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Reduced glutathione levels affect the culmination and cell fate decision in *Dictyostelium discoideum*

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Abstract

Glutaredoxins have been known to be glutathione-dependent oxidoreductases that participate in the redox regulation of various cellular processes. To understand the role of glutaredoxins in the development, we examined glutaredoxin 1 (Grx1) of *Dictyostelium discoideum*. Its mRNA was highly accumulated at the mound and the culmination stages. When Grx1-overexpressing cells were developed, their culmination was delayed, and the expression of marker genes for prespore and spore decreased. Interestingly, they had about 1.5-fold higher amount of reduced glutathione (GSH) compared with parental cells and their prolonged migration was repressed by the oxidant such as hydrogen peroxide. To confirm the effect of GSH on the culmination, glutathione reductase (Gsr) was overexpressed or underexpressed. Similar to Grx1-overexpressing cells, Gsr-overexpressing cells contained about 1.5-fold higher amount of GSH and exhibited the delayed culmination. In contrast, the knockdown mutant of Gsr had nearly 50% lower amount of GSH and showed accelerated culmination. Taken together, these data suggest that the culmination of *Dictyostelium* is controlled by GSH. In addition, the cells having higher GSH levels showed a prestalk tendency in the chimeric slugs with parental cells, indicating that the difference in the amount of GSH may affect the determination of cell fate.

Keywords: Glutaredoxin; Glutathione reductase; Glutathione; Culmination; Cell fate; *Dictyostelium discoideum*

Introduction

Dictyostelium discoideum has been considered as a useful model organism for developmental biology because of its dynamic morphogenesis during development. When starved, amoebae aggregate together in response to cAMP pulse, forming a finger-like structure. During this period, the expression of cell-type-specific genes, the tip formation and the cell sorting occur successively. At this time, the prestalk cells are localized to the anterior region and the prespore cells to the posterior region, thereby establishing spatial patterning. The prespore region also contains some anterior-like cells that express prestalk marker genes. When the finger-like structure falls down on the ground, it can migrate away as a slug. The slugs migrate to seek out the place to disperse spores effectively and finally undergo culmination, a terminal process to form fruiting bodies, under an appropriate condition (Firtel, 1996; Parent and Devreotes, 1996; Thomason et al., 1999; Weijer, 1999).

The culmination of the migrating slugs is controlled by external factors such as light, temperature and humidity and internal factors such as ammonia and cAMP (Newell et al., 1969; Schindler and Sussman, 1977). These factors have been identified by the studies of slugger mutants which are unable to culminate under normal condition (Davies et al., 1993; Fukuzawa et al., 1997; Fukuzawa and Williams, 2002; Gee et al., 1994; Nelson et al., 2000; Newell and Ross, 1982; Singleton et al., 1998).

Since glutaredoxins were originally discovered as glutathione-dependent electron donors for ribonucleotide reductase in the *Escherichia coli* mutant lacking thioredoxin (Holmgren, 1976), they have been identified and characterized in a variety of prokaryotes and eukaryotes (Luthman et al., 1979; Minakuchi et al., 1994; Padilla et al., 1995). They are small

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glutathione-dependent oxidoreductases with a conserved disulfide/dithiol group of Cys-Pro-Tyr-Cys within their active site. Besides the reduction of ribonucleotide, they participate in many cellular processes: protection against oxidative stress (Grant, 2001) and regulation of redox-sensitive transcription factors such as OxyR (Zheng et al., 1998) and nuclear factor 1 (Bandyopadhyay et al., 1998). In recent works, they appear to be a primary catalyst on the glutathionylation/deglutathionylation reaction which is important for redox regulation of many proteins (Barrett et al., 1999; Borges et al., 2002; Klatt et al., 1999; Rao and Clayton, 2002). Oxidized glutaredoxin is directly reduced by reduced glutathione (GSH), and in turn, oxidized glutathione (GSSG) is reduced back by glutathione reductase (Gsr) and NADPH.

It was reported that endogenous reactive oxygen species (ROS) can act as secondary messengers and regulate the key elements within a variety of pathways (Finkel and Holbrook, 2000; Grant, 2001; Rietsch and Beckwith, 1998). Especially, several studies have emphasized the importance of ROS in the developmental processes: the conidiation of *Neurospora crassa* (Hansberg et al., 1993), nerve growth factor-induced neuronal differentiation (Suzukawa et al., 2000) and cardiomyocyte differentiation (Sauer et al., 2000). They suggested that increased ROS levels accelerate differentiation, whereas antioxidant enzymes prevent differentiation. In *Dictyostelium*, it was reported that ROS levels increase at early stages of multicellular development (Fisher et al., 1991; Fisher and Rosenberg, 1988); in particular, superoxide radicals are generated during aggregation, and the cells, in which superoxide dismutase is overexpressed, fail to form aggregates (Bloomfield and Pears, 2003). Therefore, ROS is believed to be important for cell signaling during the development of *Dictyostelium*.

We have demonstrated that GSH levels change during the development of *Dictyostelium*, and the depletion of GSH inhibits the growth and differentiation of *Dictyostelium* (Kim et al., 2005). In the present work, we describe that the overexpression of Grx1 inhibits the culmination of migrating slugs, and a direct modulation of GSH levels through controlling Gsr levels affects the timing of *Dictyostelium* culmination. Our data strongly support the fact that the alteration in cellular redox state affects cell differentiation.

Materials and methods

Cell culture and development

Dictyostelium KAx3 cells were grown in HL5 axenic medium (Cocucci and Sussman, 1970) supplemented with 100 μg/ml streptomycin and 100 units/ml penicillin either on culture dish plates or in a shaken suspension at 22°C. 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 were plated at a density of 3×10^6 cells/cm^2 on KK_2 agar plates or nitrocellulose filters (Millipore) placed on KK_2 agar plate and then were incubated at 22°C. For dark condition, the plates were wrapped with aluminum foil.

Construction of Grx1 and Gsr overexpression strains

The complete sequence of *grxA* gene was amplified by polymerase chain reaction (PCR) using a cDNA clone SSE867 (a kind gift from the *Dictyostelium* cDNA project in Japan) as a template. PCR amplification of *gsr* gene was carried out using cDNA from 8-h-developed cells as a template. The amplified DNA fragments were cloned into pGEM-T easy vector (Promega). For constitutive overexpression in *Dictyostelium*, the full-length cDNAs of *grxA* and *gsr* were inserted into EXP4(+) expression vector, respectively. To generate inactive form of Grx1 (C21S), cysteine at position 21 was substituted with serine by PCR using site-directed mutagenesis. The full-length cDNA of the mutated *grxA* was inserted in-frame into pTX-FLAG vector for expression of FLAG-tagged protein. All vectors were introduced into KAx3 cells by electroporation (Pang et al., 1999), and stable transformants were selected with 10 μg/ml G418.

