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Glutathione is required for growth and prespore cell differentiation in *Dictyostelium*

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Abstract

Glutathione (GSH) is the most abundant non-protein thiol in eukaryotic cells and acts as reducing equivalent in many cellular processes. We investigated the role of glutathione in *Dictyostelium* development by disruption of γ-glutamylcysteine synthetase (GCS), an essential enzyme in glutathione biosynthesis. GCS-null strain showed glutathione auxotrophy and could not grow in medium containing other thiol compounds. The developmental progress of GCS-null strain was determined by GSH concentration contained in preincubated media before development. GCS-null strain preincubated with 0.2 mM GSH was arrested at mound stage or formed bent stalk-like structure during development. GCS-null strain preincubated with more than 0.5 mM GSH formed fruiting body with spores, but spore viability was significantly reduced. In GCS-null strain precultured with 0.2 mM GSH, prestalk-specific gene expression was delayed, while prespore-specific gene and spore-specific gene expressions were not detected. In addition, GCS-null strain precultured with 0.2 mM GSH showed prestalk tendency and extended G1 phase of cell cycle. Since G1 phase cells at starvation differentiate into prestalk cells, developmental defect of GCS-null strain precultured with 0.2 mM GSH may result from altered cell cycle. These results suggest that glutathione itself is essential for growth and differentiation to prespore in *Dictyostelium*.
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Keywords: Glutathione; γ-Glutamylcysteine synthetase; Cell fate; *Dictyostelium discoideum*

Introduction

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a ubiquitous tripeptide found in almost all organisms and the most abundant intracellular thiol in eukaryotic cells. Since oxidized glutathione (GSSG) is efficiently reduced by glutathione reductase (GR), intracellular ratio of GSH to GSSG is maintained high in most eukaryotic cells (Halliwell and Gutteridge, 1989). GSH plays important roles in many cellular processes including amino acid transport, synthesis of DNA, source of cysteine for protein synthesis, enzyme activity modulation, and defense against reactive oxygen species (ROS) (Janaky et al., 1999; Meister and Anderson, 1983; Sies, 1999). GSH is synthesized in two steps by γ-glutamylcysteine synthetase (GCS) and glutathione synthetase. The first step catalyzed by GCS is condensation of glutamic acid to cysteine and the rate-limiting step of GSH synthesis. In addition, GCS is subject to feedback inhibition by GSH (Meister and Anderson, 1983).

GCS-null mutants of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* show GSH auxotrophy, demonstrating that GSH plays an essential role in yeast (Baek et al., 2004; Chaudhuri et al., 1997; Grant et al., 1996; Wu and Moye-Rowley, 1994). In *Arabidopsis thaliana*, mutation of γ-glutamylcysteine synthetase (*rml1*) abolishes cell division in the root and exogenous GSH is necessary not only for initiating but also for maintaining *rml1* root growth (Vernoux et al., 2000). In mammalian cells, mutation of GCS results in embryonic lethality and

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blastocysts isolated from such mutant strain cannot grow without GSH or *N*-acetylcysteine (NAC) (Shi et al., 2000). GSH depletion causes an arrest of cell cycle at the G1 phase (Russo et al., 1995; Spector et al., 2001; Vernoux et al., 2000) and cell death via apoptosis in *S. cerevisiae* and *C. albicans* (Baek et al., 2004; Madeo et al., 1999). In addition, GSH concentration is related to differentiation in *Physarum polycephalum* and *C. albicans* (Allen et al., 1985; Thomas et al., 1991). Although GSH is important for development and growth, the reason is still unknown.

