

Activity of the Yap1 Transcription Factor in *Saccharomyces cerevisiae* Is Modulated by Methylglyoxal, a Metabolite Derived from Glycolysis

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Methylglyoxal (MG) is synthesized during glycolysis, although it inhibits cell growth in all types of organisms. Hence, it has long been asked why such a toxic metabolite is synthesized in vivo. Glyoxalase I is a major enzyme detoxifying MG. Here we show that the Yap1 transcription factor, which is critical for the oxidative-stress response in *Saccharomyces cerevisiae*, is constitutively concentrated in the nucleus and activates the expression of its target genes in a glyoxalase I-deficient mutant. Yap1 contains six cysteine residues in two cysteine-rich domains (CRDs), i.e., three cysteine residues clustering near the N terminus (n-CRD) and the remaining three cysteine residues near the C terminus (c-CRD). We reveal that any of the three cysteine residues in the c-CRD is sufficient for MG to allow Yap1 to translocate into the nucleus and to activate the expression of its target gene. A Yap1 mutant possessing only one cysteine residue in the c-CRD but no cysteine in the n-CRD and deletion of the basic leucine zipper domain can concentrate in the nucleus with MG treatment. However, substitution of all the cysteine residues in Yap1 abolishes the ability of this transcription factor to concentrate in the nucleus following MG treatment. The redox status of Yap1 is substantially unchanged, and protein(s) interaction with Yap1 through disulfide bond is hardly detected in cells treated with MG. Collectively, neither intermolecular nor intramolecular disulfide bond formation seems to be involved in Yap1 activation by MG. Moreover, we show that nucleocytoplasmic localization of Yap1 closely correlates with growth phase and intracellular MG level. We propose a novel regulatory pathway underlying Yap1 activation by a natural metabolite in the cell.

Oxygen respiration allows all types of aerobic organisms to produce ATP efficiently, although it inevitably generates harmful reactive oxygen species which consequently cause oxidative stress. Cells possess diverse defense systems against oxidative stress (for a review, see reference 6). Several antioxidant systems of the budding yeast *Saccharomyces cerevisiae* are regulated at the transcriptional level (31). The Yap1 transcription factor is a functional homologue of mammalian AP-1 in *S. cerevisiae* (20, 43), and the expression of several antioxidant genes is up-regulated by Yap1 under oxidative-stress-inducing conditions (42). Yap1 activity is considered to be regulated mainly through its nuclear localization; i.e., Yap1 is distributed in both the cytoplasm and nucleus, preferentially in the cytoplasm, under nonstress conditions, although it is concentrated in the nucleus on the exposure of cells to oxidative stress (35). Yap1 has a nuclear export signal (NES) sequence in the C terminus, and Crm1 (exportin) exports Yap1 from the nucleus to the cytoplasm under normal conditions. Under oxidative-stress-inducing conditions, the interaction between Yap1 and Crm1 is inhibited and Yap1 is consequently enriched in the nucleus, leading to elevated expression of its target genes (36, 68).

Three cysteine residues, which partially overlap the NES,

exist in the C terminus of Yap1. These cysteine residues (C-terminal cysteine-rich domain [c-CRD]) are considered to constitute a redox sensor, and modification of these cysteine residues may block the interaction with Crm1 under oxidative stress-inducing conditions (68). We have demonstrated that the disulfide bond between Cys598 and Cys620 within the c-CRD in Yap1 is preferentially formed on H₂O₂ treatment (34). In addition, Delaunay et al. (11) reported that a disulfide bond between Cys303, which is in the n-CRD (N-terminal cysteine-rich domain), and Cys598 is formed. It has been reported that Gpx3, one of the glutathione peroxidase homologues in yeast (25), functions as a redox transducer for Yap1 (12).

Regarding deactivation of Yap1, we previously found that a thioredoxin deficiency (*trx1Δ trx2Δ*) caused the constitutive activation of Yap1: i.e., Yap1 was routinely concentrated in the nucleus and the basal expression levels of Yap1 target genes were increased (30). In this case, intracellular oxidation levels were twofold higher than those in the wild type. This elevated intracellular redox potential is considered to activate Yap1, since the constitutive activation of Yap1 was suppressed if cells were cultured anaerobically. Although a direct interaction of thioredoxin and Yap1 could not be detected by a two-hybrid assay (30), thioredoxin can be formally considered to be a negative regulator for Yap1 activity. We also clarified that the oxidized Yap1 is reduced directly by thioredoxin (34).

Several genes responsible for glutathione metabolism, such as glutathione synthesis (*GSH1* and *GSH2*) and glutathione-dependent antioxidant systems (*GPX2* and *GLR1*), are up-

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regulated by Yap1 (19, 25, 55, 58, 67). In addition to its anti-oxidant function, glutathione is important in detoxifying many harmful xenobiotics. In addition, glutathione functions as a detoxicant for endogenous toxic compounds such as methylglyoxal (MG: CH₃COCHO) (27). MG is a typical 2-oxoaldehyde, and the major source of this aldehyde in eukaryotes is a β -elimination reaction carried out by triosephosphate isomerase, a glycolytic enzyme (29, 49, 50). MG was therefore once thought to be an intermediate of glycolysis. However, MG was found to inhibit the growth of various types of cells from microorganisms to mammals (14, 15, 56). MG exerts its toxicity through modification of macromolecules to diminish their biological activities. For example, MG reacts with guanine residues in both DNA and RNA to form an adduct (53). An interaction with proteins also contributes to MG toxicity (1). Detoxification of MG is performed by the glyoxalase system, which consists of the glyoxalase I and glyoxalase II enzymes. Glyoxalase I catalyzes the conversion of MG to *S*-D-lactoylglutathione in the presence of glutathione, while the glutathione thiol ester is hydrolyzed by glyoxalase II. Therefore, a deficiency of the structural gene for glyoxalase I (*GLO1*) and/or glyoxalase II (*GLO2* and *GLO4*) in *S. cerevisiae* increases susceptibility to MG (3, 28). Although glyoxalase I is involved in a glutathione-requiring reaction, expression of the *GLO1* gene is not up-regulated by Yap1 (26).

To gain insight into the cellular function of MG, we compared the gene expression profile between wild-type and *glo1* Δ cells by DNA microarray analysis. Since glyoxalase I is the major MG-metabolizing enzyme (28), the steady-state level of MG in the *glo1* Δ mutant was higher than that in the wild-type cells throughout the growth phase. We found that Yap1 is constitutively concentrated in the nucleus in the *glo1* Δ mutant. In contrast to the case involving thioredoxin deficiency, the constitutive activation of Yap1 in *glo1* Δ cells was observed under both aerobic and anaerobic conditions. Cysteine residues within the c-CRD in Yap1 are necessary for activation by MG, although no disulfide bond formation between such cysteine residues is involved in this event, because any one of the cysteine residues in the c-CRD of Yap1 is sufficient to activate this transcription factor with MG. We present evidence indicating that Yap1 activity is reversibly modulated by MG, a natural metabolite from glycolysis, without redox regulation.

MATERIALS AND METHODS

Strains, media, and growth conditions. *S. cerevisiae* YPH250 (*MATa trp1- Δ his3- Δ 200 leu2- Δ 1 lys2-801 ade2-101 ura3-52*) and its isogenic diploid strain YPH274 (*MATa/MAT α trp1- Δ 1/trp1- Δ 1 his3- Δ 200/his3- Δ 200 leu2- Δ 1/leu2- Δ 1 lys2-801/lys2-801 ade2-101/ade2-101 ura3-52/ura3-52*) were obtained from the Yeast Genetic Stock Center, University of California, Berkeley. SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids [pH 5.5]) supplemented with appropriate amino acids and bases was used for the culture of yeast cells. Unless otherwise stated, cells were cultured at 28°C with reciprocal shaking. For anaerobic culture, the medium was flushed with nitrogen gas for 2 min after subculturing of the cells, the vessel was sealed, and the cells were incubated at 28°C without shaking.

Gene disruption. The *GLO2* gene was cloned by PCR using the primers GLO2Sneo (5'-TTGGCGATGGCATTAAACGAGGATCCCGCTT-3') and GLO2Rneo (5'-GGTGCTAATGTTCGACAATGTGTTCCGCCAA-3'). The GLO2Sneo and GLO2Rneo primers were designed to contain a BamHI site and SalI site (underlined), respectively. The PCR product containing the *GLO2* gene was digested with BamHI and SalI and cloned into the BamHI and SalI sites of pUC19 to yield pUGLO2. The pUGLO2 plasmid was then digested with EcoRV and Eco47III and treated with Klenow, and the *LEU2* gene was inserted to yield

pUglo2 Δ LEU2. The resultant plasmid was digested with PvuII and NcoI, and the *glo2* Δ ::*LEU2* cassette was used to disrupt the *GLO2* gene.

The *GLO4* gene was amplified by the primers GLO4S (5'-CCATGCAGGA TCCATTATAACTATCAAAGG-3') and GLO4R (5'-GGAAAAGAGAGAG AGTTCGACGTCTGTGATG-3'). The GLO4S and GLO4R primers were designed to contain a BamHI site and SalI site (underlined), respectively. The PCR product containing the *GLO4* gene was digested with BamHI and SalI and then cloned between the BamHI and SalI sites of pUC19 to construct pUGLO4. The pUGLO4 plasmid was digested with Eco47III and treated with Klenow, and the *HIS3* gene was inserted to give pUglo4 Δ HIS3. The resultant plasmid was digested with SspI, and the *glo4*::*HIS3* cassette was used to disrupt the *GLO4* gene.