Construction of Gsr knockdown strain by RNA interference

Knockdown of Gsr was accomplished by inducible RNA interference (RNAi)-mediated gene silencing. The construct for RNAi was designed with 800-bp inverted repeat and 598-bp hairpin loop. 1398-bp full-length cDNA and 800-bp 5′ fragment of *gsr* gene were amplified by PCR and were ligated tail-to-tail into MB38 vector, a response plasmid of the tetracycline-controlled expression system for *Dictyostelium* (Blaauw et al., 2000), which was applied to conditionally repress *gsr* gene expression. The resulting RNAi construct was introduced by electroporation into MB35 cells, which already carried MB35 vector, a plasmid expressing tetracycline-controlled transcriptional activator. Transformed cells were selected with the HL5 medium containing 10 μg/ml G418, 5 μg/ml blasticidin and 10 μg/ml tetracycline. For inhibition of *gsr* gene expression, transformed cells were cultured for at least 2 days in the absence of tetracycline, prior to each experiment.

Assay of enzyme activities

Glutaredoxin activity was determined by the reduction of the mixed disulfide formed between β-hydroxylethylene disulfide (HED) and GSH as described previously (Holmgren and Åslund, 1995). Briefly, the reaction mixture containing 0.2 mM β-NADPH, 1 mM GSH, 0.7 U glutathione reductase, 2 mM EDTA and 0.7 mM HED was prepared in the cuvette. After 2 min of preincubation, glutaredoxin or cell extract was added to the cuvette, and the decrease in absorbance at 340 nm was measured using a UV–Visible Spectrophotometer (Shimadzu). Glutathione reductase activity was measured spectrophotometrically at 340 nm in a reaction mixture of 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 2 mM oxidized glutathione and 0.1 mM NADPH. One unit (U) of both glutaredoxin and glutathione reductase was defined as the oxidation of 1 μmol of β-NADPH per min using a molar absorption coefficient of 6200 M^{-1} cm^{-1}.

Western blot and Northern blot analysis

Polyclonal anti-Grx1 and anti-Gsr antibodies were raised against purified Grx1 and Gsr. The anti-Gsr antibodies were then purified using a blot-affinity purification method (Tang, 1993). For Western blot analysis, the proteins were resolved on 10–15% polyacrylamide-SDS slab gels and were transferred to a nitrocellulose membrane (Schleicher and Schuell). The blot was probed with polyclonal anti-Grx1 antibodies or polyclonal anti-Gsr antibodies or monoclonal anti-FLAG antibodies (Sigma) or polyclonal anti-Dd31 antibodies (a kind gift from Dr. W. F. Loomis) (diluted to 1:2000, 1:100, 1:10,000 and 1:10,000, respectively), followed by incubation of alkaline phosphatase-conjugated secondary antibodies. The bands were visualized through staining with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

For Northern blot analysis, 20 μg of total RNAs was separated on a 1% agarose gel containing 0.22 M formaldehyde and blotted onto a nylon membrane (Amersham Biosciences). Hybridization was performed with various probes in Rapid-Hyb buffer (Amersham Biosciences) as recommended by manufacturer. The blots were prehybridized for 1 h, hybridized for 2 h and washed twice for 20 min at 65°C. The specific probes were labeled with [α-^{32}P]-dATP by random priming (Feinberg and Vogelstein, 1983).

Slug migration test

Dictyostelium cells were washed twice with KK_2 buffer and were dropped at a density of 3×10^6 cells/cm^2 on KK_2 agar plates. The plates were wrapped with

aluminum foil for dark condition and were incubated at 22°C. The trails of slugs were stained as described previously (Wilkins et al., 2000) with some modifications: after 2 days of incubation, the foil was removed, and then slime trails were transferred to a transparency film, followed by staining with Coomassie blue dye. To test the effect of oxidants on the slug migration, cells were developed on the agar plates supplemented with various concentrations of hydrogen peroxide.

Determination of intracellular GSH levels

The intracellular GSH levels were measured as described previously (Kim et al., 2005) with some modifications: *Dictyostelium* cells were harvested and suspended in 10% trichloroacetic acid (TCA) solution. After sonication and incubation for 20 min in ice, the precipitate was collected by centrifugation. For HPLC analysis, the supernatant was separated on Agilent ZORBAX SB-C18 column using Waters Associates Liquid Chromatography System (Millipore) linked with Hewlett Packard 1049A electrochemical detector. Elution was carried out isocratically with 20 mM sodium phosphate buffer (pH 2.7). The detector potential was set at +0.7 V, and the flow rate was 1 ml/min. The amount of GSH was quantified by integration relative to the internal standard.

β-Galactosidase staining

To observe the spatial expression of *grxA* gene during development, *grxA*/*lacZ* construct, which was generated by inserting 1 kb of *grxA* upstream sequence into *Bam*HI/*Hin*dIII site of pA15Gal, was introduced into KAx3 cells. To mark Grx1-overexpressing cells with *lacZ* driven by actin15 promoter, pA15Gal-BSR construct was created through blunt-end ligation of a blasticidin resistance cassette into the *Nco*I sites within a neomycin resistant gene of the pA15Gal and then was introduced into Grx1-overexpressing cells. Transformed cells were selected with 5 μg/ml blasticidin. All cells were washed with KK_2 buffer and allowed to develop on nitrocellulose filters at 22°C. For synergy experiment, *act15/lacZ*-marked cells were mixed at a ratio of 1:3 or 1:9 with unmarked cells, just prior to deposition on filters. Developing structures were fixed and stained for β-galactosidase activity as described previously (Haberstroh and Firtel, 1990).

Results

Identification of the gene encoding glutaredoxin 1 in D. discoideum

By searching the database of Japanese *Dictyostelium* cDNA project, the clone SSE867 containing a dithiol glutaredoxin was adopted, and the gene and the protein of dithiol glutaredoxin were named *grxA* and Grx1, respectively, and characterized. The cDNA coding sequence of *grxA* gene was 300 bp long, encoding a polypeptide of 100 amino acids. As shown in Fig. 1A, deduced amino acid sequence possessed a conserved active site (Cys-Pro-Tyr-Cys), a GSH-binding site (Ile-Gly-Gly) and a hydrophobic region (Thr-Val-Pro) (Lundberg et al., 2001). Activity assay on recombinant wild type and mutated Grx1 (C21S) showed that Grx1 had a glutathione-dependent oxidoreductase activity, and Cys21 was essential for the activity as reported previously (Bushweller et al., 1992; Rouhier et al., 2002) (data not shown). These data indicate that *Dictyostelium* Grx1 is a dithiol glutaredoxin having a glutathione-dependent oxidoreductase activity.