Dictyostelium discoideum is a social amoeba that grows as a unicellular organism, feeding on soil bacteria and dividing by binary fission. When food runs out, it undergoes the multicellular developmental cycle. Approximately 10^5 cells aggregate to form multicellular slug. The slug is composed of prestalk and prespore cells and can travel to appropriate condition where it forms a mature fruiting body composed of stalk and spore cells. It has been reported that cell-type choice is influenced by cell-cycle status of vegetative growing cells before development starts. Cells in the M, S, or early G2 phase at the time of starvation preferentially differentiate into prestalk cells, while cells in the middle or late G2 differentiate to prespore cells (Azhar et al., 2001; Gomer and Ammann, 1996; Gomer and Firtel, 1987; Ohmori and Maeda, 1987; Wang et al., 1988; Weijer et al., 1984b; Wood et al., 1996).

In spite of potential importance of glutathione in many biological processes, its roles in *D. discoideum* development have not been known until recently. To find out developmental roles of GSH, we cloned the gene (*gcsA*) encoding γ-glutamylcysteine synthetase and generated mutant that could not produce glutathione by disruption of *gcsA*. GCS-null cells showed GSH auxotrophy and other thiol-containing molecules could not rescue the growth of the mutant. When GCS-null cells precultured with 0.2 mM GSH were starved, they were arrested at mound stage or formed bent stalk-like structure. Only GSH and GSSG could rescue the developmental defect of GCS-null cells. We found that GCS-null cells had prestalk tendency and could not differentiate into prespore cells. Since GCS-null cells precultured with 0.2 mM GSH showed extended G1 phase and decreased G2 phase, prestalk tendency of GCS-null cells may be related to altered cell cycle. These results demonstrate that developmental defect of GCS-null cells results from defect in prespore differentiation and therefore glutathione is essential for *Dictyostelium* growth and development.

Materials and methods

Cell culture and development

The axenic *D. discoideum* strain KAx3 was used in all experiments and was grown at 22°C in HL5 medium (Cocucci and Sussman, 1970). GCS-null cells were grown in HL5 medium supplemented with GSH. For development, exponentially growing cells were washed twice, resuspended in development buffer (DB) (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$) and placed on nitrocellulose filter or non-nutrient agar plate (DB, 1.5% agar). All developments were performed at 22°C.

Measurement of intracellular GSH

The extraction of GSH from *D. discoideum* was carried out according to the method proposed by Pogolotti and Santi (1982) with some modifications. In brief, *D. discoideum* cells were washed twice with distilled water and resuspended in 10% trichloroacetic acid (3 volumes of wet weight). This suspension was stored for 20 min at 4°C. The insoluble residue was removed by centrifugation and the extracts were subjected to analytical HPLC using a Waters Associates Liquid Chromatography System (Millipore) linked with Hewlett Packard 1049A programmable electrochemical detector. 10 μl of trichloroacetic acid soluble extracts were passed through Agilent ZORBAX SB-C18 column and eluted with 0.1% trifluoroacetic acid.

Cloning the gcsA gene and strain construction

By using the sequence from the *Dictyostelium* genomic DNA sequence database at the Sanger Center, putative *gcsA* was amplified by polymerase chain reaction using the primers AGATCTAAAAATGGGTTTTATAGCAAAA and GGATCCTTAATTTAAAATTGAAGA. PCR amplification was carried out using cDNA from 8-h-developed cells as a template. The amplified product was cloned into pGEM-T cloning vector (Promega) and sequenced. A 2-kb *Bgl*II/*Bam*HI *gcsA* fragment was inserted into the *Bgl*II site of EXP4(+) vector. This *gcsA* expression vector under the control of the actin15 promoter was used for transformation of GCS-null mutant. The *gcsA/lacZ* expression vector was made with genomic DNA 2 kb upstream of the *gcsA* coding region. PCR amplification was carried out using the primers GGATCCAAGGAAGAATTCTTATTCC and GTATTTCCTTAAGCTTTAAAACCCAT. The amplified product was cloned into pGEM-T cloning vector and sequenced. A *Bam*HI/*Hin*dIII fragment was inserted into pDdGAL17 vector between *Bam*HI and *Hin*dIII sites. For *gcsA* disruption, the former part of *gcsA* (750 bp) amplified by PCR using the primers TTAATGTTGGAGGAAAAGAG and GGATCCCTATCAATTGAATCATATCTTGA was inserted into pGEM-T vector, yielding pGCSD1. The rear part of *gcsA* (810 bp) amplified by PCR using the primers GGATCCGATAGTTATATTGGCTCAAAG and TTTCATCATTTACGATGGAATCATG was inserted into pGEM-T vector, yielding pGCSD2. An 810-bp *Bam*HI/*Nde*I fragment from pGCSD2 was inserted into *Bam*HI/*Nde*I-digested pGCSD1. The yielded pGCSD3 was digested with *Bam*HI and ligated with a 1.3-kb *Bam*HI fragment containing a blasticidin S resistant cassette from SL63 vector. The resulting disruption construct was

