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# Methylglyoxal accumulation by glutathione depletion leads to cell cycle arrest in *Dictyostelium*

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## Summary

**Reduced glutathione (GSH) serves as a primary redox buffer and its depletion causes growth inhibition or apoptosis in many organisms. In *Dictyostelium discoideum*, the null mutant (*gcsA*$^-$) of *gcsA* encoding γ-glutamylcysteine synthetase shows growth arrest and developmental defect when GSH is depleted. To investigate the mechanism by which GSH depletion induces growth arrest, a proteomic analysis was performed and aldose reductase (AlrA) was identified as the most prominently induced protein in *gcsA*$^-$ cells. Induction of AlrA was dependent on GSH concentration and was repressed by GSH but not effectively by either the reducing agent such as dithiothreitol or overexpression of superoxide dismutase. Methylglyoxal (MG), a toxic α-ketoaldehyde, strongly induced *alrA* expression and AlrA catalysed MG reduction efficiently. The *alrA* knockdown *gcsA*$^-$ cells (*gcsA*$^-$/*alrA*$^{as}$) exhibited more decreased growth rate than *gcsA*$^-$ cells, whereas the *gcsA*$^-$ cells overexpressing *alrA* (*gcsA*$^-$/*alrA*$^{oe}$) showed the recovery of growth rate. Interestingly, intracellular MG levels were significantly augmented in *gcsA*$^-$/*alrA*$^{as}$ cells compared with *gcsA*$^-$ cells following GSH depletion. By contrast, *gcsA*$^-$/*alrA*$^{oe}$ cells showed repression of MG induction. Furthermore, MG treatment inhibited growth of wild-type KAx3 cells, inducing G1 phase arrest. Thus, our findings suggest that MG accumulated by GSH depletion inhibits cell growth in *Dictyostelium*.**

## Introduction

Reduced glutathione (GSH), a tripeptide composed of glutamate, cysteine and glycine, participates as an electron donor in a number of reactions in eukaryotic and some prokaryotic cells. The high concentration and high GSH/oxidized GSH (GS-SG) ratio, maintained by glutathione reductase and NADPH, reflect that GSH functions as a cellular redox buffer. The depletion of GSH alters redox balance, resulting in an increase in the level of reactive oxygen species (ROS), which are implicated in ageing and neurodegenerative disease, such as Parkinson's disease (Kokoszka *et al.*, 2001; Zhou and Freed, 2005). In addition, GSH depletion leads to the growth arrest (Wanke *et al.*, 1999; Kim *et al.*, 2005) and apoptosis (Madeo *et al.*, 1999; Baek *et al.*, 2004) in some microorganisms, which have been widely used for model systems for study of GSH in mammalian cells. GSH contributes to the elimination of toxic compounds; peroxides with glutathione peroxidase (Avery and Avery, 2001), xenobiotics with glutathione-S transferase (Vuilleumier and Pagni, 2002) and α-ketoaldehydes with glyoxalase system (Thornalley, 1998).

Methylglyoxal (MG) is an endogenous toxic α-ketoaldehyde produced by non-enzymatic elimination of phosphate from glycolytic pathway intermediates. MG modifies DNA and proteins, thereby inhibiting cell proliferation (Kang *et al.*, 1996) or inducing apoptosis (Fukunaga *et al.*, 2005). MG is a major glycation agent to form advanced glycation end-product (AGE), which has been implicated in diabetic complications (Yim *et al.*, 2001; Fukunaga *et al.*, 2005). MG detoxification is mainly accomplished by glyoxalase system consisting of glyoxalase I and II, which catalyses the conversion of MG to lactic acid using GSH as a cofactor. Overexpression of glyoxalase system in tobacco leads to an increase in tolerance against MG (Singla-Pareek *et al.*, 2003).

In addition to glyoxalase system, it has been reported that aldose reductases take part in the MG metabolism in yeast and other microorganisms (Gomes *et al.*, 2005; Ko *et al.*, 2005; Xu *et al.*, 2006). Aldose reductase is a member of aldo-keto reductase superfamily and catalyses the reduction of various aldehydes with NADPH. Aldose reductase can convert MG to acetol (Ko *et al.*, 2005). In these systems, aldose reductase inhibits MG-mediated AGE formation (Gomes *et al.*, 2005) and its null mutant shows the accumulation of MG (Xu *et al.*, 2006) and enhanced susceptibility to MG (Ko *et al.*, 2005), indicating that aldose reductase plays a role in MG detoxification.

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*Dictyostelium discoideum* is a soil-living amoeba, feeding on bacteria. When food source is available, amoebae multiply by binary fission. Upon starvation, however, they aggregate toward cAMP pulse and finally form a fruiting body consisting of a stalk and spores (Firtel, 1996; Parent and Devreotes., 1996; Thomason *et al*., 1999). During growth in axenic culture, glucose is the major carbon source and appears to be metabolized through a glycolysis pathway, which is well conserved in *D. discoideum* (Eichinger *et al*., 2005).

Recently, we have shown that redox homeostasis is important for the growth and development of *D. discoideum* (Kim *et al*., 2005; Choi *et al*., 2006; Jeong *et al*., 2006). The null mutant (*gcsA*$^-$) of *gcsA* gene encoding γ-glutamylcysteine synthetase, a rate-limiting enzyme for GSH synthesis exhibits severe defects in the normal growth and differentiation into prespore cells when GSH is depleted (Kim *et al*., 2005). In particular, a long-term depletion of GSH induces the cell cycle arrest at G1 phase (Kim *et al*., 2005). Although deficiency of GSH has been reported to affect cell cycle (Russo *et al*., 1995; Vernoux *et al*., 2000), the precise mechanism is poorly understood. In the present work, we describe the role of aldose reductase 1 (AlrA), which is highly induced in *gcsA*$^-$ cells lacking GSH. AlrA was induced by MG and effectively used MG as a substrate. The knockdown of AlrA led to a detrimental effect on the growth of *gcsA*$^-$ cells by increasing the levels of MG. Conversely, overexpression of AlrA restored the growth of *gcsA*$^-$ cells, suppressing the increase of MG levels. Taken together, this study provides evidence that MG is an endogenous growth inhibition factor in *Dictyostelium*.

## Results

### *AlrA is a protein highly upregulated in GSH-deficient cells*

Previously, we reported that GSH is essential for normal growth of *Dictyostelium* cells (Kim *et al*., 2005). The growth rate of *gcsA*$^-$ cells, which is unable to synthesize GSH, decreases as GSH concentrations in media decreases and is completely halted by depletion of GSH as described by Kim *et al*. (2005) (Fig. 1). To address why GSH depletion induces growth arrest of *Dictyostelium*, we first performed a proteomic analysis to see the difference in the protein expression patterns between parental KAx3 cells and *gcsA*$^-$ cells lacking GSH. When proteins were separated on two dimensional (2D) gels, we found many proteins that were differentially expressed in *gcsA*$^-$ cells as compared with KAx3 cells (Fig. S1). We were especially interested in the protein spots upregulated by depletion of GSH and analysed them using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. We identified 10 proteins and presented in Table S1. Among them, two glutathione-S transferases (GST) and peroxiredoxin seems to play a role in the defence against oxidative stress, possibly invoked by GSH depletion. Likewise, elongation factor 1b has a GST domain, implying the similar role with GST protein. In addition, *S*-adenosylmethionine-dependent methyltransferase, ATP synthase β-chain and two unknown proteins were identified as proteins induced by GSH depletion.