Expression of Grx1 was developmentally regulated

To investigate the role of glutaredoxin during *Dictyostelium* development, we first examined the developmental expression pattern of *grxA* gene. Northern blot analysis showed that the amount of *grxA* mRNA increased up to the tight aggregation stage (9 h) and thereafter rapidly decreased (Fig. 1B). Its mRNA was also highly accumulated at the fruiting body stage (24 h) (Fig. 1B). To find out the spatial expression pattern of Grx1 during development, the *lacZ* expression driven by the promoter of *grxA* was examined on developing structures. When the developing structures were stained for β-galactosidase activity, the prespore region was mainly stained at the first finger stage (12 h, panel b of Fig. 1C), and the spore region was strongly stained at the mid-culminant stage (18 h, panel c of Fig. 1C). In a fruiting body (24 h, panel d of Fig. 1C), it seemed likely that all cells were stained. Therefore, the expression of *grxA* gene seemed to be developmentally regulated and be somewhat abundant in the prespore cells, although it was found in all cells of *D. discoideum*.

Constitutive expression of Grx1 led to delayed culmination

To gain an insight into the role of Grx1 during development, Grx1 was overexpressed in *Dictyostelium* using a constitutive actin 15 promoter. Overexpression of Grx1 was confirmed by Western blot analysis (Fig. 2A) and activity assay (Fig. 2B). When Grx1-overexpresssing cells ($Grx1^{OE}$) were allowed to develop, their slugs did not culminate but continued to migrate for a long period, called 'slugger phenotype' (Fig. 2C); this delayed culmination phenotype became more evident under dark condition. To confirm whether the developmental defect was due to the enhanced activity of Grx1, inactive C21S, which was tagged with FLAG, was also overexpressed in *Dictyostelium* (Fig. 2A). Since C21S had no GSH-dependent oxidoreductase activity (data not shown), it was unlikely that N-terminal FLAG epitope affects the activity. As expected, C21S-overexpressing cells ($Grx1^{C21S}$) did not show an increase in the activity compared with parental KAx3 cells (Fig. 2B) and culminated normally unlike $Grx1^{OE}$ cells (Fig. 2C). When the cells spotted on agar plate were incubated for 2 days in the dark, the slugs of $Grx1^{OE}$ migrated for much longer period than those of parental KAx3 (Fig. 2D). In addition, when the cells were grown in association with *Klebsiella pneumoniae* for 6 days, all fruiting bodies of parental KAx3 cells were developed within the plaques, whereas some fruiting bodies of $Grx1^{OE}$ cells were found on the bacterial lawn outside the plaques, as pointed with arrows (Fig. 2E). This was very similar to the plaque morphology of OSBPa null cells showing the slugger phenotype (Fukuzawa and Williams, 2002). Moreover, $Grx1^{OE}$ cells were sensitive to ammonia inhibition like other slugger mutants (Gee et al., 1994; Singleton et al., 1998) (data not shown). These results indicate that the overexpression of Grx1 causes the culmination of migrating slugs to be delayed, thereby leading to the slugger phenotype.

Expression of some prespore-specific genes was reduced in $Grx1^{OE}$ cells

The aberration of specific cell-type differentiation has been found in some slugger mutants (Fukuzawa et al., 1997; Nelson

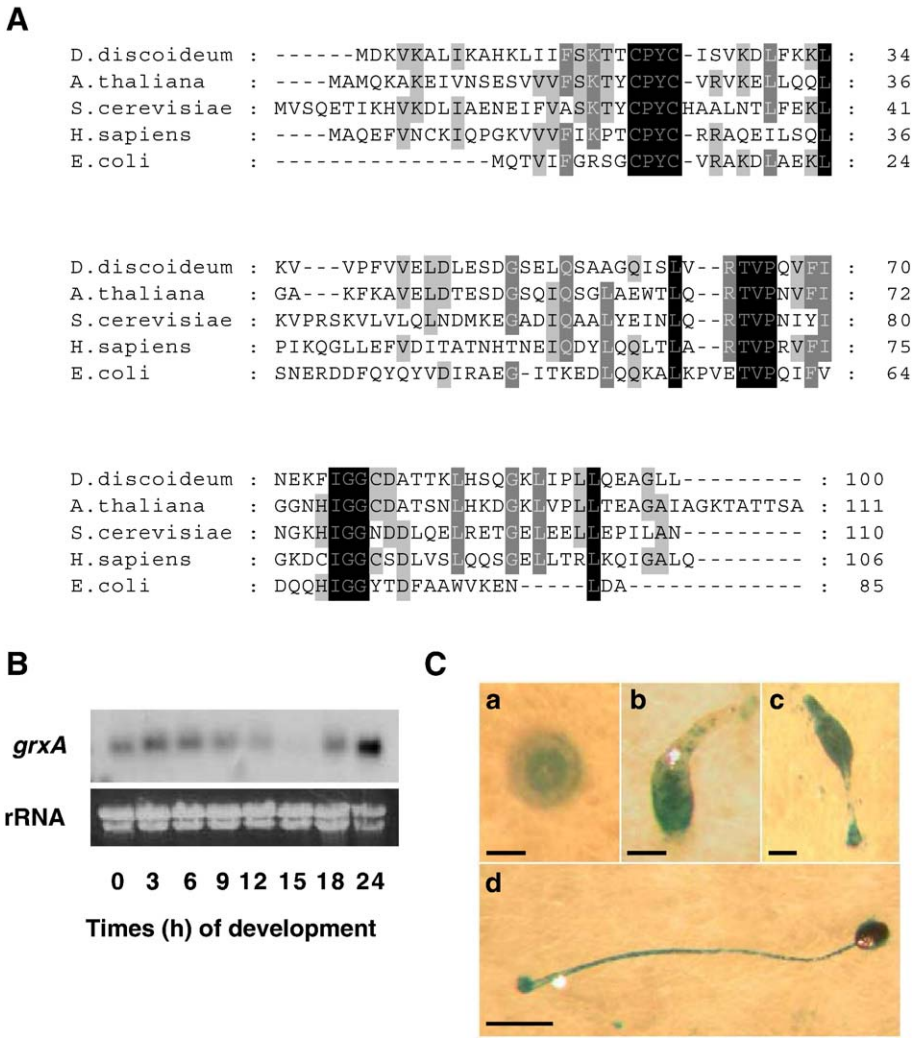

Fig. 1. Characterization of *Dictyostelium* Grx1. (A) Amino acid sequence comparison of *Dictyostelium* Grx1 with glutaredoxins in a variety of organisms. From top to bottom: *D. discoideum* Grx1 (GenBank accession no. XP_635914), *Arabidopsis thaliana* glutaredoxin (*NP_198853*), *Saccharomyces cerevisiae* glutaredoxin (CAA42381), *Homo sapiens* glutaredoxin (*CAG29308*), and *Escherichia coli* glutaredoxin (*AAA23936*). The residues which were identical in all proteins were marked with black background. Sequence alignment was displayed using ClustalW and GeneDoc. (B) Temporal expression of *grxA* gene during development. Northern blot analysis was performed with total RNAs extracted from the cells at the indicated times (h) of development. The RNA blot was probed with full-length cDNA of *grxA* labeled with ^{32}P. (C) Spatial expression of *grxA* during development. KAx3 cells carrying *grxA/lacZ* construct were allowed to develop on nitrocellulose filters and were fixed at 6 h (a), 12 h (b), 18 h (c) or 24 h (d) of development and then were stained for β-galactosidase activity. Scale bars, 0.2 mm.

et al., 2000; Singleton et al., 1998). To verify cell-type differentiation in Grx1^{OE} cells, the expression pattern of various cell-type-specific genes was examined by Northern blot analysis, as shown in Fig. 3. The expression of *ecmA*, a prestalk-specific gene and *lagC*, a post-aggregative gene in Grx1^{OE} cells was almost similar to that of parental cells (Fig. 3). In the case of prespore-specific genes, the expression of *cotB* and *cotC* decreased in Grx1^{OE} cells whereas that of *pspA* was not changed (Fig. 3). This differential expression pattern has been also shown in other slugger mutants such as *cudA*$^{-}$ and *mybC*$^{-}$ (Fukuzawa et al., 1997; Guo et al., 1999). Also, the expression of *spiA*, a spore-specific gene, was hardly detected in Grx1^{OE} cells (Fig. 3), which was consistent with their delayed culmination phenotype. This decreased expression of some prespore- and spore-specific genes may be caused by the delayed culmination of Grx1^{OE}.