digested with *Eco*RI and *Sca*I and used to transform KAx3 cells. Transformation was performed by electroporation (Pang et al., 1999). *gcsA*-null strains were selected in HL5 medium containing 10 μg/ml of blasticidin and 1 mM GSH and overexpression strains were selected in HL5 medium containing 20 μg/ml of G418. To isolate single clones, transformed cells were plated on SM agar plates (Sussman, 1987) with *Klebsiella pneumoniae*.

Southern blot and Northern blot analysis

For Southern blot analysis, 10 μg of genomic DNA extracted as described previously (Richardson et al., 1991) was digested with restriction enzymes and electrophoresed on 0.7% agarose gel. Blotting was carried out with positively charged nylon membrane. For hybridization probes, the former part of *gcsA* was labeled with DIG DNA labeling kit (Boehringer Mannheim) as described by the manufacturer. The signals were visualized with DIG nucleic acid detection kit (Boehringer Mannheim) according to the manufacturer's instructions. For Northern blot analysis, 20 μg of RNA prepared as described previously (Nellen et al., 1987) was separated on 1% agarose gel containing 0.22 M formaldehyde and blotted onto nylon membrane. The coding regions of various marker genes were generated by PCR using cDNA as template and labeled with [α-^{32}P]-dATP. All blots were prehybridized for 1 h, hybridized for 2 h, and washed twice for 20 min at 65°C.

Spore viability assay

The spore viability assay was performed as described by Dynes et al. (1994). To determine the number of viable spores, 2×10^7 cells were plated onto filters and allowed to develop. After 3 days, cells were collected from the filters and resuspended in DB with 0.4% Nonidet P-40, or incubated at 45°C for 30 min. The cells were washed three times with DB, and then spores from each sample were counted using a hemacytometer. Spores were serially diluted and plated with *K. pneumoniae* on SM agar plates.

Cell-cycle analysis

The cell-cycle analysis was performed as described by Chen et al. (2004). 2×10^7 cells were resuspended in 1.5 ml of 0.9% NaCl, 2% sucrose, 5 mM EDTA, in PDF buffer (22.2 mM potassium phosphate, pH 6.4, 20 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgSO_4$) and fixed by adding 5 ml of 90% ice-cold ethanol with gentle vortexing. The cells were incubated for 30 min at 22°C and 2×10^6 cells were collected by centrifugation. The cells were resuspended in 1 ml of propidium iodide (50 μg/ml in phosphate-buffered saline). Thirty minutes before analysis, 10 μl of 10 mg/ml DNase-free RNase A was added. The DNA content of the cells was analyzed on FACScalibur flow cytometer (Becton-Dickinson).

Results

GSH concentration increases at aggregation stage

To find out whether GSH plays important roles in *D. discoideum* development, the level of GSH during *D. discoideum* development was determined by analytical HPLC linked with electrochemical detector (Fig. 1). In initial development, the level of GSH increased and peaked at aggregation stage (8 h). The GSH level of aggregation stage was a 1.6-fold increase compared with that of vegetative growth and then the level decreased rapidly. It suggests that GSH may be important for the aggregation stage and *D. discodieum* development.