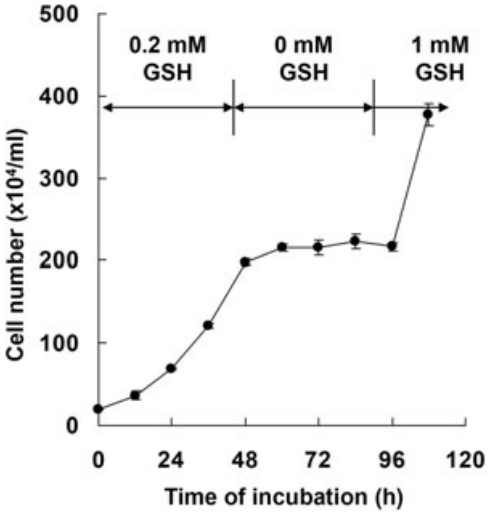


**Fig. 1.** Growth curve of *gcsA*$^-$ cells supplemented with different concentrations of GSH. *gcsA*$^-$ cells cultured with 1 mM GSH were diluted at $2 \times 10^5$ cells ml$^{-1}$ in HL5 medium with 0.2 mM GSH. After 2 days, the cells were washed and re-suspended in HL5 without GSH. Finally, 1 mM GSH was added to the medium after 4 days. Cells were counted using a haemacytometer. Each point represents the average (±standard deviation) of three independent experiments.

Interestingly, AlrA was prominently induced in *gcsA*$^-$ cells when GSH was depleted (Fig. 2A). Aldose reductase belongs to aldo/keto reductase superfamily and reduces various aldehydes to the corresponding alcohols. There are six aldose reductase genes on the *Dictyostelium* genome. To confirm the data from the 2D gel, we analysed transcript of *alrA* encoding AlrA. Northern blot analysis revealed that *alrA* level was elevated by GSH depletion whereas *alrB* was not (Fig. 2B). Interestingly, *alrA* level was inversely proportional to GSH concentrations in the media. Consistently, the aldose reductase activity in *gcsA*$^-$ cells deprived of GSH was about threefold higher than that of *gcsA*$^-$ cells cultured with 1 mM GSH (Fig. 2C). As a decrease in GSH level in the media results in a decrease in GSH level in *gcsA*$^-$ cells (Kim *et al*., 2005), these data indicate that a decrease in intracellular GSH level may be involved in *alrA* induction.

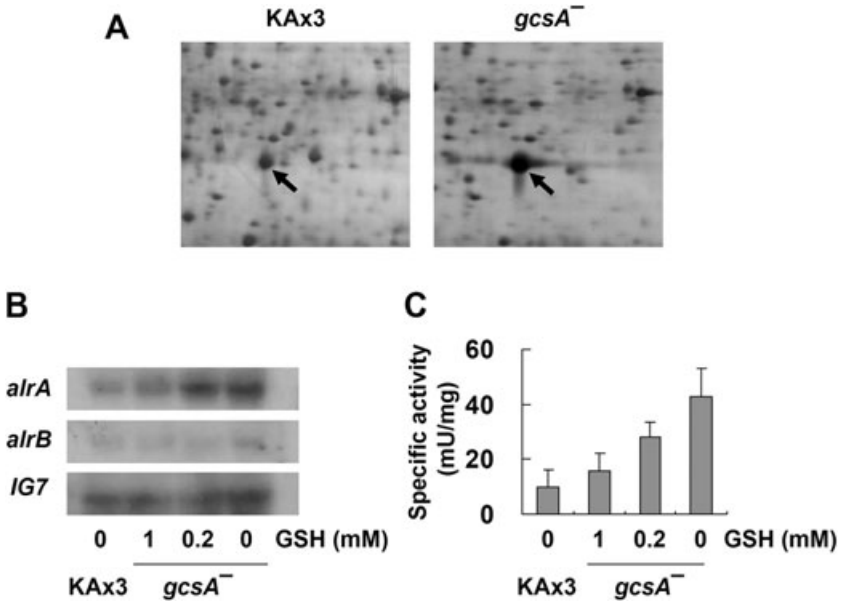


**Fig. 2.** a*lrA* is induced by GSH depletion.
A. Close-up image of 2D gel demonstrating the difference in AlrA levels.
B. Northern blot analysis. Total RNA was extracted from *gcsA*⁻ cells in the media supplemented with the indicated GSH concentrations and was hybridized with *alrA*- and *alrB*-specific probes. IG7 is shown as a reference.
C. Specific activity of aldose reductase was determined using D,L-glyceraldehyde as a substrate in *gcsA*⁻ cells prepared under the same condition stated above (See *Experimental procedures*). The values represent the average (±standard deviation) of three independent experiments.

*Induction of* alrA *is directly correlated with GSH depletion*

As GSH is a major antioxidant, it is likely that GSH depletion in *gcsA*⁻ cells induces an increase in ROS level and *alrA* expression. To test whether ROS is an inducer of *alrA* expression in *gcsA*⁻ cells, we analysed the transcript of *alrA* in *gcsA*⁻ cells cultured with reducing agents such as *N*-acetylcysteine (NAC), dithiothreitol (DTT) and GSH. The *gcsA*⁻ cells precultured with 1 mM GSH were washed with fresh media and then were re-suspended in the media supplemented with 1 mM DTT, 1 mM NAC and 1 mM GSH respectively. Northern blot analysis revealed that the level of *alrA* transcript in *gcsA*⁻ cells cultured with 1 mM GSH was 27% of that in *gcsA*⁻ cells with 0 mM GSH (Fig. 3A). In the cells cultured with 1 mM DTT or 1 mM NAC, *alrA* levels were 70% and 51%, respectively, compared with the *alrA* level in *gcsA*⁻ cells with 0 mM GSH (Fig. 3A), suggesting that the reducing agents such as NAC and DTT are not as effective as GSH in repressing *alrA* induction by GSH depletion in *gcsA*⁻ cells.

To evaluate whether ROS causes the induction of *alrA* in *gcsA*⁻ cells, we generated the *gcsA*⁻ cells mutant overexpressing Cu–Zn superoxide dismutase (SodA) (Fig. 3B). It is likely that the overexpression of SodA decreases superoxide level and increase hydroperoxide. However, because catalases that are abundant in *Dictyostelium* can remove the peroxides effectively, it is expected that an increase in overall ROS level after GSH depletion is suppressed by the overexpression of SodA.

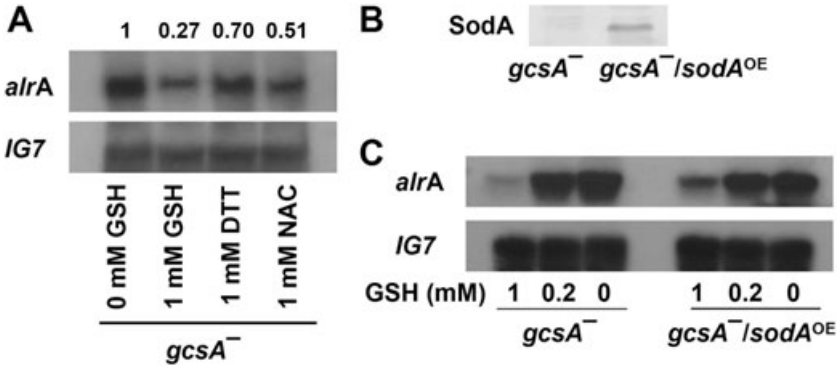


**Fig. 3.** ROS is not involved in *alrA* induction by GSH depletion.
A. *gcsA*⁻ cells exponentially cultured with 1 mM GSH were reinoculated in the media supplemented with 0 mM GSH, 1 mM GSH, 1 mM DTT and 1 mM NAC and then were cultured for 36 h. DTT, dithiothreitol; NAC, *N*-acetylcysteine. IG7 is used as a reference. Numbers indicate relative level of *alrA* normalized against IG7, which is quantified by densitometry using ImageJ.
B. Expression of SodA tagged with FLAG in *gcsA*⁻ cells was confirmed by Western blot analysis using anti-FLAG antibodies.
C. Total RNA was extracted from the *gcsA*⁻ cells and *gcsA*⁻/*sodA*$^{OE}$ cells cultured with the indicated concentrations of GSH. Transcript level of *alrA* was determined by Northern blot analysis. IG7 is shown as a reference.