Grx1 was involved in the defense against oxidants in Dictyostelium

It has been well established that glutaredoxin serves as an antioxidant enzyme in many organisms. In *Saccharomyces cerevisiae*, the expression of glutaredoxin genes is induced by oxidants, and each null mutant shows differential sensitivity toward various oxidants (Luikenhuis et al., 1998). To test whether Grx1 acts as an antioxidant in *Dictyostelium*, we examined the change of mRNA level of *grxA* after the treatment of oxidants. After the treatment with 1 mM hydrogen peroxide, the expression of its mRNA increased strongly at 2 h and thereafter maintained at a relatively high level (Fig. 4A). We also obtained the same result from the treatment with 0.1 mM menadione (data not shown). When treated with various concentrations of menadione during cell

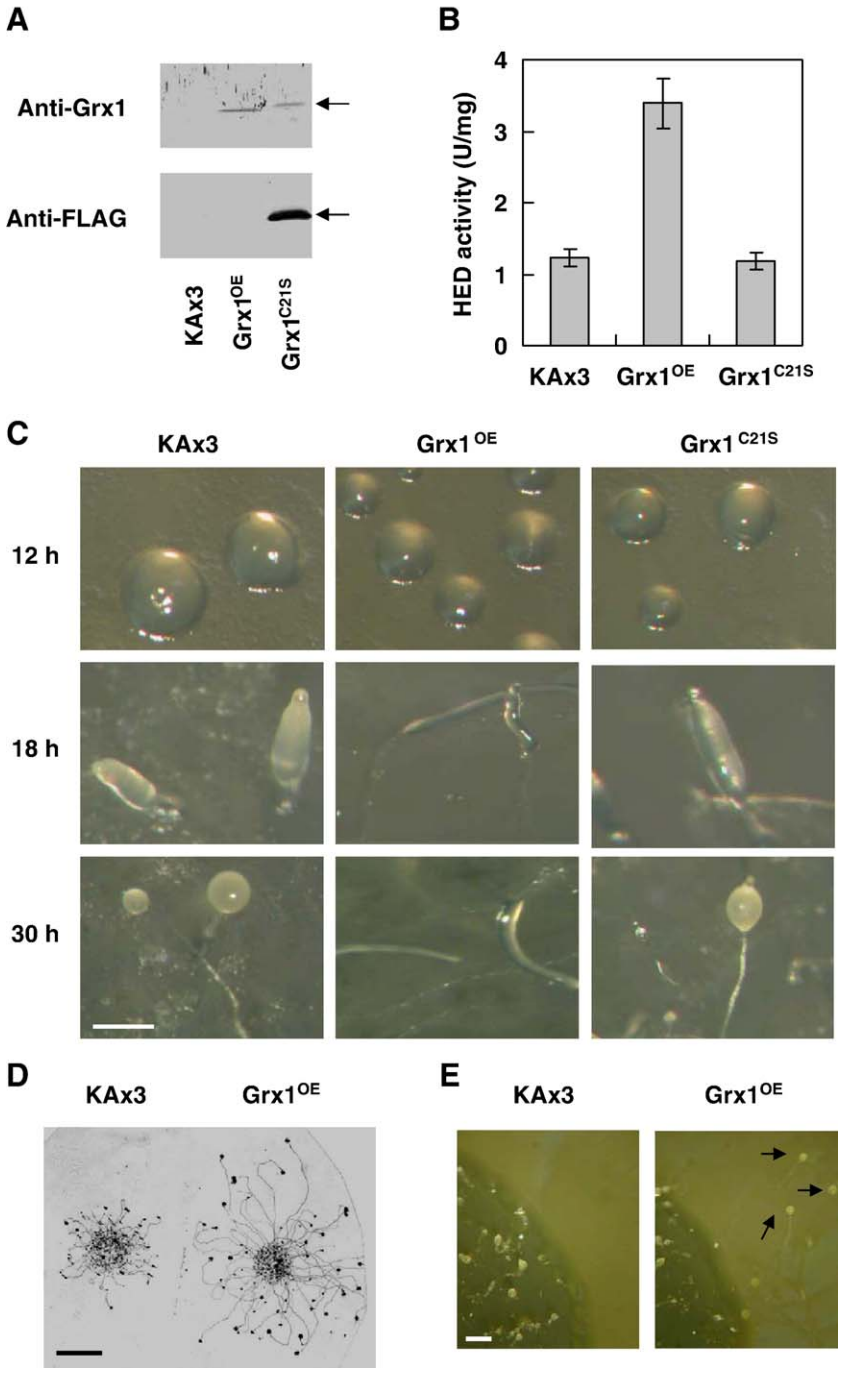

Fig. 2. Developmental morphology of Grx1^{OE} cells. (A) Overexpression of Grx1 was confirmed by Western blot analysis. Total protein extracts from KAx3, Grx1^{OE} and Grx1^{C21S} were probed with the anti-Grx1 antibody and anti-FLAG antibody, respectively. The same amounts of proteins were loaded in each lane. (B) From the cell extracts prepared from KAx3, Grx1^{OE} and Grx1^{C21S}, the glutaredoxin activity was measured as described in Materials and methods. The values represent the mean ± standard deviation of three independent experiments. (C) KAx3, Grx1^{OE} and Grx1^{C21S} cells were allowed to develop on KK_2 agar plate and were photographed at the indicated times (h) of development. Scale bar, 0.5 mm. (D) After KAx3 and Grx1^{OE} cells were developed on agar plate for 40 h in the dark, their slug trails were transferred to transparency film, stained with Coomassie blue. Scale bar, 10 mm. (E) KAx3 and Grx1^{OE} cells were plated in association with *Klebsiella pneumoniae* on SM/5 agar plates. Morphology of their plaques was observed after 6-day incubation. Arrows indicate the fruiting bodies of Grx1^{OE} which were formed outside the plaque on bacterial lawn. Scale bar, 1 mm.