The *xpo1-1* allele was introduced to an *xpo1* Δ ::*LEU2* mutant in the YPH250 background as follows. The *XPO1/CRM1* gene of YPH274 (*MATa/MAT α*) was disrupted by replacement with *LEU2* as described by Stade et al. (54). A plasmid carrying the *xpo1-1* allele (pKW457) and pKW430 bearing NLS^{SV40}-NES^{PK1-2} \times GFP (54) were introduced into the resultant mutant (*MATa/MAT α XPO1/xpo1* Δ ::*LEU2*). Tetrad dissection was performed to isolate a strain in the same genetic background of YPH250, except for *xpo1* Δ ::*LEU2*, with plasmid-borne *xpo1-1* and the NLS^{SV40}-NES^{PK1-2} \times GFP reporter gene.

The *glo1*::*URA3*, *glo1* Δ ::*HIS3*, *yap1* Δ ::*HIS3*, *gpx3* Δ ::*LEU2*, *tsa1* Δ ::*TRP1*, *gsh1* Δ ::*LEU2*, *trx1* Δ ::*URA3*, and *trx2* Δ ::*HIS3* mutants in the YPH250 background have been described previously (25, 26, 28, 30).

Reporter gene. The Yap1-dependent reporter gene, which carried three AP-1 binding sites followed by an upstream activator site-less *CYCI* gene and *lacZ* (37), was subcloned into YCp50. We designated this reporter gene AP-1-*CYCI-lacZ*. The DNA fragment containing AP-1-*CYCI-lacZ* was also cloned into a low-copy-number vector, pRS415. The *FLR1-lacZ* reporter gene (Ylp368/FLR1) was obtained from M. Raymond (45). The *GLO1-lacZ* gene (pRSGLac415) was described previously (26).

Enzyme assay. β -Galactosidase activity was measured by the method of Miller (40). One unit of the activity was expressed as ($A_{420} \times 1,000$) per hour (where A_{420} is the absorbance at 420 nm). Glyoxalase I activity was measured as described previously (24). One unit of glyoxalase I activity was defined as the amount of enzyme forming 1 μ mol of *S*-D-lactoylglutathione per min at 25°C. The amount of protein was determined by the method of Bradford (4).

Measurement of intracellular MG content. Cells were cultured in SD minimal medium with appropriate amino acids and bases. Cells were collected by centrifugation, washed once with 0.85% NaCl solution, and suspended in 1.0 ml of distilled water. They were boiled for 5 min and centrifuged at 1,500 \times g for 5 min, and the resultant supernatants were used as an MG source. MG concentration was measured enzymatically (glyoxalase I; Sigma, St. Louis, Mo.). To measure cellular MG content in the MG-treated cells, cells were cultured in SD medium until the A_{610} was 0.5 and 8 mM MG was added. The cells were collected periodically, and cellular MG levels were measured as described above.

Western blotting. To determine the effect of *GLO1* deficiency on the steady-state levels of Yap1, cells of *yap1* Δ and *yap1* Δ *glo1* Δ mutants carrying pRS315-Yap1-9Myc, which was donated by M. Toledano, were cultured in SD medium until they reached an A_{610} of 1. They were disrupted with glass beads in 10 mM Tris-HCl buffer (pH 7.0) containing 1 mM β -mercaptoethanol. Homogenates were centrifuged at 12,000 \times g for 15 min at 4°C. Cellular proteins (40 μ g) in the supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electrically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). Yap1-9Myc protein was detected by an anti-c-Myc monoclonal antibody (9E 10; Roche Diagnostics GmbH, Mannheim, Germany). To determine the effect of exogenously added MG on the levels of Yap1, *yap1* Δ cells carrying pRS315-Yap1-9Myc were cultured in SD medium until an A_{610} of 1 was reached and 8 mM MG was added. Cells were collected periodically (0 to 3 h), and Yap1-9Myc protein levels were compared by Western blotting as described above.

To detect the biochemical changes in Yap1, the *yap1* Δ cells carrying pRS314 cp HA YAP1 (HA-tagged Yap1) (35) were treated with 8 mM MG for 60 min or 0.4 mM H₂O₂ for 15 min. Cellular proteins were extracted using trichloroacetic acid as described by Delaunay et al. (11), and the resultant proteins were subjected to nonreducing SDS-PAGE. The HA-tagged Yap1 was detected by anti-HA monoclonal antibody (Roche Diagnostics GmbH).

Fluorescence microscopy analysis. Cells were cultured in SD minimal medium until an A_{610} of 0.5 was reached, and localization of green fluorescent protein (GFP)-tagged Yap1 was observed by fluorescence microscopy. To visualize DNA, the cells were stained with 1 μ g of 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) per ml.

Cloning and expression of *gloA*. The structural gene for *Escherichia coli* glyoxalase I (*gloA*) (9) was amplified by PCR with the primers gloA-F (5'-AGCCAT TTTGAGGATCCAAAGATGCGTCTT-3') and gloA-R (5'-CGGGCGCGAT

GAGTCGACGCCCGCAGGAG-3'). The *gloA*-F and *gloA*-R primers were designed to contain a BamHI site and SalI site, respectively (underlined). The PCR product was digested with BamHI and SalI, and the resultant fragment was cloned into the BamHI and SalI sites of the 2 μ m-based plasmid pG-1 (51), which contains the *GAP* promoter, *PGK* terminator, and *TRP1* marker. The resultant plasmid (pG-1-*gloA*) was introduced into the *glo1Δ* mutant, and expression of *gloA* was confirmed by measurement of glyoxalase I activity.

Coimmunoprecipitation. Cells of *yap1Δ* and *yap1Δ glo1Δ* mutants carrying the GFP-Yap1^{WT} fusion protein were cultured to log phase, and cell extracts were prepared. Immunoprecipitation was carried out using a kit (Roche Molecular Biochemicals) with anti-glyoxalase I antibody (23) or anti-GFP antibody (Clontech) and protein A-agarose. Detailed procedures for immunoprecipitation were performed as specified by the manufacturer. After SDS-PAGE, Western blot analysis was conducted using anti-glyoxalase I antibody or anti-GFP antibody as the primary antibody and anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (New England Biolabs) as the secondary antibody. Immunoreactive protein was visualized with 4-chloro-1-naphthol and H₂O₂.

Cys-substituted mutants of Yap1. GFP-Yap1 plasmids were based on the plasmid pRS cp-GFP-HA YAP1 (GFP-Yap1^{WT}) (35). Detailed procedures for the construction of pRS cp-GFP-HA yap1 3Cys (GFP-Yap1^{3Cys}), where three cysteine residues were mutated to Thr (C303T, C310T, and C315T), were described previously (34). To construct pRS cp-GFP-HA yap1 6Cys (GFP-Yap1^{6Cys}), in which all six cysteine residues are substituted (C303T, C310T, C315T, C598T, C620A, and C629T), the c-CRD region of pRS cp-GFP-HA yap1 cm46A5 (35) (*yap1* gene with mutation of C598T, C620A, and C629T; GFP-Yap1^{cm46A5}) was isolated with BsmI and BstEII and replaced by the c-CRD region of pRS cp-GFP-HA yap1 3Cys. The construction of single-cysteine-substituted c-CRD mutants (GFP-Yap1^{C598T}, GFP-Yap1^{C620A}, and GFP-Yap1^{C629T}) was described previously (35). GFP-Yap1 mutants that possess only one cysteine residue were constructed by a PCR-based method as described previously (35), using *yap1* genes carrying a C598T, C620A, or C629T mutation as templates. The c-CRD regions of the resulting PCR fragments were digested with BsmI and SalI and cloned into pRS314 cp-GFP-HA yap1 3Cys to yield an expression vector for GFP-HA-fused Yap1^{3Cys} cm56, Yap1^{3Cys} cm46, and Yap1^{3Cys} cm45. To construct pRS cp-GFP Yap1^{daH}, the region of the *yap1* gene corresponding to bZIP (amino acids 73 to 121) was replaced with a SmaI restriction site (CCCGGG encoding Pro-Gly) by the PCR-based method described above. To construct pRS cp-GFP yap1^{daH 3Cys} cm46, the AfeI-SalI restriction fragment of pRS cp-GFP Yap1^{daH} was replaced with the corresponding fragment of GFP-Yap1^{3Cys} cm56.

DNA microarray analysis. Cells of the wild-type strain and the *glo1Δ::HIS3* mutant were cultured in SD minimal medium until they reached an A₆₁₀ of 1. Total RNA was extracted by the hot-phenol method (52), and poly(A)⁺ mRNA was isolated from total RNA by using a kit (Oligotex-dT30 <Super> mRNA purification kit; Takara Shuzo, Kyoto, Japan). cDNAs labeled with Cy3-dCTP or Cy5-dCTP were generated by using reverse transcriptase and the Cyscribe first-strand cDNA labeling kit (Amersham Pharmacia, Piscataway, N.J.). Fluorescent probes were purified using Qiaquick PCR purification columns (Qiagen GmbH, Hilden, Germany) and concentrated by a Microcon-30 filter (Amicon, Charlotte, N.C.). Hybridization was carried out at 37°C for 14 h in DIG Easy Hybe solution (Roche Diagnostics GmbH, Mannheim, Germany) containing 100 μg of salmon sperm DNA per ml. After hybridization, microarray slides (Eurogentec, Seraing, Belgium) were washed with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS and rinsed with 0.2× SSC at room temperature. Slides were scanned using an Affymetrix 417 Arrayer (Affymetrix, Santa Clara, Calif.). Images were processed using the microarray spot finding and quantification software ImaGene (BioDiscovery, Marina del Rey, Calif.). Fluorescence intensity values were normalized so that the medians of all Cy3 and Cy5 intensities were equal.