 

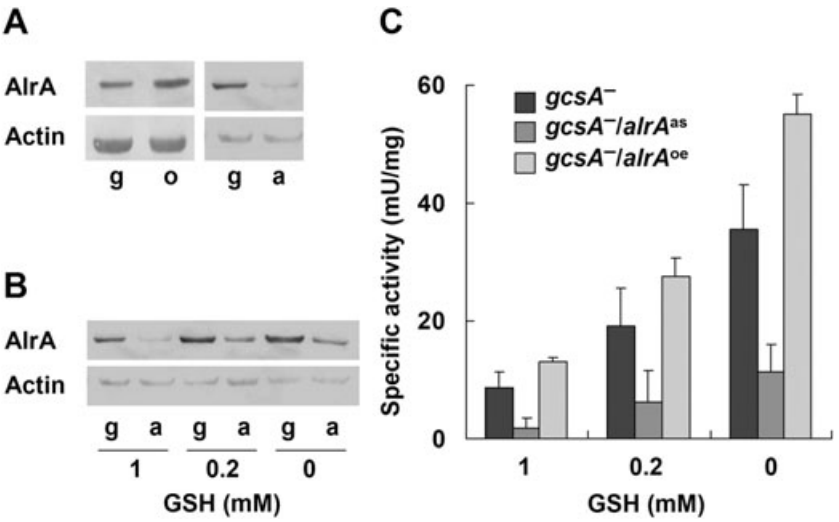


**Fig. 4.** Overexpression and knockdown of *alrA* in *gcsA*⁻ cells.
A. Overexpression and knockdown of *alrA* were confirmed by Western blot analysis using anti-AlrA antibody. Actin is shown as a loading control. g, *gcsA*⁻ cells; o, *gcsA*⁻/*alrA*$^{oe}$ cells; a, *gcsA*⁻/*alrA*$^{as}$ cells. The each Western blot is a separate experiment.
B. AlrA expression was repressed in *gcsA*⁻/*alrA*$^{as}$ cells during GSH depletion. Cells cultured with the indicated concentrations of GSH were harvested and cell lysates were prepared for Western blot analysis using with anti-AlrA antibody.
C. Specific activity of aldose reductase was measured in *gcsA*⁻, *gcsA*⁻/*alrA*$^{oe}$ and *gcsA*⁻/*alrA*$^{as}$ cells cultured with the indicated concentrations of GSH. The values represent the average (±standard deviation) of three independent experiments.

Activity staining on the native gel confirmed that FLAG-tagged SodA is active in *gcsA*⁻ cells (data not shown). Northern blot analysis showed that in *gcsA*⁻ cells overexpressing SodA, *alrA* level increased by GSH depletion like the parental *gcsA*⁻ cells (Fig. 3C). When thioredoxin, a small antioxidant protein was overexpressed in *gcsA*⁻ cells, there was also little difference in *alrA* induction by GSH depletion (data not shown). Thus, these data suggest that the induction of *alrA* expression is directly correlated with GSH depletion, but not with ROS.

### *Overexpression or repression of AlrA affects the growth of* gcsA⁻ *cells*

To find out the role of AlrA in *gcsA*⁻ cells lacking GSH, *alrA* was overexpressed using actin promoter (Fig. 4A) or underexpressed by antisense expression in *gcsA*⁻ cells (Fig. 4B). When GSH was depleted, Western blot showed that AlrA was more induced in *alrA*-overexpressing cells (*gcsA*⁻/*alrA*$^{oe}$) and was strongly repressed in *alrA*-knockdown cells (*gcsA*⁻/*alrA*$^{as}$) compared with the parental *gcsA*⁻ cells (Fig. 4A and B). In accordance with the protein level, enzyme activity increased by about 50% in *gcsA*⁻/*alrA*$^{oe}$ and decreased by about 70% in *gcsA*⁻/*alrA*$^{as}$ as compared with the parental *gcsA*⁻ cells (Fig. 4C).

Next, we examined the effect of overexpression and knockdown of AlrA on the growth of *gcsA*⁻ cells. In the media containing 1 mM GSH, the growth rates of *gcsA*⁻, *gcsA*⁻/*alrA*$^{oe}$ and *gcsA*⁻/*alrA*$^{as}$ cells were indistinguishable from that of wild-type KAx3 cells (data not shown). As GSH concentration decreased, the growth rate of *gcsA*⁻ cells decreased whereas the growth rate of *gcsA*⁻/*alrA*$^{oe}$ cells was comparable to that of wild-type KAx3 cells even at 0.2 mM GSH, suggesting AlrA induction might rescue the defective growth of *gcsA*⁻ cells. By contrast, the growth of *gcsA*⁻/*alrA*$^{as}$ cells was severely inhibited and finally they stopped growth at the relatively low cell density (Fig. 5A). We previously reported that the cell cycle of *gcsA*⁻ cells is arrested at G1 phase when GSH is depleted (Kim *et al*., 2005). To see the effect of *alrA* knockdown on cell cycle progress, we prepared *gcsA*⁻ and *gcsA*⁻/*alrA*$^{as}$ cells cultured with 1 mM GSH or without GSH and analysed the DNA content by flow cytometry. It has been well established that vegetative cells of *Dictyostelium* are mainly in G2 phase of cell cycle and spore cells are in G1 phase (Chen *et al*., 2004) (Fig. 5B). The depletion of GSH increased the population of *gcsA*⁻ cells at G1 phase from 9.9% to 84.3% (Fig. 5B). And 98.4% of *gcsA*⁻/*alrA*$^{as}$ cells remained in G1 phase after GSH depletion, indicating that *alrA* knockdown in *gcsA*⁻ cells may stimulate the cell cycle arrest by GSH depletion. One concern in interpreting this experiment is that during axenic growth in liquid media, up to 40% of the cells have more than two nuclei due to a delay in cytokinesis after karyokinesis (Zada-Hames and Ashworth, 1978). We considered the possibility therefore that the reduction in DNA content observed upon GSH depletion might reflect a decrease in the proportion of binucleated cells in the culture. To address this possibility, we determined the number of nuclei per cell in GSH replete and depleted cultures, using DAPI (4′,6-diamidino-2-phenylindole) staining. We found there was little change in the number of nuclei per cell for KAx3, *gcsA*⁻ or *gcsA*⁻/*alrA*$^{as}$ cells cultured under GSH replete or GSH-depleted conditions (data not shown). These data suggest strongly that GSH depletion leads to a G1 arrest rather that having any effect on cytokinesis.