growth, Grx1^{OE} cells were more resistant to menadione than parental cells, especially at 0.5 mM (Fig. 4B). In addition to growth inhibition, we observed the inhibition of development by menadione. When developed on the agar plates supplemented with menadione, Grx1^{OE} cells formed mounds better than parental cells (Fig. 4C). Assuming that the resistancy of Grx1^{OE} cells to the oxidants may be due to altered redox state, we measured the levels of protein thiols and GSH to determine cellular redox state. There was no significant difference in the levels of protein thiols between KAx3 and

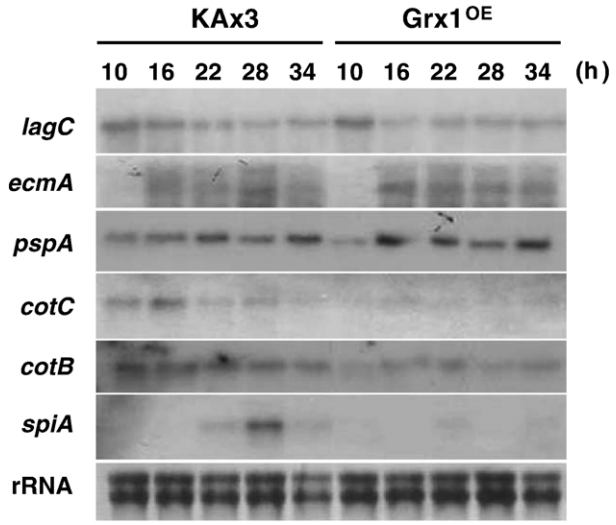

Fig. 3. Expression of cell-type-specific genes in Grx1^{OE} cells during development. Total RNAs were extracted from the cells harvested at the indicated times (h) of development and were transferred to a nylon membrane. The blot was probed with ^{32}P-labeled DNA fragments specific for the post-aggregative gene marker *lagC*, the prestalk gene marker *ecmA*, the prespore markers *pspA*, *cotB*, and *cotC*, and the spore marker *spiA*. Ethidium bromide-stained rRNA is shown as a loading control.

Grx1^{OE} cells, but GSH levels were approximately 50% higher in Grx1^{OE} cells than those in the parental KAx3 (Table 1). Thus, these data suggest that the increased amount of Grx1 affects the cellular redox state, thereby leading to an increase in resistancy against oxidants.

Table 1
Levels of GSH in KAx3 and the mutant strains

Strain	GSH (nmol/10^8 cells)
KAx3	74.2 ± 3.98
Grx1^{OE}	109 ± 14.6
Gsr^{OE}	110 ± 11.6
MB35	70.6 ± 6.24
Gsr-RNAi	39.4 ± 3.85

GSH was extracted from exponentially growing cells of each strain and its amounts were measured as described in Materials and methods. The values represent the mean ± standard deviation of three independent experiments.

There was a correlation between an increase in GSH levels and delayed culmination

To test whether the altered redox state can be involved in the slugger phenotype, we observed the effect of oxidants on the migrating slugs. Preliminary experiment showed that the treatment of the oxidants such as hydrogen peroxide and menadione led to a rapid decline in GSH levels in vegetative cells (data not shown). When cells were deposited on the agar plates supplemented with various concentrations of hydrogen peroxide, the prolonged migration of Grx1^{OE} cells was inhibited in a concentration-dependent manner (Fig. 5A); especially at 2 mM hydrogen peroxide, the slug

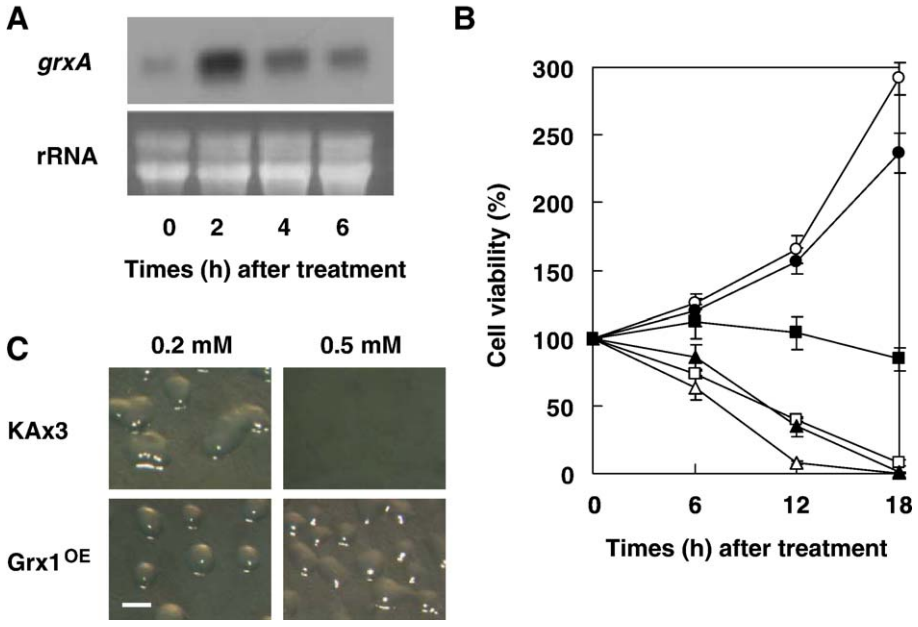

Fig. 4. Role of Grx1 in the defense against oxidants. (A) Induction of *grxA* mRNA by hydrogen peroxide was confirmed by Northern blot analysis. From KAx3 cells treated with 1 mM hydrogen peroxide for the indicated times (h), the total RNAs were extracted and probed with ^{32}P-labeled full-length cDNA of *grxA*. Ethidium bromide-stained rRNA is shown as a loading control. (B) Overexpression of Grx1 contributed to the defense against menadione. Exponentially growing KAx3 (open symbols) and Grx1^{OE} (closed symbols) cells were treated with menadione and only viable cells were counted with a hemacytometer at intervals of 6 h. Final concentrations of menadione were 0 mM (circles), 0.5 mM (squares), and 1 mM (triangles), respectively. To distinguish the viable cells from dead cells, 0.01% phloxine B was added to each medium. The cell viability is expressed relative to the cell number at the initiation of experiment and is the mean ± standard deviation of three independent experiments. (C) The aggregation of Grx1^{OE} cells was more resistant to menadione. Cells were allowed to develop on the KK_2 agar plates supplemented with the indicated concentrations of menadione. After 9-h incubation, the aggregates were photographed. Scale bar, 0.5 mm.