RESULTS

Yap1 is constitutively activated in a *glo1Δ* mutant. MG is a natural metabolite derived from glycolysis, although it inhibits cell growth and in some cases it induces apoptosis (33). In addition, MG is likely to be involved in some pathological processes such as cancer and diabetes (32, 57). However, the intrinsic function of MG is not yet fully understood. To gain insight into the physiological function of MG in the cell, we performed DNA microarray analysis to compare the gene ex-

TABLE 1. Effect of glyoxalase I deficiency on the expression of Yap1 target genes

Gene ^a	Product	Fold up-regulation
<i>GSH1</i>	γ-Glutamylcysteine synthetase	2.6
<i>GPX2</i>	Glutathione peroxidase	6.4
<i>GTT2</i>	Glutathione S-transferase	3.7
<i>TRX2</i>	Thioredoxin	3.5
<i>TRR1</i>	Thioredoxin reductase	3.4
<i>CYS3</i>	Cystathionine γ-lyase	4.7
<i>SSA1</i>	Heat shock protein 70	2.9
<i>HSP78</i>	Mitochondrial heat shock protein	2.4
<i>FLR1</i>	ABC transporter, fluconazole resistance	3.2
<i>YCF1</i>	ABC transporter, cadmium resistance	2.6
<i>GRE2</i>	Methylglyoxal reductase	13.0
<i>TAL1</i>	Transaldolase	3.0
<i>PGM2</i>	Phosphoglucomutase	3.6
<i>PDII</i>	Protein disulfide isomerase	3.1
<i>YNL134c</i>	Similarity to alcohol dehydrogenase	2.1

^a The basis of some Yap1 targets is from the report by Lee et al. (38).

pression pattern between wild-type and *glo1Δ* cells. Because glyoxalase I is the major enzyme for the detoxification of MG, the steady-state level of MG in the *glo1Δ* mutant (5.25 ± 1.17 nmol/10⁹ cells) was higher than that in the wild type (2.61 ± 0.39 nmol/10⁹ cells). As a result of analysis; an increase (more than twofold) in the expression of approximately 10% of the entire set of yeast genes was observed in *glo1Δ* cells, with the genes for energy metabolism and stress response being among the 10%. Details of the microarray analysis will be discussed elsewhere.

We noted that many genes whose expression is regulated by Yap1 transcription factor were up-regulated in the *glo1Δ* mutant (Table 1). Yap1 is distributed predominantly in the cytoplasm under nonstress conditions, whereas it is concentrated in the nucleus under oxidative-stress-inducing conditions (35). Interestingly, Yap1 was constitutively concentrated in the nucleus in the *glo1Δ* mutant. We confirmed that GFP-Yap1 was observed in the nuclei of more than 87.6% of *glo1Δ* cells ($n = 1,419$). Consequently, the basal expression levels of the Yap1-dependent artificial reporter gene (*AP-1-CYCI-lacZ*) were increased (Fig. 1A and B), although this was not the case for mutants defective in cytosolic glyoxalase II (*glo2Δ*), mitochondrial glyoxalase II (*glo4Δ*), and both of these enzymes (*glo2Δ glo4Δ*) (data not shown).

Previously, we observed a similar phenotype in terms of the nucleocytoplasmic localization of Yap1 in a cytosolic thioredoxin-deficient (*trx1Δ trx2Δ*) mutant (30). In this case, the constitutive activation of Yap1 was observed only when the mutant was cultured aerobically. In contrast, however, Yap1 activation in the *glo1Δ* mutant was also seen when cells were cultured anaerobically (Fig. 1B).

Recently, Delaunay et al. (12) reported that Gpx3, one of the glutathione peroxidase homologues in *S. cerevisiae* (25), is a redox transducer for Yap1. They proposed a model whereby Gpx3 is first oxidized by H₂O₂ and the oxidized Gpx3 (Gpx3-Cys-SOH) forms a mixed disulfide with Cys598 in Yap1, which is then reduced by Cys303 in Yap1 to form an intramolecular disulfide bond (Cys303-Cys598). Thus, Yap1 activation by H₂O₂ does not occur in a *gpx3Δ* mutant. However, the constitutive nuclear accumulation of Yap1 in the *glo1Δ* mutant was

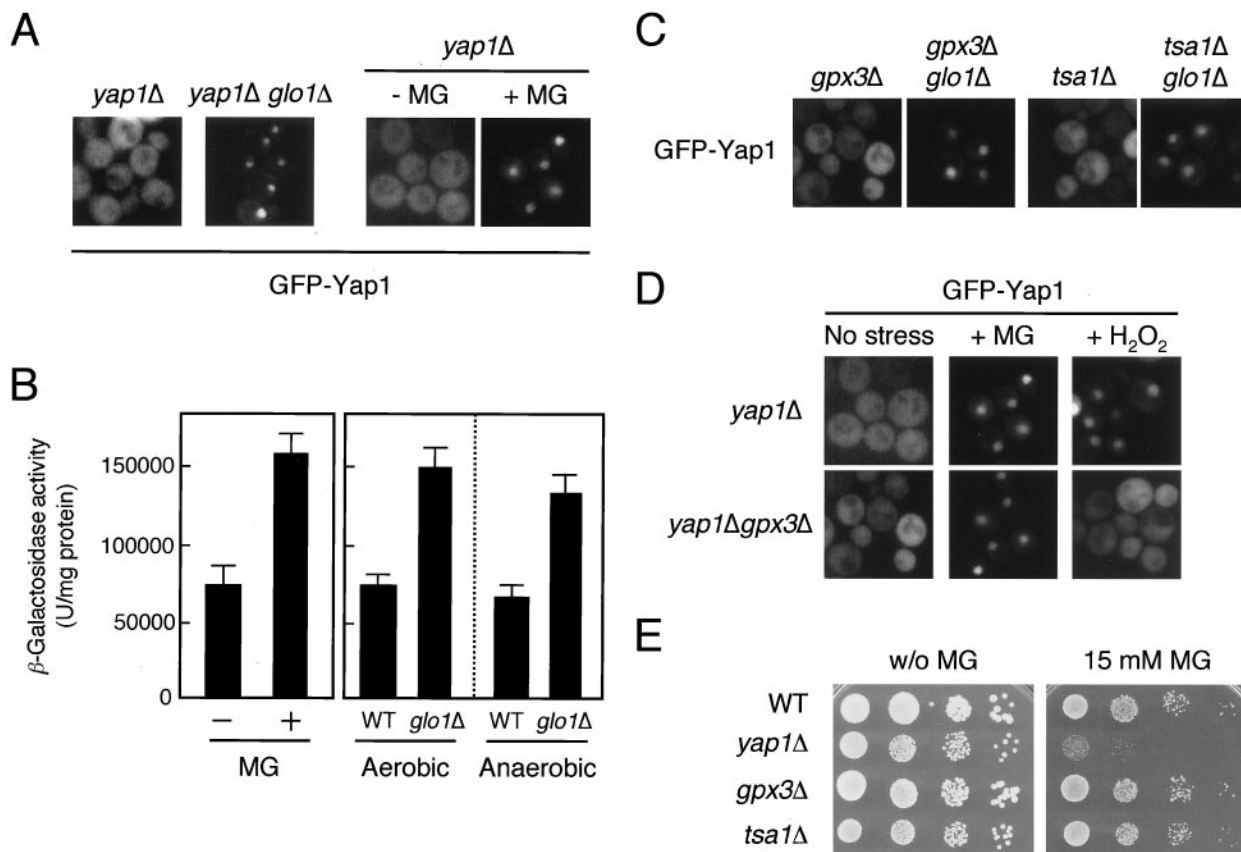


FIG. 1. Effect of MG on activation of Yap1. (A) Cells of *yap1Δ* and *yap1Δ glo1Δ* carrying the GFP-Yap1 plasmid were cultured in SD medium to an A_{610} of 0.5, and localization of GFP-Yap1 was observed. (B) (Left panel) Cells of the wild type (WT) carrying the AP-1-*CYC1-lacZ* reporter plasmid were cultured in SD medium to an A_{610} of 0.5, and 8 mM MG was added. After a 3-h incubation, β -galactosidase activity was measured. (Right panel) For anaerobic culture, after cells of the wild type and the *glo1Δ* mutant with reporter gene were inoculated into SD medium, nitrogen gas was flushed through the flask, which was then sealed, and the cells were cultured without shaking at 28°C. (C) GFP-tagged Yap1 was expressed in each mutant. All mutants have a *yap1Δ* background. (D) GFP-Yap1 was expressed in the *yap1Δ* and *yap1Δ gpx3Δ* mutants. Cells were cultured in SD medium to an A_{610} of 0.5, and 8 mM MG or 0.4 mM H₂O₂ was added. Localization of GFP-Yap1 was monitored for 60 min (for MG) or 15 min (for H₂O₂) after the addition of each chemical. (E) Cells were cultured in YPD medium to an A_{610} of 0.5, diluted with 0.85% NaCl, and spotted onto YPD agar plates with or without 15 mM MG. The cells were cultured at 28°C for 2 days.

seen even in the *gpx3Δ* background (Fig. 1C). In addition, exogenously added MG allowed Yap1 to accumulate in the nucleus of the *gpx3Δ* cell (Fig. 1D). Furthermore, the *gpx3Δ* mutant did not exhibit hypersensitivity to MG (Fig. 1E).