Characterization of the gcsA gene encoding γ-glutamylcysteine synthetase

Since γ-glutamylcysteine synthetase is rate-limiting enzyme in GSH synthesis and is subject to feedback inhibition by GSH, the level of GSH is directly related to GCS. We confirmed the sequence of the *gcsA* gene (DDB0186120) described by the genome sequencing project (http://www.dictybase.org) by cloning and sequencing a full-length cDNA.

The deduced amino acid sequence of γ-glutamylcysteine synthetase from *D. discoideum* is 48%, 47%, and 43% identical to those of γ-glutamylcysteine synthetase from *Drosophila melanogaster*, *Homo sapiens*, and *S. pombe*, respectively. The amino acids predicted to form the catalytic center of the active site are well conserved among different species (Fig. 2A). Among them, Cys-256 has been proposed as a putative active site residue of rat GCS (Griffith and Mulcahy, 1999). In addition, glycine-rich loop (Gly-249, Gly-251, and Gly-253) is a common motif found in phosphate-binding sites (Saraste et al., 1990).

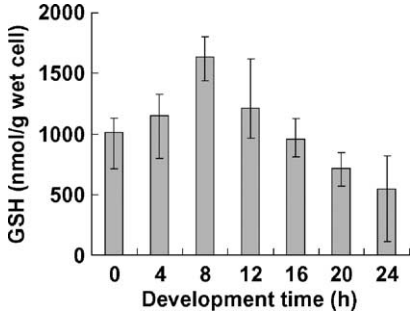

Fig. 1. GSH concentration during development. *Dictyostelium* cells were harvested at each stage and resuspended in 10% trichloroacetic acid. The insoluble residue was removed by centrifugation and the extracts were subjected to the analytical HPLC linked with electrochemical detector. During initial development, the level of GSH increased and peaked at aggregation stage (8 h). Then, the level decreased rapidly thereafter.

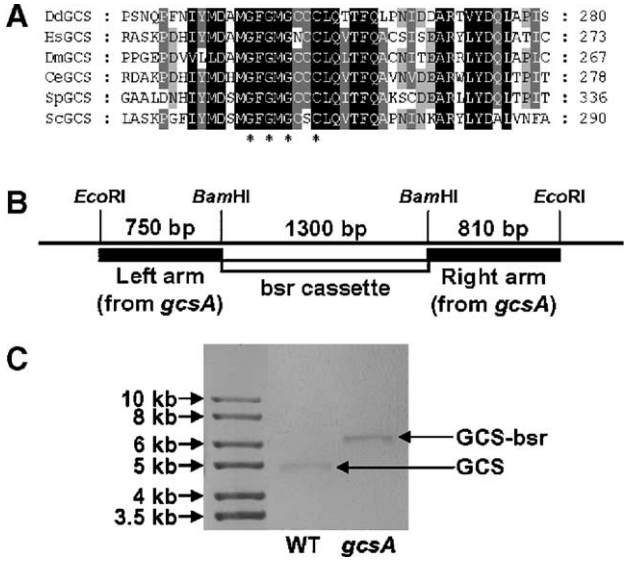

Fig. 2. Comparison of GCS amino acid sequence and disruption of GCS. (A) GCS sequence alignment in proposed active site region. From top to bottom: *D. discoideum* GCS, *H. sapiens* GCS (AAH39894), *D. melanogaster* GCS (Q9W3K5), *C. elegans* GCS (CAA90955), *S. pombe* GCS (Q09768), *S. cerevisiae* GCS (P32477). Amino acids identical to column consensus are indicated with black backgrounds whereas amino acids similar to the column consensus are indicated with shaded backgrounds. Putative active site residue (Cys-256) and glycine-rich loop (Gly-249, Gly-251, and Gly-253) are indicated by asterisks below the alignment. (B) A *gcsA*-disruption construct. A blasticidin S resistant cassette was inserted into *gcsA* open reading frame. (C) Southern blot analysis of genomic DNA isolated from KAx3 and *gcsA*-disruption mutant cells. The genomic DNA digested with *Eco*RI was separated on a gel and transferred to a nylon membrane. The Southern blot was probed with a radiolabeled 750-bp former fragment of *gcsA* open reading frame. In KAx3, the *Eco*RI digest yielded 4.9 kb band. The band of *gcsA*-disruption mutant was increased to 6.2 kb by the insertion of 1.3 kb blasticidin S resistant cassette.