In *Dictyostelium*, it was shown that *alrA* gene is regulated by counting factor (CF) and *alrA* knockout cells form huge aggregates during early development because of reduced cell motility (Ehrenman *et al*., 2004). We tested

 

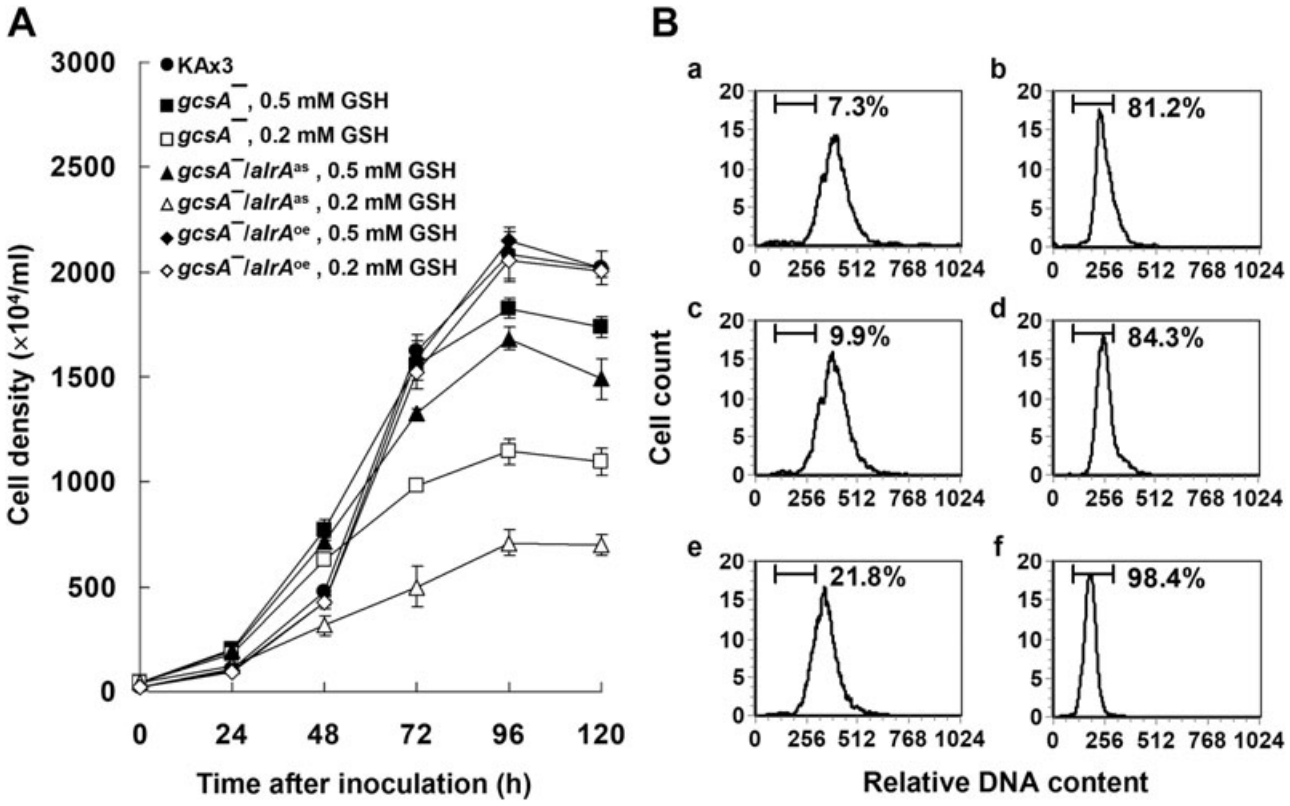


**Fig. 5.** Growth of *gcsA*⁻, *gcsA*⁻/*alrA*$^{oe}$ and *gcsA*⁻/*alrA*$^{as}$ cells.
A. Growth curves. Wild-type KAx3 (closed circle), *gcsA*⁻ (squares), *gcsA*⁻/*alrA*$^{oe}$ (diamonds) and *gcsA*⁻/*alrA*$^{as}$ cells (triangles) were inoculated at a density of $2 \times 10^5$ cells ml$^{-1}$ in the HL5 media respectively. The media were supplemented with 0.2 mM GSH (open symbols) and 0.5 mM GSH (closed symbols). Cells were counted using a haemacytometer at the indicated times (h). Each point represents the average (±standard deviation) of three independent experiments.
B. Cell cycle analysis. Vegetative cells cultured with different GSH concentrations were harvested and fixed with ethanol. DNA content of cells was analysed by flow cytometry (see *Experimental procedures*). Data are representatives of three independent experiments. Spore cells are used for G1 control. Numbers indicate the percentage of G1 phase cells. a. KAx3 cells; b. KAx3 spores; c. *gcsA*⁻ cells cultured with 1 mM GSH; d. *gcsA*⁻ cells deprived of GSH; e. *gcsA*⁻/*alrA*$^{as}$ cells cultured with 1 mM GSH; f. *gcsA*⁻/*alrA*$^{as}$ cells deprived of GSH.

the development of *gcsA*⁻ cells and *gcsA*⁻/*alrA*$^{as}$ cells, but there was no significant difference between two strains (data not shown); the *alrA* knockdown in *gcsA*⁻ cells did not affect the group size and *gcsA*⁻/*alrA*$^{as}$ cells were arrested at the aggregation stage like *gcsA*⁻ cells (Kim *et al*., 2005), although they showed a slightly delayed stream formation.

### *Methylglyoxal is an inducer of* alrA *expression*

To understand why *alrA* is induced by GSH depletion, we challenged wild-type KAx3 cells with various compounds depleting intracellular GSH. Total RNAs were extracted after 3 h of treatment and were analysed by Northern blot. As shown in Fig. 6A, *alrA* expression was induced by menadione and paraquat, which are known to generate superoxide radical anion via redox cycling (Comporti, 1989), but not by $H_2O_2$ and diamide, a thiol-oxidizing agent (O'Brien *et al*., 1970). Several reports have shown that aldose reductase is induced by MG, its substrate, and malonaldehyde, a product of lipid peroxidation (Koh *et al*., 2000; Chang *et al*., 2002). We observed that *alrA* expression was induced by MG, but not by malonaldehyde (Fig. 6A). As MG generates free radicals on binding proteins (Lee *et al*., 1998), it is likely that the induction of *alrA* may be due to free radicals generated by MG. The time-course of *alrA* induction after the treatment of menadione and MG showed that *alrA* increased rapidly after 2 h of treatment of both reagents, whereas *sodA* gene was induced by menadione, but by MG (Fig. 6B). Thus, *alrA* induction may be not due to ROS, which is consistent with the result that SodA overexpression failed to repress induction of *alrA* by GSH depletion (Fig. 3C). To address why *alrA* is induced by menadione and paraquat, we measured intracellular MG level in the wild-type KAx3 cells challenged by these reagents. MG were derivatized into 2-methylquinoxaline and its concentration was determined using reverse-phase high-performance liquid chromatography (HPLC). Interestingly, MG level was elevated in the cells treated with menadione and paraquat, but not

 