On the other hand, Veal et al. (61) reported that Ybp1 functions in the Gpx3 pathway. They demonstrated that Ybp1 binds to Yap1 and stimulates the nuclear localization of Yap1 on exposure to H₂O₂ stress. Interestingly, W303-1A, a laboratory yeast strain, possesses a *ybp1-1* mutation that abolishes the function of Ybp1 (61). We confirmed that the constitutive activation of Yap1 occurs in a *glo1Δ* mutant in a W303-1A background (data not shown).

Delaunay et al. (12) also reported that Gpx3 functions as a thioredoxin peroxidase. If an oxidized Gpx3 (Gpx3-Cys-SOH) is condensed with another cysteine residue in Gpx3 intramolecularly, an intramolecular disulfide bond is formed and thioredoxin reduces it, thereby accomplishing a thioredoxin-dependent H₂O₂ reducing reaction. *S. cerevisiae* possesses *TSA1* coding for cytosolic thioredoxin peroxidase (7, 44). Recently, involvement of Tsa1 in H₂O₂-induced activation of Yap1 has been suggested (46). We determined whether Tsa1 is involved

in the activation of Yap1 by MG, although Yap1 was constitutively concentrated in the nucleus of *tsa1Δ* cells in the *glo1Δ* background (Fig. 1C). Yap1 was found to be concentrated in the nucleus of *tsa1Δ* cells following treatment with MG (data not shown), and the *tsa1Δ* mutant did not exhibit susceptibility to MG (Fig. 1E). These results suggest that the underlying mechanism of Yap1 activation by MG is different from that by H₂O₂.

The intracellular MG level correlates with activation of Yap1. The subcellular localization of Yap1 is regulated at the stage of nuclear export; i. e., Crm1 exports Yap1 from the nucleus to the cytoplasm under nonstress conditions, but interaction between Yap1 and Crm1 is inhibited under oxidative-stress conditions, thereby allowing Yap1 to remain in the nucleus (36, 68). We examined the function of Crm1 in the *glo1Δ* mutant by using the GFP-tagged Crm1-recognizable NES derived from the human protein kinase inhibitor α (PKI α) (64) with the nuclear localization signal (NLS) of the simian virus 40 (SV40) large T antigen (54). The resultant reporter protein (NLS^{SV40}-NES^{PKI-2} × GFP) is translocated to the nucleus but is immediately exported from the nucleus to the cytoplasm by

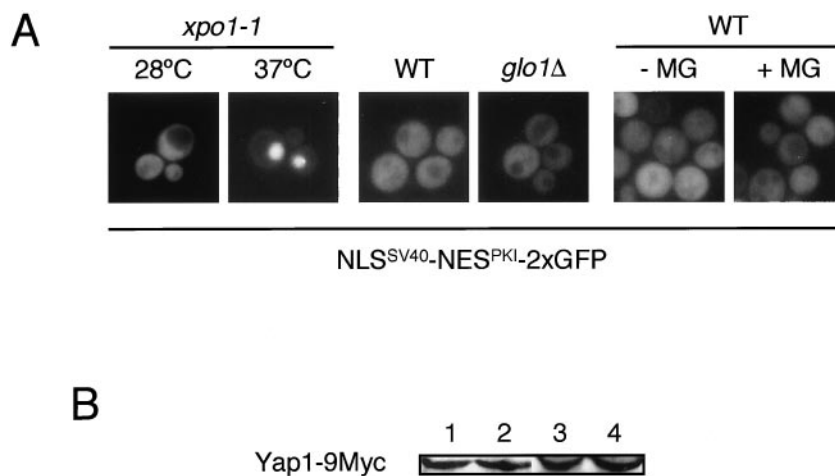


FIG. 2. The function of the Yap1 nuclear export machinery and the Yap1 protein level are normal in the *glo1Δ* mutant. (A) Temperature-sensitive *xpo1-1* cells carrying pKW430 (NLS^{SV40}-NES^{PK1}-2xGFP) were cultured at 28°C (permissive temperature) to an A_{610} of 0.5 and shifted to 37°C (nonpermissive temperature). After 15 min, the localization of NLS^{SV40}-NES^{PK1}-2xGFP was monitored. Cells of the wild type (WT) and the *glo1Δ* mutant carrying pKW430 were cultured in SD medium to an A_{610} of 0.5, and localization of NLS^{SV40}-NES^{PK1}-2xGFP was observed. To determine the effect of exogenously added MG on the function of Crm1, wild-type cells carrying pKW430 were treated with 8 mM MG. No change in the localization of NLS^{SV40}-NES^{PK1}-2xGFP was seen at least up to 120 min after the addition of MG. The pictures shown in the figure were taken 60 min after the addition of MG. (B) To compare the steady-state level of Yap1 protein, cells of the *yap1Δ* (lane 1) and *yap1Δ glo1Δ* (lane 2) mutants carrying pRS315-Yap1-9Myc were cultured in SD medium to an A_{610} of 1.0, and cell extracts were prepared. To determine the effect of MG on the Yap1 protein level, *yap1Δ* cells carrying pRS315-Yap1-9Myc were cultured in SD medium to an A_{610} of 0.5. After the addition of 8 mM MG, the cells were incubated at 28°C and collected periodically. Lane 3, without MG for 1 h; lane 4, with 8 mM MG for 1 h. We confirmed that the Yap1 protein level did not change at least up to 3 h after MG treatment.

Crm1, thereby allowing the cytoplasmic localization. If the function of Crm1 is impaired in *glo1Δ* cells, this reporter protein will remain in the nucleus.

First, to verify whether Crm1 in YPH250 background functions normally, NLS^{SV40}-NES^{PK1}-2xGFP was expressed in a temperature-sensitive mutant of Crm1 (*xpo1-1*) and the resultant transformant was shifted to the nonpermissive temperature. As a result, this reporter protein was distributed in the cytoplasm at the permissive temperature but was concentrated in the nucleus after a shift to the nonpermissive temperature; therefore, the function of Crm1 was confirmed to be normal in the strain used in this study (Fig. 2A). Next, we determined the localization of NLS^{SV40}-NES^{PK1}-2xGFP in the *glo1Δ* mutant. As shown in Fig. 2A, this reporter protein was distributed predominantly in the cytoplasm in *glo1Δ* cells. Similarly, exogenously added MG did not affect the localization of NLS^{SV40}-NES^{PK1}-2xGFP (Fig. 2A), indicating that Crm1 functions normally in the *glo1Δ* mutant.

Thus, regarding the constitutive nuclear localization of Yap1 in the *glo1Δ* mutant, we considered three possible models. The first model is that glyoxalase I functions as a negative regulator for Yap1 through direct protein-protein interaction; i.e., glyoxalase I, which exists in the cytoplasm, may serve as an anchor protein for Yap1. A second model is that glyoxalase I deficiency influences the Yap1 protein level. A third model is that MG directly modulates the Yap1 activity.

To assess whether direct interaction occurs between Yap1 and glyoxalase I, we performed a coimmunoprecipitation assay. The GFP-Yap1^{WT} fusion protein was expressed in the *yap1Δ* and *yap1Δ glo1Δ* mutants, and cell extracts were immunoprecipitated with anti-glyoxalase I antibody and then Western blotted with anti-GFP antibody. However, no interaction

between Yap1 and glyoxalase I was observed. Similarly, no direct interaction was observed when the anti-GFP antibody was used for the immunoprecipitation (data not shown).

Next, we compared the steady-state level of the Yap1 protein in wild-type and *glo1Δ* cells, because overexpression of *YAP1* is reported to cause a similar phenotype in terms of Yap1 activation (63). The Yap1-9Myc protein was expressed by its own promoter in the *yap1Δ* and *yap1Δ glo1Δ* mutants. We confirmed that Myc-tagged Yap1 is functional in *yap1Δ* cells (data not shown). As shown in Fig. 2B, no difference was seen in the protein levels of Yap1 between the wild-type and *glo1Δ* cells. We confirmed that MG treatment did not affect the Yap1 protein levels (Fig. 2B).

Then we investigated whether MG is involved in the activation of Yap1. The steady-state level of MG in the *glo1Δ* mutant was higher than that in the wild type. Accumulation of MG was also observed in the cells cultured anaerobically (wild type, 3.33 ± 0.24 nmol/10⁹ cells; *glo1Δ*, 6.68 ± 0.24 nmol/10⁹ cells). Although we have confirmed that the yeast glyoxalase I per se does not function as a negative regulator for Yap1 through protein-protein interaction, we introduced the *gloA* gene coding for the glyoxalase I of *E. coli* (9) into the *glo1Δ* mutant to reduce the intracellular MG level. On expression of the *gloA* gene, the cellular MG content decreased to the normal level (vector control, 5.31 ± 0.86 nmol/10⁹ cells; *gloA* expression, 2.14 ± 0.37 nmol/10⁹ cells), and concomitantly the basal expression level of the Yap1-dependent reporter gene, AP-1-*CYC1-lacZ*, was reversed to the wild-type level (data not shown).