gcsA gene expression increases in prespore region at aggregation and culmination stages

To reveal the developmental regulation of *gcsA*, we investigated the temporal and spatial expression patterns of *gcsA* during *D. discoideum* development. Total RNA was extracted at every 4 h and applied to Northern blot analysis. As shown in Fig. 3A, *gcsA* expression level was low in vegetative cells. However, the level increased during aggregation (8 h) and decreased rapidly thereafter. The level of *gcsA* showed second peak during culmination (20 h). These results are in good agreement with the previous microarray data (Iranfar et al., 2001).

To investigate the spatial pattern of *gcsA* gene expression, *gcsA* upstream sequence was found out from the genome sequencing project (http://www.dictybase.org). Using this upstream sequence (Chromosome 4, position 2294952–2296000), the vector containing the *lacZ* reporter gene driven by *gcsA* upstream region (1.8 kb) was prepared and KAx3 was transformed with this vector. During development, β-galactosidase expression was examined in KAx3 cells transformed with this construct (Fig. 3B). When tipped aggregate was formed, $lacZ^+$ cells were localized in the prespore region and tip region showed no detectable *lacZ* expression. At slug stage, *lacZ* staining was found predominantly in the posterior prespore region. While weak staining was observed in PstO region at slug stage, β-galactosidase expression was not detected in PstA region of the slug. When fruiting body was formed, β-galactosidase expression was detected in basal disk and spore region containing upper cup and lower cup. These results demonstrate that GCS is induced mainly in prespore region during development and GSH could play a role in prespore cells.

GCS-null cells absolutely require GSH for growth

Since *gcsA* expression is regulated during *D. discoideum* development, GSH-deficient strain was generated in order to examine developmental roles of GSH. To make GSH-deficient strain, *gcsA*-disruption vector was prepared by insertion of a blasticidin S resistant cassette into *gcsA* ORF (Fig. 2B). The linearized DNA was used to transform KAx3 cells and transformants were selected on HL5 medium containing 10 μg/ml of blasticidin and 1 mM GSH. A *gcsA* disruptant was obtained by repeating selection and disruption of *gcsA* was confirmed by Southern blot analysis (Fig. 2C). In addition, intracellular GSH concentration in mutant was proportional to GSH supplemented in media and especially that in GCS-null cells cultured with 1 mM GSH was similar to that in parental KAx3 cells (Table 1).

When GSH was withdrawn from HL5 medium, GCS-null cells underwent growth arrest after 1 day and then died 5 days later. At least 0.2 mM GSH could support growth of GCS-null cells in shaking culture but GCS-null cells could grow at 0.05 mM GSH in plate culture. GCS-null strain cultured with lower concentration than 1 mM GSH showed lower growth rate than parental KAx3. To determine

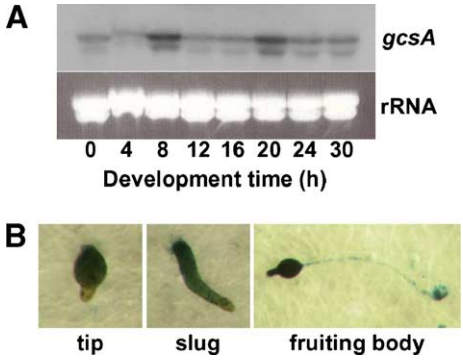

Fig. 3. Expression of *gcsA* during development. (A) Northern blot analysis of *gcsA*. Total RNA was extracted at 4-h intervals during development and Northern blot analysis was probed with a radiolabeled fragment of *gcsA* cDNA. rRNA was used as the loading control. *gcsA* expression level was at a maximum during aggregation (8 h) and decreased rapidly thereafter. The level of mRNA increased again during culmination (20 h). (B) Spatial pattern of *gcsA* expression during development. KAx3 cells carrying an *gcsA/lacZ* reporter construct were stained for β-galactosidase activity at different developmental stages. Those cells expressing *lacZ* from the *gcsA* promoter were stained blue.