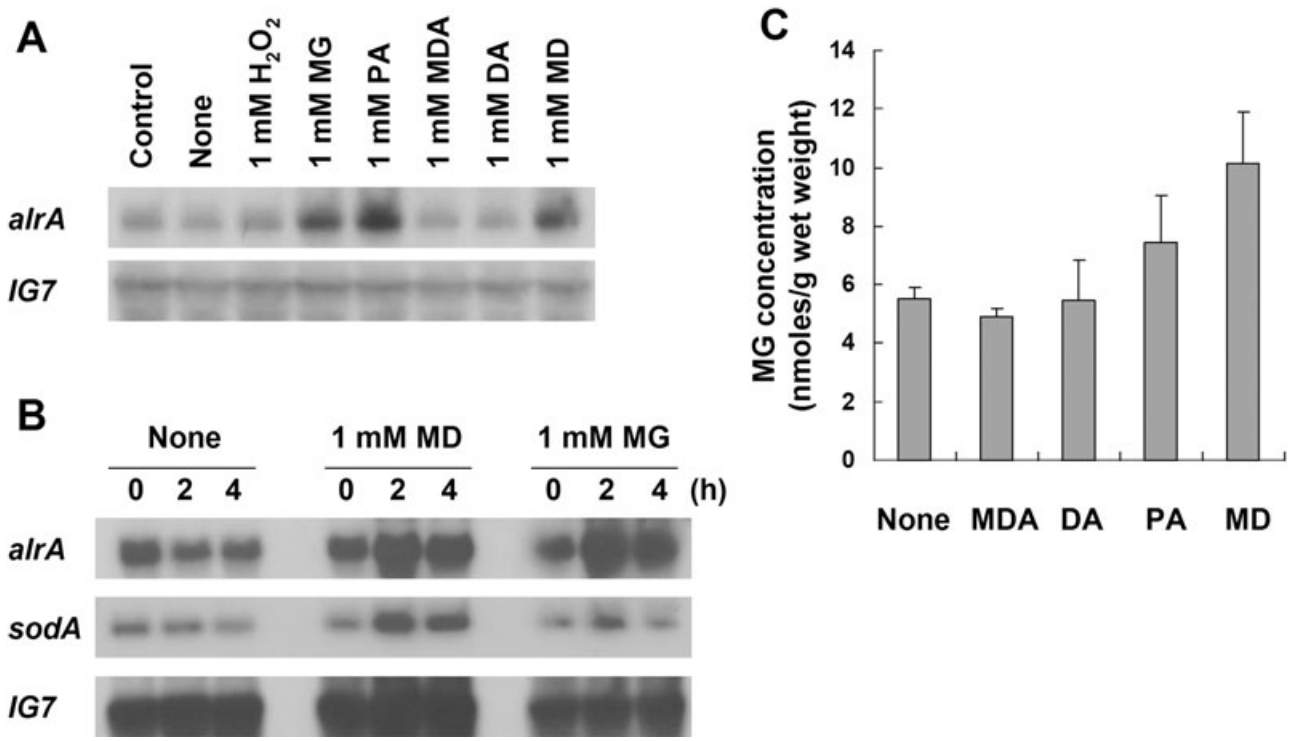


**Fig. 6.** Induction of *alrA* in response to various kinds of stress.
A. Northern blot analysis of *alrA* induction. Wild-type KAx3 cells were incubated with the indicated chemicals for 3 h and total RNA was extracted from the cells. MG, methylglyoxal; MDA, malonaldehyde; MD, menadione. IG7 is shown as a reference.
B. Time-course of *alrA* induction by menadione and MG. KAx3 cells were harvested at the indicated times (h) after treatment and total RNA was analysed by Northern blot using *alrA* and *sodA* as probes. IG7 is shown as a reference.
C. Determination of MG level after treatment of MDA, DA, PA and MD. Wild type KAx3 cells were treated with 1 mM MDA, DA, PA and MD for 3 h and then collected by centrifugation. Then, MG was extracted with 0.5 M perchloric acid and quinoxaline derivative concentration was determined by reverse HLPC (see *Experimental procedures*). The values represent the average (±standard deviation) of three independent experiments.

with malonaldehyde and diamide (Fig. 6C). Thus, these data suggest that MG may play a role as an inducer of *alrA* expression in *Dictyostelium*.

### *Accumulation of methylglyoxal by GSH depletion results in growth arrest*

It has been known that GSH participates in removal of toxic α-ketoaldehydes such as MG; it serves as a cofactor of glyoxalase system that can convert MG to lactic acid (Thornalley, 1998). Thus, it is possible that GSH depletion may lead to accumulation of MG. Our result that MG treatment strongly induced *alrA* expression in *Dictyostelium* (Fig. 6A) prompted us to examine a difference in intracellular MG levels between the mutants cultured with the different concentration of GSH. In *gcsA*$^-$ cells, MG level gradually increased as GSH concentration decreased and dropped slightly at 24 h after GSH depletion (Fig. 7A). Surprisingly, *gcsA*$^-$/*alrA*$^{as}$ cells had above 20% higher MG level at all different concentrations of GSH and MG level dramatically augmented at 24 h after GSH depletion (Fig. 7A). By contrast, MG level in *gcsA*$^-$/*alrA*$^{oe}$ decreased rather than increased despite a decrease in GSH concentration (Fig. 7A). These data suggest that depletion of GSH in cells induces MG accumulation and AlrA plays an important role in regulating intracellular MG level. Actually, when we tested the enzyme activity using recombinant AlrA, MG showed threefold lower $K_m$ and twofold higher $k_{cat}$ than D,L-glyceraldehydes, a substrate normally used for aldose reductase activity assay (Table 1), suggesting that AlrA can catalyse MG very efficiently. Our hypothesis that AlrA detoxifies intracellular MG is also sup-

**Table 1.** Kinetic parameters of AlrA from *Dictyostelium*.

| Substrates | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) |
|---|---|---|---|
| Methylglyoxal | 51.1 ± 4.8 | 167.3 ± 9.5 | $3.28 \times 10^6$ |
| D,L-Glyceraldehyde | 163.0 ± 5.6 | 71.7 ± 1.5 | $4.40 \times 10^5$ |

AlrA was incubated with concentrations of substrate ranging from 25 to 100 μM in 0.1 M sodium phosphate buffer, pH 6.2, containing 0.15 mM NADPH at 30°C. The numbers represent the average of at least three independent measurements.

 