To determine the correlation between the intracellular MG level and Yap1 activity more directly, we added MG to the culture medium. As shown in Fig. 3, the intracellular MG level

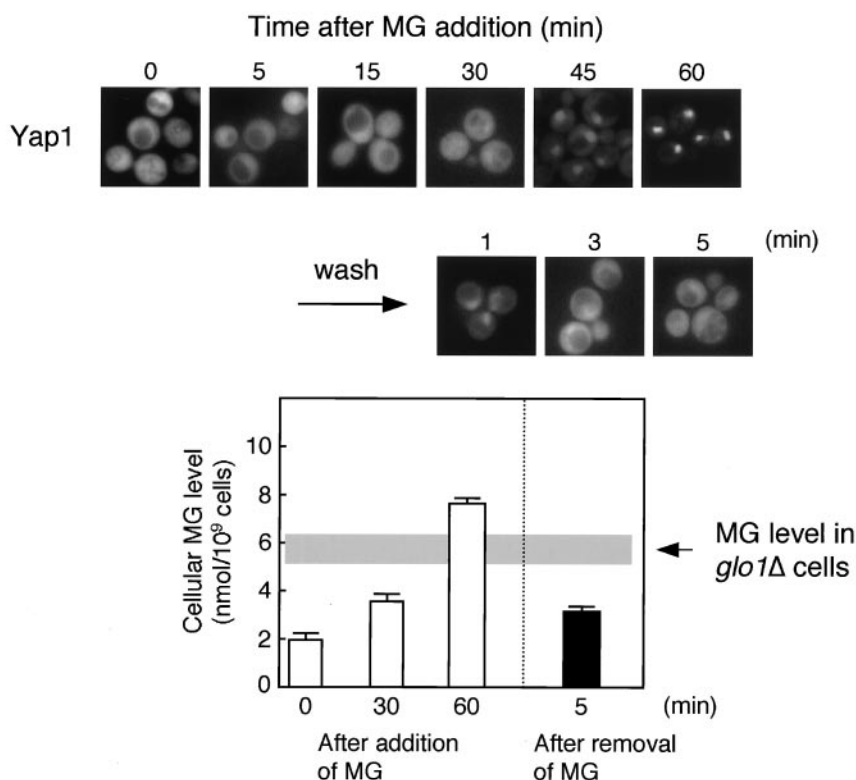


FIG. 3. Yap1 activation by MG is reversible. GFP-Yap1 was expressed in the *yap1Δ* mutant and cultured in SD medium to an A_{610} of 0.5. At this point, the localization of GFP-Yap1 was monitored (time, 0 min), 8 mM MG was added to the culture medium, and the cells were incubated to monitor the localization of GFP-Yap1 periodically (time, 5 to 60 min). After 60 min, cells were collected by centrifugation, washed with 0.85% NaCl, and resuspended in fresh SD medium, and localization of GFP-Yap1 was monitored periodically (time, 1 to 5 min). The cellular MG content was measured when the A_{610} had reached 0.5, and 8 mM MG was added. The MG level was monitored after the addition of MG (30 and 60 min) and also after the removal of MG (5 min).

increased and subsequently Yap1 was concentrated in the nucleus, but the MG level reverted to normal when the cells were washed (Fig. 3). Consequently, Yap1 was immediately redistributed in the cytoplasm (Fig. 3). Since glyoxalase I is an efficient catalyst ($k_{cat}/K_m = 1.89 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) (28), the turnover of intracellular MG is high. These results indicate that Yap1 activation directly correlates with the intracellular MG level. Importantly, these observations also suggest that activation of Yap1 by MG is reversible.

Importance of Cys residues in the c-CRD for Yap1 activation by MG. Yap1 has six cysteine residues, three in the trans-activation domain (Cys303, Cys310, and Cys315) (Coleman et al. [10] named it the N-terminal cysteine-rich domain [n-CRD]) and three at the C terminus (c-CRD; Cys598, Cys620, and Cys629) (Fig. 4A). Since the c-CRD overlaps the NES, deletion of the c-CRD causes the constitutive nuclear localization of Yap1 (35). Kuge et al. (35) reported that cysteine residues in the c-CRD are important for the regulation of the subcellular localization of Yap1. On the other hand, Coleman et al. (10) reported that the n-CRD contains trafficking information for Yap1. MG generates irreversible adducts with the arginine and lysine residues of protein (65). The c-CRD/NES of Yap1 contains Arg604, Lys610, Lys626, Lys628, and Arg632, and therefore it is conceivable that modification of these amino acid residues with MG might abolish the NES function and inhibit interaction with Crm1, leading to concen-

tration of Yap1 in the nucleus of the *glo1Δ* mutant. However, as we have shown in Fig. 3, Yap1 activation by MG is reversible, and thus the possibility of modification of arginine and/or lysine residues scattered within the c-CRD/NES is lowered.

To determine whether the cysteine residues that exist in the n-CRD and/or c-CRD are involved in the activation of Yap1 in the *glo1Δ* mutant, we constructed several cysteine-substituted mutants of Yap1 (Fig. 4A). In the Yap1^{3Cys} mutant, three cysteine residues in the n-CRD (Cys303, Cys310, and Cys315) were changed to threonine. Yap1^{cm46A5} contains the substitutions C598T, C620A, and C629T in the c-CRD. This mutant is insensitive to oxidative stress and is distributed in the cytoplasm even under oxidative-stress-inducing conditions (35). In Yap1^{6Cys}, the configurations of Yap1^{3Cys} and Yap1^{cm46A5} were combined. These cysteine-substituted *yap1* mutant genes were fused to *GFP* gene, and the resultant GFP-Yap1 mutant proteins were expressed in the *yap1Δ* and *yap1Δ glo1Δ* mutants carrying the *FLR1-lacZ* reporter gene. The *FLR1* gene is one of the Yap1 targets, and its promoter contains three Yap1 response elements (45).

As shown in Fig. 4B, the GFP-Yap1^{cm46A5} protein was present in the cytoplasm in the *glo1Δ* mutant. In addition, the GFP-Yap1^{6Cys} was not concentrated in the nucleus. These results indicate that (i) the c-CRD contains trafficking information for the constitutive nuclear localization of Yap1 in the *glo1Δ* mutant, (ii) cysteine residues in the c-CRD are critical,

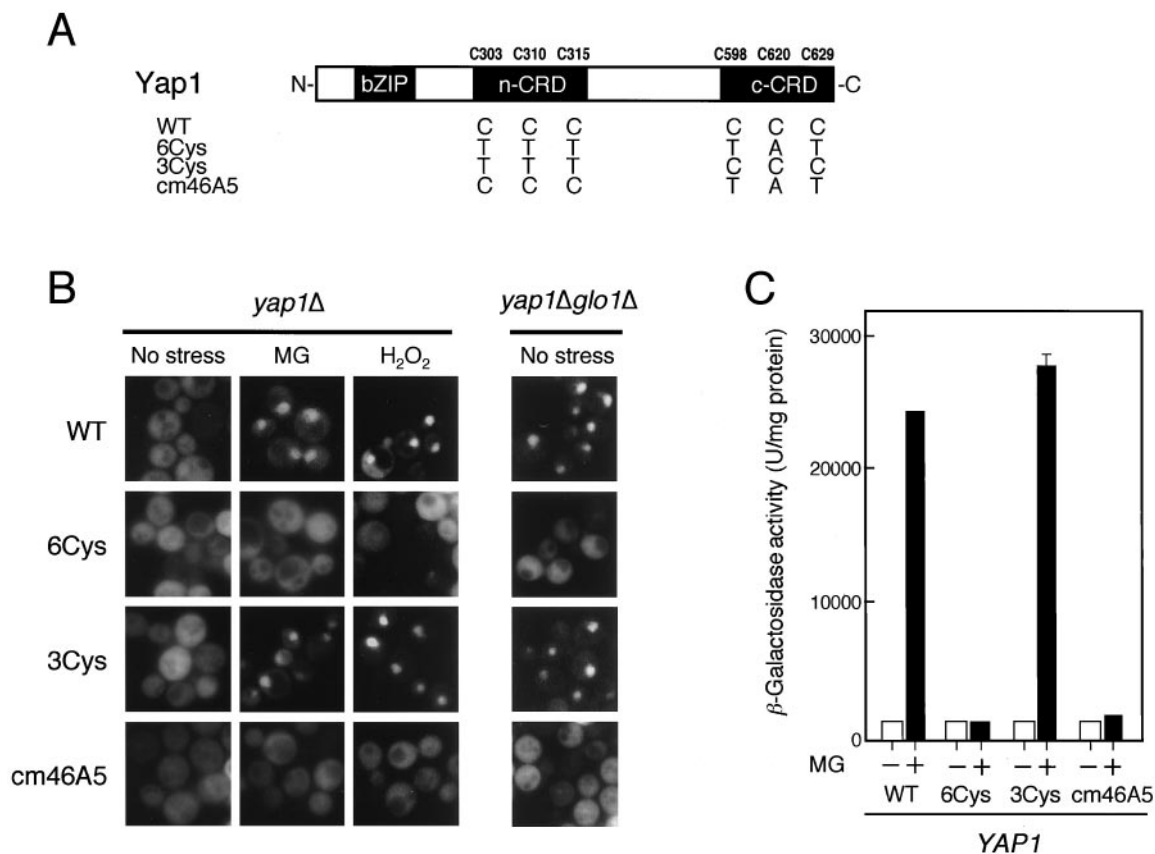


FIG. 4. Importance of Cys residues in Yap1 for activation by MG. (A) Schematic of the Yap1 mutant protein. (B) GFP-tagged Yap1 of the wild type (WT) and each mutant was expressed in the *yap1Δ* and *yap1Δ glo1Δ* mutants. Cells were cultured in SD medium to an *A*₆₁₀ of 0.5, and localization of GFP-Yap1 in *yap1Δ glo1Δ* cells was monitored. For *yap1Δ* cells, 8 mM MG or 0.4 mM H₂O₂ was added to the culture medium, the cells were incubated for a further 60 min (MG) or 15 min (H₂O₂), and localization of GFP-Yap1 was monitored. (C) Each GFP-tagged protein was also expressed in the *yap1Δ* mutant carrying the *FLR1-lacZ* reporter gene. After treatment with 8 mM MG for 3 h, the β-galactosidase activity was measured.

and (iii) arginine and/or lysine residues in the c-CRD are less feasible in the regulation of the nucleocytoplasmic localization of Yap1 in the *glo1Δ* mutant.