Table 1
Levels of GSH in GCS-null strain and BSC strain

Cell type	GSH (nmol/g wet cell)
KAx3	1012 ± 263
gcsA-null (0.2 mM GSH)	201 ± 28.5
gcsA-null (0.5 mM GSH)	392 ± 83.2
gcsA-null (1 mM GSH)	973 ± 241
gcsA-null (2 mM GSH)	1161 ± 206
gcsA-null (0.2 mM GSSG)	327 ± 69.3
gcsA-null (1 mM GSSG)	890 ± 302
BSC9	870 ± 298
BSC10	1702 ± 271

The GSH levels of *gcsA*-null strain were determined as described in Materials and methods. Results are the average of separate experiments ± the standard deviation.

whether GCS-null mutant has an absolute requirement for GSH, mutant cells were cultured with various thiol-containing molecules (Fig. 4). Although *N*-acetylcysteine (NAC) delayed growth arrest of GCS-null cells, thiol compounds such as NAC and dithiothreitol (DTT) could not recover the growth of GCS-null cells. To distinguish between a simple slowing of growth and a delay to arrest, we attempt to culture GCS-null cells upon serial passage in the medium with DTT or NAC. However, GCS-null cells could not grow in the medium containing NAC or DTT for 20 days. In addition, mutant cells could not grow in the medium containing α-lipoic acid that has been shown to elevate total glutathione level (Han et al., 1995). GCS-null cells could grow in the medium containing only GSH, GSSG, or γ-glutamylcysteine (data not shown), suggesting that GSH itself is indispensable for the growth of *Dictyostelium*.

GCS-null cells exhibit morphogenetic defects

To examine the developmental phenotype of GCS-null cells, GCS-null cells were grown in HL5 medium supplemented with 0.2 mM GSH, washed twice with DB and then plated on black filter under overhead light. GCS-null cells formed mounds with normal kinetics (8 h), but they were arrested at mound stage (Fig. 5A). Most of aggregates could not progress further development. Some aggregates could form fingers and their terminal structures were bent stalk-like structures (Fig. 5B). These terminal structures consisted of enlarged bases and twisted transparent stalks with no spore mass. When GCS-null cells cultured with 0.2 mM GSH were plated on non-nutrient agar plates in the dark, some aggregates could form slugs and finally formed bent stalk-like structures (Fig. 5C). When GCS-null cells depleted of GSH for more than 1 day were plated on black filter or non-nutrient agar plate, they could not aggregate. GCS-null cells cultured with 0.5 mM GSH displayed a delay at slug stage, but most of slugs formed fruiting bodies. Although sori appeared glassy, GCS-null cells precultured with more than 1 mM GSH could form fruiting bodies with spores (Fig. 5D). We examined whether thiol compounds could rescue the developmental defect of GCS-null cells (Fig. 5E). When they were plated on agar plate containing 0.2 mM GSH or 1 mM GSH, GCS-null cells could form fruiting bodies with spore masses. When GCS-null cells were plated on plates containing GSSG, some aggregates could form fruiting bodies with spore masses, but most aggregates could not progress further. When plated on plates containing NAC or DTT, they failed to form a spore mass at the end of morphogenesis regardless of thiol-containing molecule concentration. These results suggest that GSH itself rather than redox properties of GSH is indispensable for *Dictyostelium* development.

