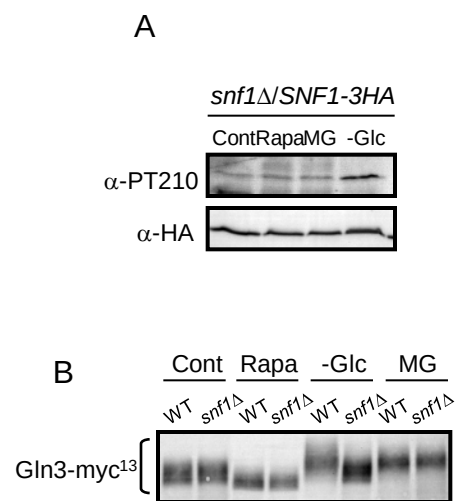


Fig. 6 Nomura et al.