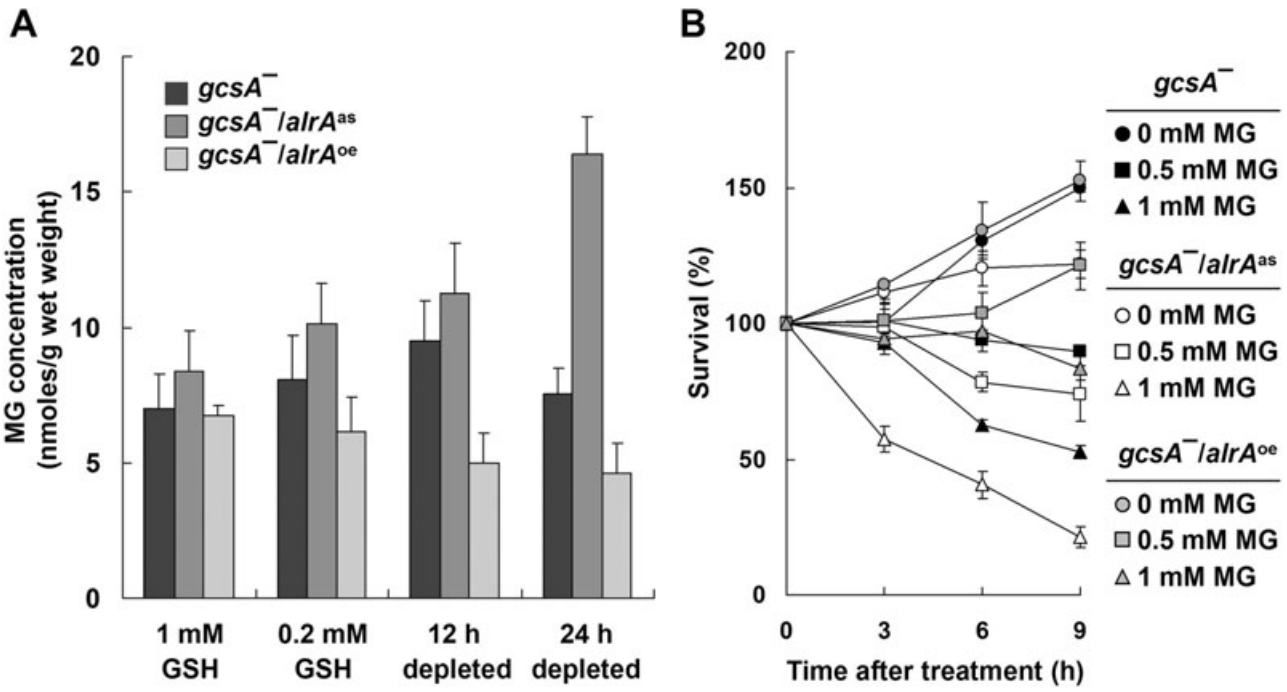


**Fig. 7.** Role of AlrA in MG detoxification.
A. MG levels in the *gcsA*⁻, *gcsA*⁻/*alrA*$^{oe}$ and *gcsA*⁻/*alrA*$^{as}$ cells. Cells were cultured with 1 mM or 0.2 mM GSH. were inoculated in the media with 1 mM GSH or without GSH. After 1 day culture, cells were harvested and MG levels were measured (see *Experimental procedures*). The values represent the average (±standard deviation) of three independent experiments.
B. Survival test against external MG. *gcsA*⁻ (closed black symbols), *gcsA*⁻/*alrA*$^{oe}$ (closed grey symbols) and *gcsA*⁻/*alrA*$^{as}$ cells (open symbols) were cultured with 0.2 mM GSH. The exponentially growing cells were treated with 0 mM MG (circles), 0.5 mM MG (squares) or 1 mM MG (triangles) respectively. Dead cells were discriminated using 0.01% phloxine B. Each point represents the average (±standard deviation) of three independent experiments.

ported by the result that *gcsA*⁻/*alrA*$^{as}$ cells were more sensitive to toxicity of MG than the parental *gcsA*⁻ cells, whereas *gcsA*⁻/*alrA*$^{oe}$ cells were more resistant to MG (Fig. 7B).

Methylglyoxal has been known to be an endogenous metabolite inhibiting cell growth (Szent-Györgyi *et al*., 1967; Kang *et al*., 1996). To confirm whether the accumulation of MG inhibits growth of *Dictyostelium*, we cultured wild-type KAx3 and *alrA*-knockdown KAx3 cells (KAx3/*alrA*$^{as}$) in the media supplemented with different concentrations of MG. Interestingly, KAx3 cells cultured with 0.2 mM MG showed prolonged lag phase (Fig. 8A), indicating that MG has the ability to retard cell growth of *Dictyostelium*. After 1 day of treatment with 0.5 mM MG, most cells died. Moreover, KAx3/*alrA*$^{as}$ cells showed more delayed growth (Fig. 8A), supporting that AlrA is required for detoxification of MG. Interestingly, when cultured with 0.2 mM for 3 days, KAx3 cells overcame MG toxicity and finally recovered the normal growth rate (Fig. 8A). Next, we tested the effect of MG on cell cycle progress. Whereas most KAx3 cells were in G2 phase, the cells treated with hydroxyurea, a G1/S phase blocking reagent, for 20 h, had low DNA content (Fig. 8B). The treatment of 0.2 mM MG for 24 h increased the proportion of cells having low DNA content as the treatment with hydroxyurea (Fig. 8B). Interestingly, after 36 h treatment of MG, the population of cells at G1 phase decreased from 98.2% to 61.8% (Fig. 8B), which is consistent with the growth recovery of KAx3 cells after MG treatment (Fig. 8A). These data support our hypothesis that MG accumulated by GSH depletion is a primary factor inducing cell cycle arrest of *gcsA*⁻ cells and induction of *alrA* is correlated with increased MG level.

## Discussion

In the present study, we tried to elucidate the mechanism by which GSH depletion causes growth arrest of *Dictyostelium* cells. It has been reported that GSH depletion induces the arrest of cell cycle in human (Russo *et al*., 1995), plant (Vernoux *et al*., 2000), yeast (Sharma *et al*., 2000) and slime mold (Kim *et al*., 2005), but the detailed mechanism remains unknown. The *gcsA*⁻ mutant requires GSH for axenic growth (Kim *et al*., 2005). It is interesting that its growth rate is proportion to GSH concentration in the media and growth of the mutant arrested

 

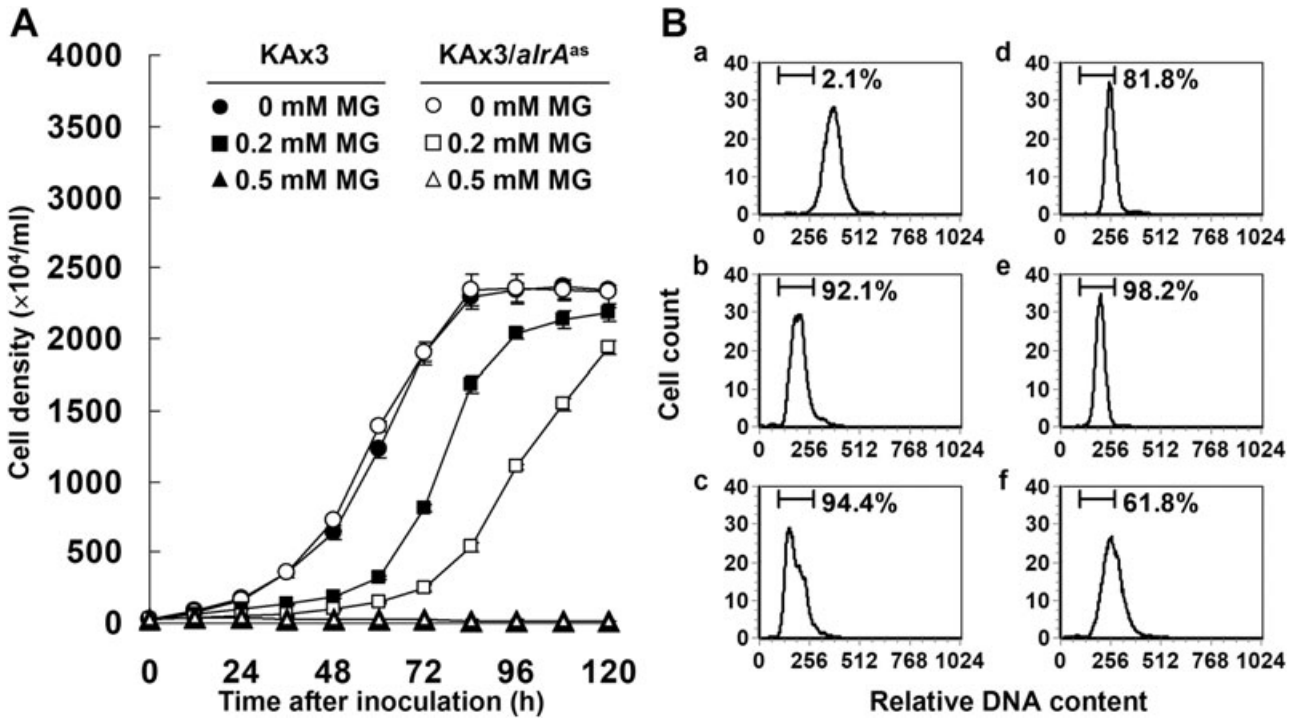


**Fig. 8.** MG is a growth-inhibiting factor in *Dictyostelium*.
A. Growth curves of wild-type KAx3 cells challenged by MG. Exponentially growing wild-type KAx3 cells (closed symbols) and *alrA* knockdown KAx3 cells (open symbols) were inoculated at $2 \times 10^5$ cells $ml^{-1}$ in the media without MG (circles) or with 0.2 mM (squares) or 0.5 mM MG (triangles). Viable cells were counted using a haemacytometer.
B. Cell cycle analysis. Wild-type KAx3 cells were cultured in the media with 2 mM hydroxyurea for 12 and 24 h, and with 0.2 mM MG for 12, 24 and 36 h. DNA content of cells was analysed by flow cytometry (see *Experimental procedures*). Data are representatives of three independent experiments. The cells treated with hydroxyurea are used for G1 control. Numbers indicate the percentage of G1 phase cells.
a. no treatment; b. 2 mM hydroxyurea for 12 h; c. 2 mM hydroxyurea for 20 h; d. 0.2 mM MG for 12 h; e. 0.2 mM MG for 24 h; f. 0.2 mM MG for 36 h.