We confirmed the importance of the c-CRD for the constitutive nuclear localization of Yap1 in the *glo1Δ* mutant by using a fusion protein of the Gal4 DNA binding domain containing an NLS followed by GFP and the c-CRD (Gal4dbd-GFP-CRD) (Fig. 5A). This construct was proven to be exported from the nucleus by Crm1 and to be sensitive to oxidative stress (35). The Gal4dbd-GFP-CRD fusion protein was concentrated in the nucleus in the *glo1Δ* mutant under normal conditions (Fig. 5B). Additionally, this reporter protein accumulated in the nucleus following MG treatment (Fig. 5B). It does not contain the n-CRD, indicating that the c-CRD is sufficient for the constitutive nuclear localization of Yap1 in the *glo1Δ* mutant.

Next, we examined the effect of exogenously added MG on the localization of these Yap1 mutant proteins and evaluated their activity by using the *FLR1-lacZ* reporter gene. As shown in Fig. 4B, GFP-Yap1^{3Cys} was concentrated in the nucleus following MG treatment and the expression of *FLR1-lacZ* was induced. Neither GFP-Yap1^{6Cys} nor GFP-Yap1^{cm46A5} accumulated in the nucleus, and therefore no induction of the

reporter gene was observed (Fig. 4C). In the case of H₂O₂ treatment, GFP-Yap1^{3Cys} was also concentrated in the nucleus (Fig. 4B); however, this mutant did not induce the reporter gene expression following H₂O₂ treatment for 1 h (data not shown). Previously, we demonstrated that a disulfide bond between Cys598 and Cys620 within the c-CRD of Yap1 is formed following H₂O₂ treatment (34). Although this GFP-Yap1^{3Cys} mutant protein was able to stay in the nucleus briefly (approximately 15 min), it redistributed to the cytoplasm rapidly compared with wild-type Yap1; i.e., GFP-Yap1^{WT} continued to be present in the nucleus for 30 to 45 min under the same conditions. The disulfide bond between Cys303 in the n-CRD and Cys598 seems necessary for the stable nuclear localization of Yap1 (11, 34). Hence, one possible explanation for the absence of Yap1 target gene induction in cells expressing the GFP-Yap1^{3Cys} following H₂O₂ treatment is that this mutant Yap1 is not present in the nucleus long enough to induce target gene expression.

Any one of three Cys residues in c-CRD is sufficient for Yap1 activation by MG. To determine whether disulfide bond formation is involved in the activation process of Yap1 by MG, we constructed a series of mutants that contains only one cysteine residue in the c-CRD, with all of the cysteine residues in the

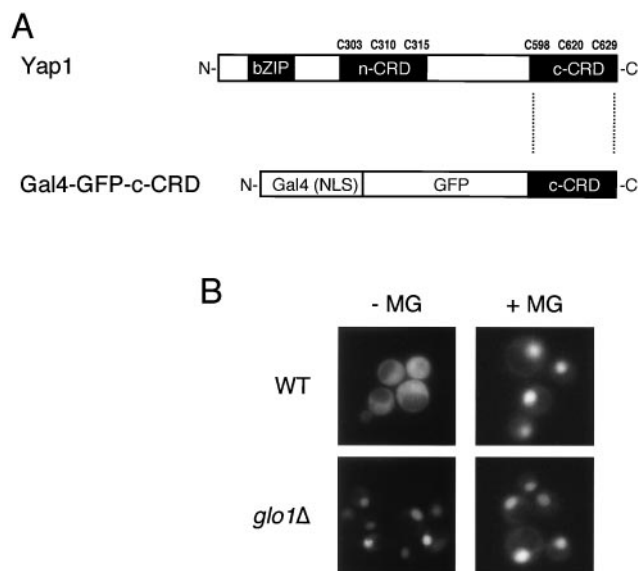


FIG. 5. Importance of c-CRD for the nuclear localization of Yap1 following MG treatment. (A) Schematic of Yap1 and the Gal4-GFP-c-CRD reporter protein. (B) The Gal4-GFP-c-CRD reporter gene was expressed in wild-type (WT) and *glo1Δ* cells. Cells were cultured in SD medium to an A_{610} of 0.5, and localization of the reporter protein was monitored. Then 8 mM MG was added, and the cells were incubated for 60 min, at which time the localization of the reporter protein was again determined.

n-CRD being simultaneously substituted (GFP-Yap1^{3Cys cm56}, GFP-Yap1^{3Cys cm46}, and GFP-Yap1^{3Cys cm45}) (Fig. 6A). Interestingly, these Yap1 mutant proteins were able to concentrate in the nucleus following MG treatment (Fig. 6B), and, consequently, expression of the Yap1 target gene was induced (Fig. 6C). The nuclear localization of these single-cysteine mutants of Yap1 by MG was reversible; i.e., they were rapidly redistributed in the cytoplasm when MG was removed from the medium (data not shown). On the other hand, these mutants were no longer able to respond to H₂O₂ (Fig. 6B), because the proteins cannot form any intramolecular disulfide bonds. These results suggest that disulfide bond formation between the cysteine residues in Yap1 is not necessary for Yap1 activation by MG.

Finally, we deleted the bZIP domain of Yap1 (GFP-Yap1^{ΔbZIP}) (Fig. 7A). Since the bZIP proteins usually interact physically with each other through the bZIP domain, such a bZIP domain-deleted Yap1 mutant is supposed to be difficult to approach closely, and therefore intermolecular disulfide bonds between cysteine residues, if any, seem less feasible. However, an intramolecular disulfide bond can be formed, and thus GFP-Yap1^{ΔbZIP} was concentrated in the nucleus following H₂O₂ treatment (Fig. 7B). We then substituted the cysteine residues of this bZIP domain-deleted Yap1 mutant protein so that it contained only one cysteine in the c-CRD (GFP-Yap1^{ΔbZIP 3Cys cm46}) (Fig. 7A). If the Yap1 NES was masked through an intermolecular disulfide bond formation between residual cysteine of GFP-Yap1^{ΔbZIP 3Cys cm46}, this reporter protein would be expected to concentrate in the nucleus following H₂O₂ treatment. However, this mutant Yap1 protein was not concentrated in the nucleus after H₂O₂ treatment, and there-

fore, the intermolecular disulfide bond is thought to be formed only slightly even under conditions of oxidative stress. On the other hand, intriguingly, this mutant protein was able to concentrate in the nucleus following treatment with MG (Fig. 7B). Collectively, our data indicate that any one of three cysteine residues in the c-CRD is sufficient for Yap1 activation of MG.

The redox status of Yap1 is not changed by MG. The data we have demonstrated thus far suggest that not only intramolecular but also intermolecular disulfide bond formation is less feasible in the regulatory mechanism of the nucleocytoplasmic localization of Yap1 by MG. Nevertheless, it remains possible that there are some MG sensor proteins in the cell, and that such proteins interact with cysteine residues in the c-CRD to mask the Yap1 NES and consequently block interaction with Crm1, leading to the nuclear localization of Yap1.

To address this possibility, we determined the biochemical changes in Yap1 following treatment with MG. Wild-type cells carrying HA-tagged Yap1 were treated with MG or H₂O₂, and cellular proteins were subjected to nonreducing SDS-PAGE. As shown in Fig. 8, the redox state of Yap1 was virtually unchanged by treatment with MG, whereas the oxidized form of Yap1 (disulfide bond between Cys303 in the n-CRD and Cys598 in the c-CRD) (11) emerged on H₂O₂ treatment. In addition, a band with low mobility on nonreducing SDS-PAGE appeared in the H₂O₂-treated sample, which is presumably the Yap1-Gpx3 complex (12). However, the low-mobility band was hardly detected in the MG-treated cells. Taken together, these results show that MG does not affect the redox state of Yap1. Furthermore, the possibility of the existence of an MG sensor protein(s), which masks the Yap1 NES to block the interaction with Crm1, can be excluded.

Physiological relevance of MG-dependent Yap1 activation. Although MG is a ubiquitous natural metabolite, it causes disorders of cellular functions due to its high reactivity. Glyoxalase I is the major enzyme responsible for MG detoxification; nevertheless, *GLO1* is not a target of Yap1 (26). However, as shown in Table 1, expression of *GRE2* coding for methylglyoxal reductase (8), an alternative enzyme for MG detoxification (27), was strongly induced in *glo1Δ* cells. Hence, yeast cells seem armed with a Yap1-dependent MG-metabolizing enzyme (Gre2) as a backup system to prepare for glyoxalase I deficiency.