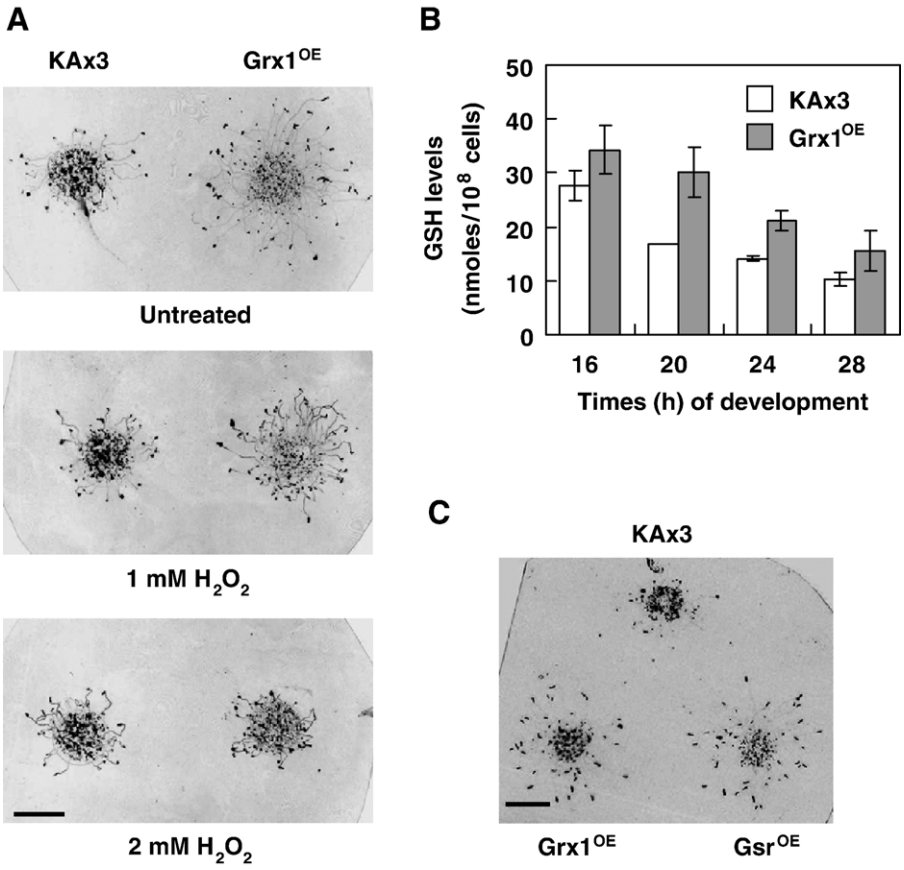

Fig. 5. Relationship between altered GSH levels and delayed culmination. (A) The prolonged migration of Grx1^{OE} slugs was inhibited by hydrogen peroxide. KAx3 and Grx1^{OE} cells were allowed to develop on the KK_2 agar plates supplemented with 0 mM, 1 mM, or 2 mM hydrogen peroxide for 40 h. Their slug trails were transferred into a transparency film, stained with Coomassie blue. Scale bar, 10 mm. (B) Comparison of GSH levels in KAx3 and Grx1^{OE} cells during later development. Cells were harvested at the indicated times (h) of development and were resuspended in 10% TCA. GSH levels were analyzed using HPLC system coupled with an electrochemical detector as described in Materials and methods. The values represent the mean ± standard deviation of three independent experiments. (C) Analysis on the slug migration of KAx3, Grx1^{OE}, and Gsr^{OE}. The cells developed for 40 h in the dark were transferred into a transparency film and were stained with Coomassie blue. Scale bar, 10 mm.

migration of Grx1^{OE} cells was indistinguishable from that of KAx3 cells. Since fruiting bodies were normally formed regardless of the concentrations of hydrogen peroxide, it seemed unlikely that the inhibition of slug migration could be attributed to the inhibition of other developmental stages (data not shown). Therefore, these data suggest that the prolonged migration of Grx1^{OE} slugs may be involved in the altered redox state.

In *Dictyostelium*, GSH levels increase at the aggregation stage, but decrease steadily during later development (Kim et al., 2005). The determination of GSH levels revealed that Grx1^{OE} cells contained higher GSH levels than parental KAx3 cells throughout later development, although GSH levels of two strains decreased gradually (Fig. 5B). To confirm whether the increase in GSH levels was related to the slugger phenotype, glutathione reductase (Gsr), an enzyme which can reduce GSSG, was also overexpressed in *Dictyostelium* in the same manner as Grx1 (data not shown). Gsr-overexpressing cells (Gsr^{OE}) had enhanced GSH levels, which were similar to those of Grx1^{OE} (Table 1). When three strains, KAx3, Grx1^{OE}, and Gsr^{OE} cells were allowed to develop on a KK_2 agar plate, the slugs of Gsr^{OE} cells exhibited the prolonged migration like those of Grx1^{OE} cells (Fig. 5C). Thus, these findings suggest that there is a close correlation between the increase in GSH levels and the delayed culmination.

A decrease in GSH levels accelerated the culmination

Since the increase in GSH levels made the culmination delayed, we examined whether a decrease in GSH levels promoted the culmination of *Dictyostelium*, conversely. To lower GSH levels, we generated the knockdown mutant of *gsr* by RNAi. In order to avoid lethality, the *gsr* gene silencing was conditionally accomplished using MB35/MB38 system, a tetracycline-controlled expression system (Blaauw et al., 2000). Culture in the absence of tetracycline for 2 days caused the protein levels of Gsr in Gsr knockdown cells (Gsr-RNAi) to be significantly reduced when compared with parental MB35 cells (Fig. 6A). Also, the expression of Gsr was successfully inhibited during development (Fig. 6A). In agreement with the decrease of protein levels, glutathione reductase activity in Gsr-RNAi cells dropped below 50% of that in MB35 cells (data not shown). As expected, GSH levels of Gsr-RNAi cells decreased by nearly 50% compared with MB35 cells (Table 1). When