by GSH depletion was restored when GSH was replenished again. Thus, our findings provide the possibility that *Dictyostelium* may be a good model system for the study about the relationship between GSH and cell cycle. As GSH is the best-known antioxidant molecule and exposure to oxidant induces cell cycle arrest (Russo *et al*., 1995; Wanke *et al*., 1999), it was expected that the growth arrest of *Dictyostelium* by GSH depletion may result from oxidative stress. However, our previous data demonstrated that the growth arrest is hardly rescued by other reducing agents such as DTT and NAC (Kim *et al*., 2005), suggesting that GSH plays special roles other than antioxidant in *Dictyostelium*.

In an attempt to unravel the role of GSH in the growth of *Dictyostelium*, we first investigated the proteins expressed differentially in GSH-depleted cells. Through comparative proteomic analysis, we identified 10 major proteins induced by GSH depletion. The list included two GST proteins and elongation factor 1b having a GST domain. In mammalian cells, it was shown that GST is induced by oxidative stress through activation of AP-1 (Pinkus *et al*., 1995). Therefore, the induction of GST proteins by GSH depletion may be closely related to increased ROS levels. In addition, peroxiredoxin was induced by GSH depletion, which appears to contribute to remove peroxides with a peroxidase activity. These data imply that GSH depletion causes oxidative stress, resulting in induction of these antioxidant enzymes. This could explain the reason why *gcsA*$^-$ cells were not sensitive to external oxidants (data not shown).

AlrA is one of the proteins strongly upregulated by GSH depletion. Previous reports have described that aldose reductase is induced by glucose, lipid peroxide product and MG (Koh *et al*., 2000; Chang *et al*., 2002). These are commonly all good substrate for aldose reductase, indicating that there may be a positive feedback loop for the expression of aldose reductase. The chemicals generating superoxide radicals induced the expression of *alrA*, but the expression of biological radical scavenger failed to repress the induction of *alrA* expression after GSH depletion. Instead, MG, which is a good substrate of AlrA, strongly induced the expression of *alrA*. Moreover, the induction of *alrA* expression by menadione seemed to be due to elevated MG level (Fig. 6C), proposing that MG

may be a good candidate for the induction of *alrA* expression by GSH depletion.

Herein, we report for the first time the effect of MG, a toxic endogenous metabolite, on *Dictyostelium* growth. The MG formed during axenic culture is expected to be effectively eliminated by glyoxalase system together with GSH because two putative genes encoding glyoxalase I (DDB0230987) and II (DDB00230988) are well conserved in *Dictyostelium* genome (Eichinger *et al*., 2005; http://www.dictybase.org). MG reacts with GSH nonenzymatically, forming hemithioacetal, which then is converted into *S*-D-lactoylglutathione by glyoxalase I. Finally glyoxalase II recovers GSH with producing D-lactic acid. However, in GSH-deficient cells, it is likely that free MG accumulates. Considering that AlrA was induced by MG, we hypothesized that increased concentration of MG by GSH depletion may be involved in the growth arrest. This was proven by the result that the *alrA* overexpression repressed MG induction during GSH depletion, resulting in recovery of the growth rate. By contrast, *alrA* knockdown stimulated MG accumulation and lowered cell growth rate. In addition, the treatment of sublethal dose of MG inhibited wild-type KAx3 cell proliferation. Thus, our findings suggest that endogenous MG is a growth inhibiting factor and AlrA has a crucial role in the elimination of MG in GSH-deficient cells.

We have emphasized the importance of AlrA on MG detoxification in GSH-deficient cells, but the glyoxalase system seems to act actively in the cells, in which GSH is not depleted. AlrA protein level in *gcsA*$^-$/*alrA*$^{as}$ cells without GSH is roughly equivalent to that in *gcsA*$^-$ cells with 1 mM GSH (Fig. 4B), but MG level between the two different mutants has a significant difference (Fig. 7A). This discrepancy can be explained by the role of glyoxalase system. At 1 mM GSH, MG level maintains at low level due to the cooperation of both glyoxalase and AlrA. However, when GSH is depleted, glyoxalase system cannot function and MG level could be mainly controlled by AlrA. Therefore, after 24 h of GSH depletion, MG was slightly decreased by strongly induced AlrA in *gcsA*$^-$ cells, whereas MG was largely accumulated in *gcsA*$^-$/*alrA*$^{as}$ cells (Fig. 7A). In addition, a little difference in MG level between *gcsA*$^-$ cells and *gcsA*$^-$/*alrA*$^{oe}$ cells with 1 mM GSH indicates that glyoxalase system may be more preferable to AlrA when GSH is enough in the cells (Fig. 7A). On the one hand, in wild-type KAx3 cells, AlrA knockdown delayed the recovery of growth rate after MG treatment (Fig. 8A) and *alrA* is much more strongly induced than *gloA* encoding glyoxalase I in response to sudden MG treatment and GSH depletion (data not shown). Taken together, these data suggest that AlrA may act as a major detoxifier under the conditions, which MG is highly accumulated in the cells.

Methylglyoxal accumulation results in the modification of macromolecules, such as DNA and proteins. MG reacts with guanine residue in DNA and RNA and modifies lysine and arginine residues in proteins. An increase in the level of MG-modified proteins has been used as a good indicator for diabetic complications and ageing (Ramasamy *et al*., 2005). There are several reports demonstrating that some proteins are specifically modified by MG under physiological conditions. Heat shock protein 27 is one of major MG-modified proteins and its modification causes the repression of apoptosis in tumour cells (Sakamoto *et al*., 2002; van Heijst *et al*., 2006). Unfortunately, we tried to identify MG-modified proteins after GSH depletion, but our attempt failed because anti-argpyrimidine antibody did not work on our cells (data not shown). There are several interesting data that MG exerts its effect on transcription factors directly. MG modulates the activity of Yap1 in *Saccharomyces cerevisiae* (Maeta *et al*., 2004) and Pap1 in *Schizosacchromyces pombe* (Zuin *et al*., 2005), thereby acting as a signal initiator for stress response cascade (Takatsume *et al*., 2006). Although it is clear that MG activates the transcription of *alrA*, the mechanism remains unknown. It will need to be further investigated how MG activates the induction of *alrA*.

Szent-Györgyi suggested that glyoxal derivative acts as a growth-inhibiting factor, called retine, whereas glyoxalase promotes cell growth, called promine (Szent-Györgyi *et al*., 1967). Since then, there have been many publications, but the precise mechanism by which MG functions as a retine is poorly understood. Here, we show that MG induces cell cycle arrest in *Dictyostelium*. Elevated intracellular MG level by *alrA* knockdown stimulated the cell cycle arrest of the cells lacking GSH. Moreover, external MG treatment attenuated cell growth and induced cell cycle arrest temporarily. Thus, a further study will be to determine the effect of MG on cell cycle regulators and it will provide a better understanding of how MG affects cell growth.