To gain further insights into the physiological correlation between Yap1 activation and MG metabolism, we monitored cellular MG level with respect to cell growth. As shown in Fig. 9, the cellular MG level increased with cell growth and reached its maximum during the transition period from log phase to stationary phase, which is referred to as the diauxic shift. We examined the intracellular localization of Yap1 periodically. As expected, the proportion of cells with the nuclear Yap1 increased synchronously in accordance with the cellular MG level, reaching maximum when the cellular MG level was also maximum. The steady-state level of MG in *glo1Δ* cells in the log phase was comparable to that of the maximal level of wild-type cells in the diauxic shift. The cellular MG level decreased gradually thereafter, which may be due to the increased expression of *GLO1*, although the induction mechanism of *GLO1* expression in the diauxic shift has not yet been elucidated. Consequently, the proportion of cells with nuclear Yap1 decreased. Wiatrowski and Carlson (66) reported that

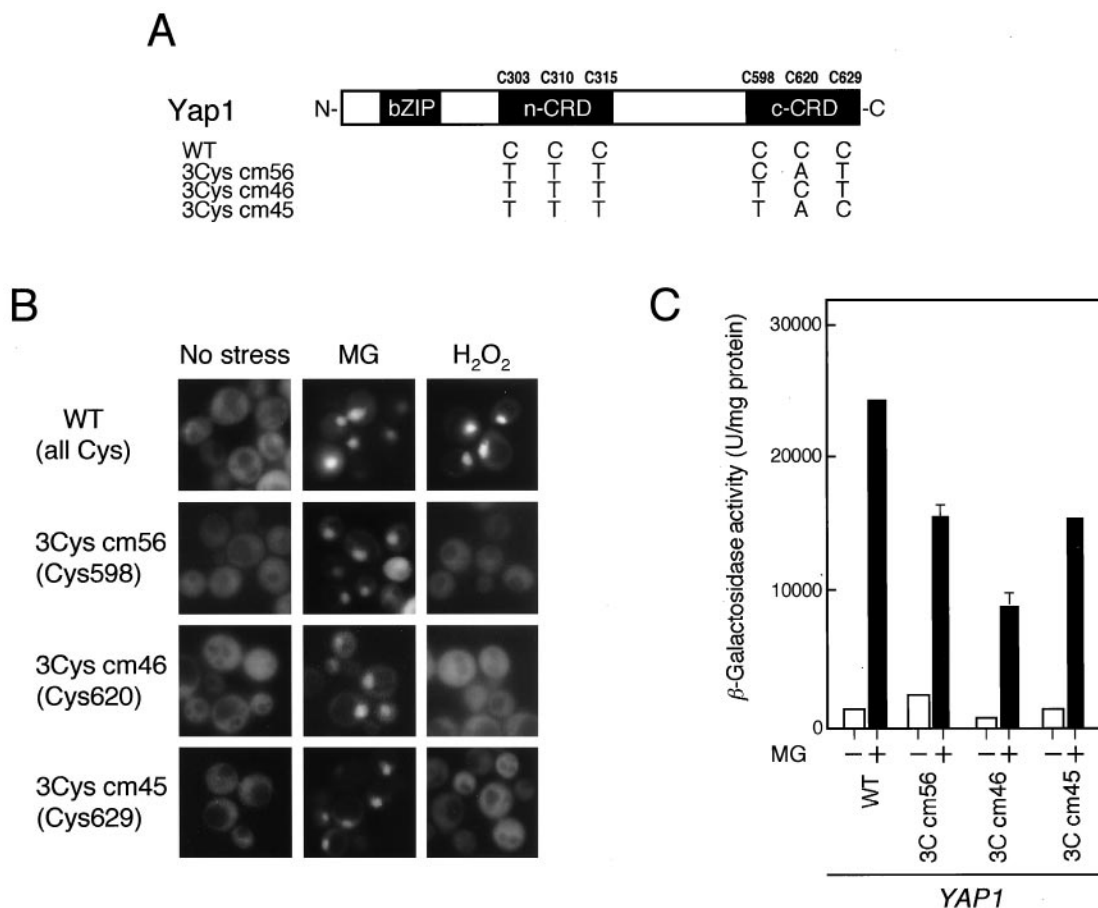


FIG. 6. Any one of three Cys residues in the c-CRD is sufficient for Yap1 activation by MG. (A) Schematic of the Yap1 mutant protein. (B) GFP-tagged Yap1 of the wild type (WT) and each mutant was expressed in *yap1Δ* cells. When the *A*₆₁₀ of the culture reached 0.5, 8 mM MG or 0.4 mM H₂O₂ was added to the culture medium, the cells were incubated for a further 60 min (MG) or 15 min (H₂O₂), and localization of GFP-Yap1 was then monitored. (C) Each GFP-tagged protein was also expressed in the *yap1Δ* mutant carrying the *FLR1-lacZ* reporter gene. After treatment with 8 mM MG for 3 h, the β-galactosidase activity was measured.

carbon deprivation caused the nuclear localization of Yap1; however, although the glucose in the medium was almost exhausted after the diauxic shift, cells with the nuclear Yap1 were not observed, as far as we have examined. Thus, the changes in the proportion of cells with the nuclear Yap1 are probably linked to the cellular MG level. We also monitored the localization of GFP-tagged Yap1^{3Cys cm46} and Yap1^{6Cys} mutants with respect to cell growth. Essentially, the result obtained with GFP-Yap1^{3Cys cm46} was the same as that obtained with GFP-Yap1^{WT}, whereas GFP-Yap1^{6Cys} was present in the cytoplasm throughout the growth phase (data not shown). Collectively, our data suggest that MG-dependent Yap1 activation occurs in yeast cells under normal growth conditions.

DISCUSSION

A sudden environmental change, such as in temperature, osmotic pressure, or redox conditions, induces the expression of a number of stress-responsive genes in both prokaryotes and eukaryotes, allowing the cells to become tolerant to these stress conditions. Each stress signal is sensed by a specific sensor molecule in the cells. In terms of oxidative-stress sens-

ing, *E. coli* has two distinct transcription factors, OxyR and SoxR, that act as sensors for reactive oxygen species (31, 70). The former has two critical cysteine residues (Cys199 and Cys208), which form a disulfide bond when cells are exposed to oxidative stress elicited by H₂O₂ (69). Glutaredoxin reduces the disulfide bond of OxyR to down-regulate its activity (69). On the other hand, SoxR has a [2Fe-2S] cluster to sense superoxide anion radicals (13). In both cases, the transcription factor serves as a sensor and transducer of oxidative stress. Importantly, the oxidized form is the active form of the transcriptional regulator. In contrast, Ref-1 and thioredoxin are required to reduce an intermolecular disulfide bond in the mammalian AP-1 DNA binding domain to allow activation of the expression of its target genes under oxidative-stress-inducing conditions (22). This indicates that the reduced form of AP-1 is the active form. Recently, we found that cysteine residues in the c-CRD function as a redox sensor for Yap1 (34). A disulfide bond is formed between Cys598 and Cys620 when cells are challenged by H₂O₂ stress. Delaunay et al. (11) reported that a disulfide bond between Cys303 and Cys598 is also formed on exposure to H₂O₂. These disulfide bonds are reduced by thioredoxin in vitro. Intramolecular disulfide bond

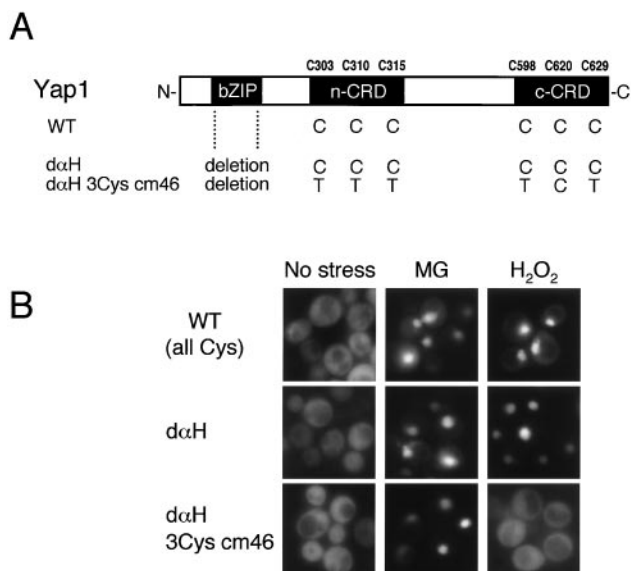


FIG. 7. No disulfide formation is involved in Yap1 activation by MG. (A) Schematic of the Yap1 mutant protein. (B) GFP-tagged Yap1 of the wild type (WT) and each mutant was expressed in *yap1Δ* cells. When the A_{610} of the culture reached 0.5, 8 mM MG or 0.4 mM H_2O_2 was added to the culture medium, cells were incubated for a further 60 min (MG) or 15 min (H_2O_2), and localization of GFP-Yap1 was then monitored.

formation in the c-CRD may mask the NES, which subsequently blocks the interaction between Yap1 and Crm1 to allow Yap1 to concentrate in the nucleus (34). Yan et al. (68) reported that the interaction between Yap1 and Crm1 is strengthened by dithiothreitol in vitro. Presumably, thioredoxin reduces the disulfide bond(s) of Yap1 in vivo, and so thioredoxin deficiency causes constitutive nuclear localization of Yap1 (30). In addition, we found that a deficiency of thi-