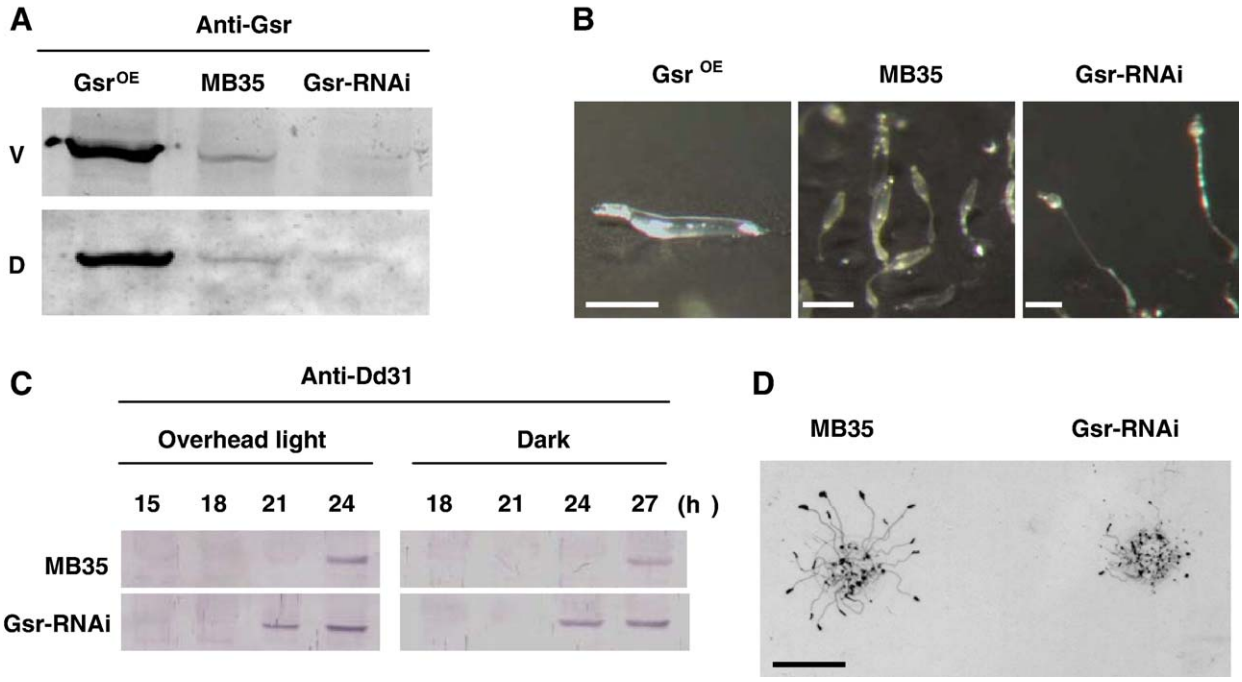

Fig. 6. Acceleration of culmination by a decrease in GSH levels. (A) Comparison of Gsr level in parental MB35, Gsr-RNAi, and Gsr^{OE} cells. To inhibit the expression of *gsr* gene, Gsr-RNAi cells were cultured for 2 days in the absence of tetracycline. Western blot analysis was performed using anti-Gsr antibodies. V, vegetative cells; D, 18-h-developed cells. (B) Developmental phenotype of MB35, Gsr-RNAi and Gsr^{OE}. After 20-h development, the structures were photographed. Scale bars, 0.5 mm. (C) Comparison of Dd31 expression pattern during development of MB35 and Gsr-RNAi. Cells were allowed to develop on nitrocellulose filters under overhead light and dark condition, respectively. Total protein extracts were prepared from cells harvested at the indicated times (h) of development. Dd31 expression was detected using Western blot analysis with anti-Dd31 antibodies. (D) Analysis on the slug migration of Gsr-RNAi and parental MB35 cells. Cells were developed for 40 h on KK_2 agar plate in darkness, and their slug trails were stained as described above. Scale bar, 10 mm.

developed, Gsr-RNAi cells formed fruiting bodies earlier than MB35 cells did (Fig. 6B); there was no difference until finger-like structures were formed. In contrast, Gsr^{OE} cells remained as a slug state while MB35 cells underwent culmination (Fig. 6B). To assure the early culmination of Gsr-RNAi cells, the expression pattern of Dd31, which was specifically expressed during culmination, was monitored. Western blot analysis showed that the Dd31 expression of Gsr-RNAi cells was initiated at least 3 h earlier than that of parental cells (Fig. 6C). Because overhead light triggered the culmination, the expression of Dd31 was earlier under overhead light condition than under dark condition (Fig. 6C). In addition, the slug migration of Gsr-RNAi cells was strongly repressed when they were allowed to develop on agar plate (Fig. 6D). Therefore, these findings indicate that the decrease in GSH levels leads to the early culmination of migrating slugs.

A difference in GSH levels affected cell fate in chimeras

Based on the observation that the expression of some prespore genes in Grx1^{OE} cells was impaired, we examined the possibility that the Grx1^{OE} cells might have a different cell fate in chimeras with control cells. The Grx1^{OE} cells marked with *act15/lacZ* were mixed at a ratio of 1:9 with unmarked parental cells and then were co-developed. Interestingly, histochemical staining for β-galactosidase activity exhibited that the marked Grx1^{OE} cells were predominantly localized in the front prestalk zone of the slugs (Fig. 7A, left panel). As a reference, when mixed with unmarked Grx1^{OE} cells, the marked Grx1^{OE} cells were evenly distributed throughout the chimeric slugs (Fig. 7A, middle panel). Furthermore, in chimeras of the marked Grx1^{OE} and the unmarked Gsr^{OE}, the stained cells were evenly distributed like reference experiment (Fig. 7A, right panel). Since GSH levels of Gsr^{OE} were similar to those of Grx1^{OE}, the prestalk tendency of Grx1^{OE} cells may result from their altered redox state.

To ensure an importance of GSH levels in cell fate decision, we performed the chimera test with Gsr^{OE} and Gsr-RNAi cells. When *act15/lacZ*-marked KAx3 cells were co-developed with unmarked Gsr^{OE} cells, they were entirely excluded from apical prestalk region in the chimeric slugs (Fig. 7B, left panel). In contrast, when Gsr-RNAi cells were used as unmarked cells, the *lacZ*-marked KAx3 cells were enriched in the apical prestalk region (Fig. 7B, middle panel). They became uniformly distributed in the chimeric slugs with unmarked KAx3 cells (Fig. 7B, right panel). Thus, these data suggest that GSH levels may affect the determination of cell fate in the chimeras with parental cells.

Discussion

Many recent studies have demonstrated that fine tuning of redox homeostasis is one of the mechanisms controlling many intracellular signaling pathways (Chiarugi and Cirri, 2003; Shelton et al., 2005; Toledano et al., 2004). The redox homeostasis is regulated and maintained by cellular redox