## Experimental procedures

### *Culture, development and transformation of* Dictyostelium *cells*

*Dictyostelium* KAx3 cells were cultured in an HL5 axenic medium (Cocucci and Sussman, 1970) at 22°C. *gcsA*$^-$ cells were maintained in HL5 media supplemented with 1 mM GSH. Depletion of intracellular GSH was accomplished by washing twice with fresh HL5 media. For development, cells were washed twice with $KK_2$ buffer (16.5 mM $KH_2PO_4$, 3.8 mM $K_2HPO_4$, pH 6.2) and plated at a density of $3 \times 10^6$ cells cm$^{-2}$ on $KK_2$ agar plates. Transformation was performed by electroporation (Pang *et al*., 1999). Transformants were selected and maintained in an HL5 medium containing 10 μg ml$^{-1}$ of G418.

### Strain construction

The full-length cDNAs of *alrA* and *sodA* gene were isolated by polymerase chain reaction based on the sequences from *Dictyostelium* genomic sequence project (http://www.dictybase.org). To inhibit the expression of *alrA* in *gcsA*$^-$ cells, *alrA* were inserted in the antisense orientation into BglII site of EXP4(+) vector and the resulting construct was introduced into *gcsA*$^-$ cells by electroporation. To overexpress *sodA* in *gcsA*$^-$ cells, *sodA* was subcloned into BamHI/XhoI site of pTX-FLAG vector, followed by transformation into *gcsA*$^-$ cells. For the recombinant AlrA, *alrA* cDNA was ligated into BamHI/XhoI site of pGEX4T-1 vector (Amersham Biosciences). The sequences of each construct were confirmed by automated DNA sequencing.

### 2-Dimensional electrophoresis

Approximately 400 μg of total proteins from KAx3 and *gcsA*$^-$ cells deprived of GSH was separated by 2D-PAGE. In first dimension, the proteins were separated by isoelectric focusing in a pH gradient ranging from 4 to 7. The second dimension was performed using an Ettan Dalt II system according to the manufacturer's instructions (GE Healthcare). Silver staining was performed as described previously (Yan *et al*., 2000).

### Protein identification by MALDI-TOF

Protein spots, excised from silver-stained gels, were destained in 15 mM potassium ferricyanide and 50 mM sodium thiosulphate. The gel pieces were rinsed several times with water and then dehydrated with acetonitrile. After removal of the acetonitrile, 30–50 μl of digestion buffer (25 mM ammonium bicarbonate and 100 ng of trypsin) was added. Trypsin digestion of the protein was performed at 37°C overnight. The supernatant containing the generated peptides was recovered, and the gel pieces were extracted twice with 50% acetonitrile and 0.1% trifluoroacetic acid for 30 min. The volume of the combined extracts was reduced to 10 μl in a Speedvac.

The peptides were analysed using a Voyager-DE STR Biospectrometry workstation (Applied Biosystems). Masses were calibrated with internal tryptic autodigestion ion peaks at *m/z* 842.5094 and *m/z* 2211.1040. Protein identification was performed against the NCBI database by using Mascot search program.

### Enzyme assay and kinetic studies

Aldose reductase activity was determined in a reaction mixture containing 0.1 M sodium phosphate buffer, pH 6.2, 0.15 mM NADPH and 10 mM D,L-glyceraldehyde at 30°C (Nishimura *et al*., 1990). The reaction was initiated by the addition of enzyme or cell extract and the decrease in $A_{340}$ was recorded using a UV-Visible Spectrophotometer (Shimadzu). One unit of enzyme was defined as the oxidation of 1 μmol of β-NADPH per minute using a molar absorption coefficient of 6200 $M^{-1}$ $cm^{-1}$.

To determine kinetic parameters, enzyme was incubated with the various concentrations of D,L-glyceraldehyde and MG in the same reaction buffer as above. The Michaelis constant ($K_m$) and the catalytic turnover constant ($k_{cat}$) were determined by the Lineweaver–Burk plot.

### Western blot and Northern blot analysis

For Western analysis, the proteins were resolved on 10–15% polyacrylamide-SDS slab gels and were transferred to nitrocellulose membrane (Schleicher and Schuell). The blot was probed with polyclonal anti-AlrA antibodies (a kind gift from Dr R. H. Gomer) or monoclonal anti-FLAG antibodies (Sigma), followed by the incubation of alkaline phosphatase-conjugated secondary antibodies. The bands were detected by staining with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Anti-actin antibodies (Sigma) are used as a loading control.

For Northern analysis, total RNAs (30 μg) were extracted using TRIZOL (Invitrogen) according to manufacture's instructions. Total RNAs (30 μg) were separated on a 1% agarose gel containing 0.22 M formaldehyde and were transferred to nylon membranes (Amersham Biosciences). Hybridization was performed with specific probes labelled with [α-$^{32}$P]-dATP in Rapid-Hyb buffer (Amersham Biosciences) as recommended by manufacturer. All blots were pre-hybridized for 1 h, hybridized for 2 h and washed twice for 20 min at 65°C.

### Cell cycle analysis

The cell cycle analysis was performed according to protocol (Haase and Reed, 2002) with some modifications. Approximately $1 \times 10^7$ cells were collected and re-suspended in 1.5 ml of $KK_2$ buffer. Then, cells were fixed by adding 5 ml of 100% ethanol. Fixed cells were collected and re-suspended in 0.5 ml of RNase solution (2 mg $ml^{-1}$ of RNase A in 50 mM Tris-HCl pH 8.0, 15 mM NaCl). After incubation for 2 h at 37°C, cells were re-suspended in 0.5 ml of 50 mM Tris-HCl pH 7.5. Just prior to FACS analysis, 50 μl of cell suspension was mixed with 1 ml of dye solution (1 μM SYTOX GREEN in 50 mM Tris-HCl, pH 7.5). FACScanto flow cytometer (BD Biosciences) was used for analysis.

### Cytokinesis assay

Cytokinesis assay was determined according to the method of Tsujioka *et al*. (2008) with some modifications. *Dictyostelium* cells were washed with HL5 and were dropped at a density of $2 \times 10^5$ cells $ml^{-1}$ on cover slip. The dropped cells were fixed with 1% formaldehyde in methanol for 5 min on ice. After the cells were washed three times with phosphate-buffered saline (PBS), fixed cell were incubated in PBS containing 0.1 μg $ml^{-1}$ DAPI. After incubation for 15 min, cells were washed three times with PBS, and the number of nuclei was counted using an Axiolab microscope (Carl Zeiss).

### Measurement of MG

Intracellular MG level was determined according to the method of Chaplen *et al*. (1996) with some modifications.

 

Exponentially growing cells were harvested and suspended in 0.5 M perchloric acid. After incubation for 10 min, the pellet was removed by centrifugation and the supernatant was incubated with 1 mM 1,2-diaminobenzene at 60°C for 4 h in the dark. The sample was passed through C-18 solid phase extraction column which was equilibrated with 10 mM $KH_2PO_4$ (pH 2.5) and then was eluted with acetonitrile. The sample was evaporated and dissolved in the mobile phase buffer [68% (v/v) 10 mM $KH_2PO_4$, pH 2.5, and 32% (v/v) acetonitrile].

The sample was separated on ZORBAX SB-C18 column using Agilent 1200 series HPLC system. Quinoxaline derivatives were detected at 336 nm. The amount of 2-methyquinoxaline, a quinoxaline derivative of MG, was quantified by integration relative to the internal standard.

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