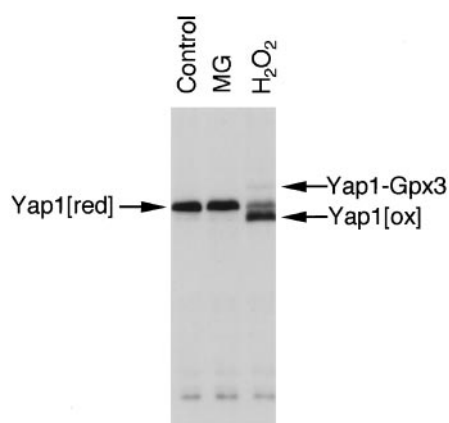


FIG. 8. Analysis of biochemical changes in Yap1. *yap1Δ* cells carrying HA-tagged Yap1 were cultured in SD medium to an A_{610} of 1.0 and then treated with 8 mM MG for 60 min or 0.4 mM H_2O_2 for 15 min. Cellular proteins were extracted as described in the text and subjected to nonreducing SDS-PAGE followed by Western blotting with anti-HA monoclonal antibody to detect HA-Yap1. The immunoreactive protein was detected using the ECL kit (Amersham Biosciences). The low-mobility band that appeared in H_2O_2 -treated samples was assumed to be a Yap1-Gpx3 complex.

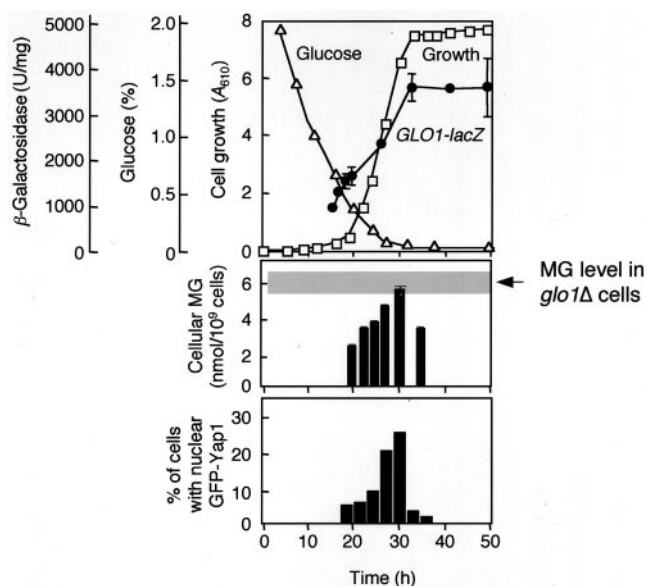


FIG. 9. Correlation between cell growth, cellular MG level, and Yap1 localization. Cells were cultured in SD minimal medium, and cell growth (A_{610}), glucose concentration in the medium, β -galactosidase activity driven by *GLO1-lacZ*, intracellular MG content, and subcellular localization of GFP-Yap1 were monitored periodically. The glucose concentration was determined using a kit (Glucose B-test Wako; Wako Pure Chemical Industries, Tokyo, Japan). The number of cells counted in each observation for localization of GFP-Yap1 was 500 to 1,000.

oredoxin reductase (*trr1Δ*) yielded the same phenotype in terms of the activation of Yap1 (5; S. Izawa and Y. Inoue, unpublished results). Taking into account the property of thioredoxin as a thiol-disulfide oxidoreductase, the oxidized form of Yap1 is the active form. Nevertheless, the present study has demonstrated that Yap1 activity seems to be regulated without redox modification in a *glo1Δ* mutant.

The intracellular MG content was increased in the *glo1Δ* mutant, although treatment of yeast cells with H_2O_2 did not increase the MG level (untreated, 2.14 ± 0.33 nmol/ 10^9 cells; treated, 2.17 ± 0.27 nmol/ 10^9 cells). Additionally, in a *gsh1Δ* mutant, intracellular oxidation levels were higher than those in the isogenic wild-type strain and basal expression levels of AP-1-*CYC1-lacZ* increased 3.5-fold; consequently, the majority of Yap1 was accumulated in the nucleus (data not shown), although the MG content was not increased (wild type, 2.60 ± 0.14 nmol/ 10^9 cells; *gsh1Δ*, 2.55 ± 0.06 nmol/ 10^9 cells), even though glutathione is required for the glyoxalase I reaction. Similarly, an increase in the MG content was not observed in a *trx1Δ trx2Δ* mutant (wild type, 2.60 ± 0.14 nmol/ 10^9 cells; *trx1Δ trx2Δ*, 2.70 ± 0.19 nmol/ 10^9 cells). These results, together with the fact that overexpression of *TRX1* and *TRX2* did not reverse the activation of Yap1 in the *glo1Δ* mutant (data not shown), suggest that there is no correlation between intracellular redox balance and the MG level in yeast cells.

We have demonstrated that (i) any one of the three cysteine residue(s) is sufficient for Yap1 to translocate into the nucleus and activate the expression of its target gene following MG treatment, (ii) the redox state of Yap1 per se is virtually unchanged with MG treatment, and (iii) the possibility of the

existence of an MG sensor protein(s) that competes with Crm1 for binding to the Yap1 NES can be excluded. These observations lead us to propose that Yap1 may be a direct target of MG.

The occurrence of several adducts has been detected when MG and *N*-acetyl-L-arginine or *N*-acetyl-L-lysine are incubated in vitro (47). The c-CRD/NES of Yap1 contains two arginine residues (Arg604 and Arg632) and three lysine residues (Lys610, Lys626, and Lys628). Since we have demonstrated that cysteine-substituted mutant proteins GFP-Yap1^{cm46A5} and GFP-Yap1^{6Cys} were not able to locate to the nucleus and activate the expression of Yap1 target genes (Fig. 4), these arginine and/or lysine residues proximal to the cysteine residues in the c-CRD are not likely to be involved in the trafficking regulation of Yap1 by MG. We do not rule out the possibility of the modification of these amino acids by MG in vivo. Nevertheless, such modifications, if any, are less feasible as contributing at least to the MG-dependent localization of Yap1.

The most important aspect of Yap1 activation by MG is that it is a reversible reaction (Fig. 3). Lo et al. (39) reported that MG can form an adduct with *N*-acetyl-L-cysteine. However, Oya et al. (47) reported that 78% of the arginine and 27% of the lysine residues were lost if bovine serum albumin was incubated with high concentrations of MG for 24 h but that the levels of other amino acid residues were not changed. Therefore, arginine and lysine residues in protein may be preferentially modified by MG compared to cysteine residues, and modification of cysteine residues by MG may be labile and/or reversible. The chemical reaction between a thiol group and aldehyde at physiological pH yields hemithioacetal (62). For example, MG nonenzymatically condenses with glutathione to form hemithioacetal reversibly ($K_d = 1 \times 10^{-3}$ M) (60). One of the possible models that may account for the reversible activation of Yap1 by MG is that cysteine residues in the c-CRD of Yap1 may be modified by MG to form some adducts, presumably hemithioacetal, which may block the interaction between Yap1 and Crm1 which results in Yap1 concentrating in the nucleus. However, an in vivo adduct derived from MG and the cysteine residue of proteins has not yet been detected owing to its unstable and reversible nature (39).

Recently, Azevedo et al. reported that cysteine residues in the c-CRD can be modified by a cysteine-modifying chemical, *N*-ethylmaleimide, that allows Yap1 to accumulate in the nucleus (2). However, this is not surprising when the nature of the chemical (cysteine modifier) is considered. Furthermore, such a chemical is xenobiotic and not a natural metabolite. In addition to the reversibility of Yap1 activation by MG, attention should be focused on the fact that MG is a natural metabolite synthesized by the ubiquitous energy-generating system, glycolysis.

To explore the physiological relevance of MG-dependent Yap1 activation under normal yeast growth conditions, we monitored MG level with respect to cell growth. The cellular MG level reached maximum during the diauxic shift, and the proportion of cells with the nuclear Yap1 rose concomitantly with the increase in the cellular MG level (Fig. 9). The steady-state level of MG in the log phase for the *glo1Δ* mutant, in which cell Yap1 is constitutively concentrated in the nucleus, was almost comparable to that for wild-type cells during the

diauxic shift (Fig. 9). Energy metabolism markedly changes before and after the diauxic shift; i.e., ATP production depends largely on glycolysis before the shift (fermentative growth), whereas it depends on oxidative phosphorylation in mitochondria during a postdiauxic shift (respiratory growth) (17). Additionally, the production of several stress proteins increases after the diauxic shift, and thus cells in the stationary phase usually exhibit higher tolerance to several environmental stresses (21). Yap1 is one of the transcription factors responsible for the stress response in yeast.

The alterations in protein function due to the posttranslational modification can be provoked by phosphorylation, redox regulation (disulfide bond formation), glutathionylation, ADP-ribosylation, acetylation, and so on (16, 18, 41, 48, 59). To our knowledge, this is the first report presenting the possibility of the modulation of a transcription factor function due to potential modification by MG. This study provides new insights into the processes underlying the activation of Yap1. Importantly, intracellular MG levels vary depending on the growth phase, and therefore, the metabolic competence of cells seems to be a factor in this regulatory system. Considering that MG is derived from glycolysis, which is the root of metabolism, we would hypothesize that MG may function as a metabolic signaling molecule.

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