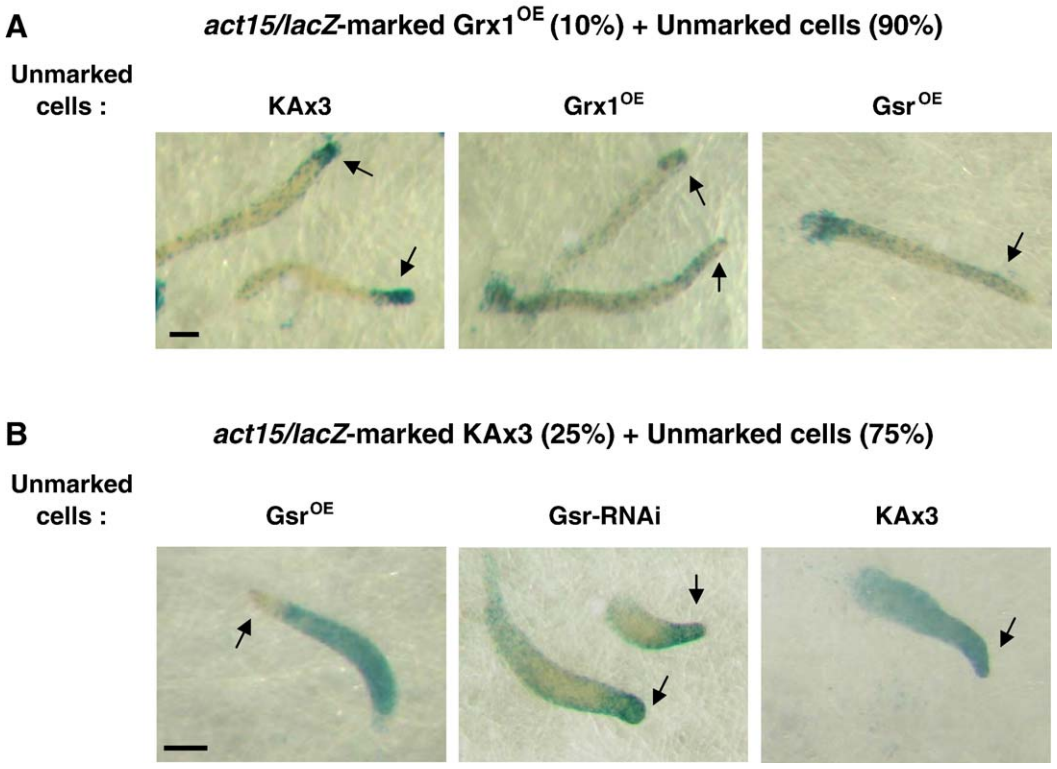

Fig. 7. Synergy experiments. (A) Localization of Grx1^{OE} cells in chimeric slugs with control cells. The Grx1^{OE} cells marked with *act15/lacZ*, which is expressed in all cell, were mixed at a ratio of 1:9 with unmarked KAx3, Grx1^{OE} and Gsr^{OE} cells, respectively, and then were allowed to form migrating slugs on nitrocellulose filters. The chimeric slugs were fixed and stained for β-galactosidase activity. Representative slugs are shown, and the arrows indicate the anterior prestalk region of slugs. Scale bars, 0.2 mm. (B) KAx3 cells marked with *act15/lacZ* were co-developed at a ratio of 1:3 with unmarked KAx3, Gsr^{OE}, and Gsr-RNAi cells, respectively. The developing cells were fixed at the slug stage and stained for β-galactosidase activity. The arrows indicate the anterior prestalk region of the slugs. Scale bars, 0.2 mm.

system such as GSH/glutaredoxin system and thioredoxin system. Since GSH is required for the development of *Dictyostelium* (Kim et al., 2005), we identified *Dictyostelium* Grx1 and investigated GSH-mediated redox signaling in the development. Grx1 belongs to a dithiol glutaredoxin family with conserved Cys-Pro-Tyr-Cys sequence and its expression was developmentally regulated. Likewise, *Dictyostelium* thioredoxins are differentially expressed during development, but their expression pattern is different from that of Grx1 (Wetterauer et al., 1992), indicating that Grx1 may have a non-overlapping function with thioredoxins.

Gain-of-function approach revealed that increased activity of Grx1 caused culmination of migrating slugs to be delayed. Especially, when the trails of slugs formed in the dark were stained, they represented a significant increase in the slug migration. However, when the slugs were exposed to a lateral light source, the migration distance of Grx1^{OE} slugs was indistinguishable from that of KAx3 slugs (data not shown), indicating that the increased migration of Grx1^{OE} slugs may be due to the delayed culmination rather than increased mobility. Previous studies have demonstrated that many slugger mutants display hypersensitivity toward ammonia and have a problem in prestalk cell differentiation (Gee et al., 1994; Hopper et al., 1993; Nelson et al., 2000; Singleton et al., 1998). Although Grx1^{OE} cells were also sensitive to ammonia inhibition (data not shown), they formed fruiting bodies normally under overhead light, which can trigger culmination, and showed the defective expression of some prespore genes. In this regard, they were different from other known slugger mutants. Unfortunately, all attempts to downregulate Grx1 levels using various methods including anti-sense expression and RNA interference have been failed, indicating that Grx1 may be essential for the growth of *Dictyostelium* like other mammalian Grxs (Chrestensen et al., 2000).

We have shown here that Grx1 not only served as an antioxidant but also maintained the cellular redox homeostasis in *Dictyostelium*. Interestingly, the overexpression of Grx1 resulted in an increase in GSH levels. Judging from the result that the cells overexpressing Gsr had higher GSH levels and showed the slugger phenotype like Grx1^{OE} cells, it seemed likely that the culmination was attenuated when GSH levels became high. However, while high GSH has been implicated in the inhibition of culmination, there is no direct evidence for this effect. It is also possible that overexpression of Grx1 acts independently to inhibit culmination. In contrast, a delay in the culmination of Grx1^{OE} slugs was reversed by the oxidants such as hydrogen peroxide, which diminishes GSH levels; thus, a decrease in GSH levels triggered the culmination. This view is well supported by the result that the Gsr knockdown, which led to a decrease in GSH levels, resulted in the early culmination. Previously, many studies have demonstrated that oxidants promote differentiation (Hansberg et al., 1993; Lara-Ortíz et al., 2003). Especially, a decrease in GSH levels during differentiation has been observed in some microorganisms such as *N. crassa* (Toledo

et al., 1995), *Candida albicans* (Thomas et al., 1991), and *Physarum polycephalum* (Allen et al., 1985). Likewise, GSH levels in *Dictyostelium* decrease steadily during later development, although they increase up to the aggregation stage (Kim et al., 2005). The increase in GSH during the early development may result from their response to the oxidants such as superoxide radicals, which are generated as a signaling molecule for aggregation (Bloomfield and Pears, 2003). Consequently, our findings provide evidence that the decrease in GSH levels may be required for the terminal differentiation of *Dictyostelium*.

Regulation of cell fate is a key step in the development of multicellular organisms. In *Dictyostelium*, aggregated cells differentiate into two major specialized cell types, prestalk and prespore cells, and a variety of activating and inhibiting signals are implicated in a decision between the cell types (Brown and Firtel, 1999). Interestingly, $Grx1^{OE}$ cells showed the prestalk tendency in the chimeric slugs with parental KAx3 cells. However, when mixed with Gsr^{OE} cells, they were not enriched in the apical prestalk region. Moreover, Gsr^{OE} cells, which had the similar GSH levels to $Grx1^{OE}$ cells, were localized in anterior prestalk zone of chimeric slugs with KAx3 cells, whereas Gsr-RNAi cells did not. Therefore, these data suggest that a difference in GSH levels may be a new determinant of cell fate in *Dictyostelium*.

Since glutaredoxin uses GSH as a reducing equivalent, it was somewhat surprising that overexpression of Grx1 resulted in an increase, not a decrease, in GSH levels. It has shown in mice that upregulation of γ-glutamylcysteine synthetase (γ-GCS), an essential enzyme in GSH synthesis, was concurrent with the induction of glutaredoxin during the recovery of mitochondrial complex I following neurotoxic insult (Kenchappa and Ravindranath, 2003). However, Northern blot analysis showed that the amount of γ-GCS mRNA in $Grx1^{OE}$ was similar to that in KAx3 cells (data not shown), suggesting that the increase in GSH levels may be not caused by transcriptional activation of γ-GCS, but by other unknown positive feedback mechanism. Taken together, our findings suggest that GSH levels are important signals for the development of *D. discoideum*, and further analysis on regulation of GSH levels will help us to understand GSH-mediated redox regulation of development.

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