To examine whether spores in glassy sorus are normal, numbers and viabilities of spores of GCS-null strain precultured with different GSH concentration were measured (Table 2). The numbers of mutant spores were dependent on GSH concentration that the mutant had been

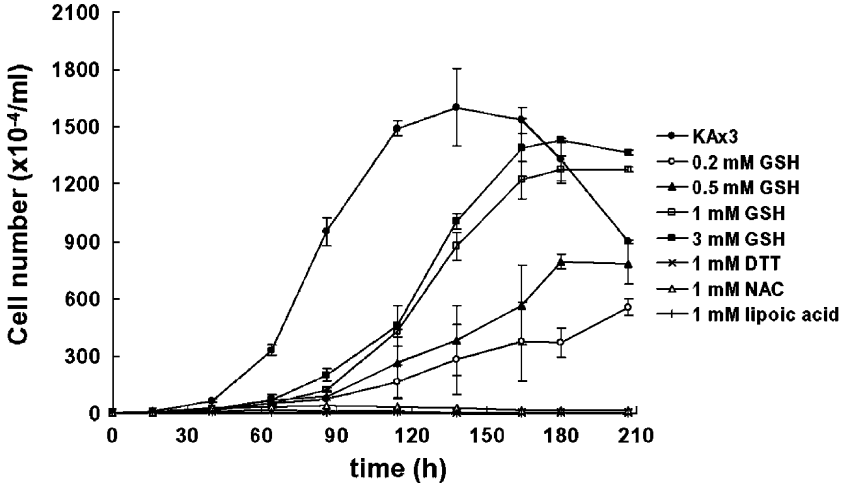

Fig. 4. Growth of GCS-null cells. Cells were cultured in shaking culture in HL-5 medium containing various thiol-containing molecules at 22°C. Cells were counted with a hemacytometer. Only GSH can support GCS-null cell growth.

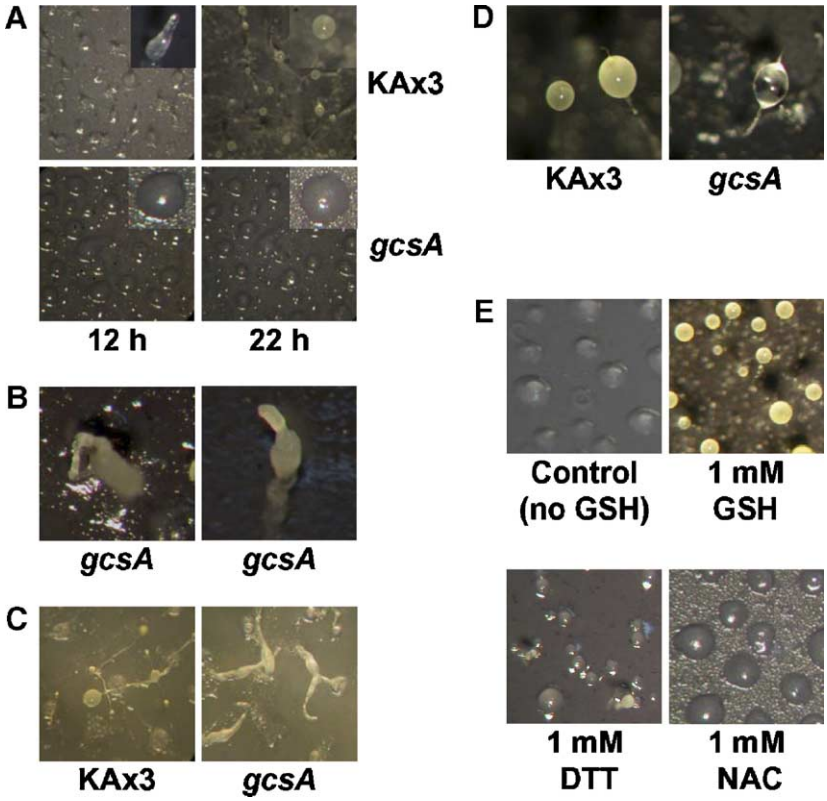

Fig. 5. Developmental morphology of KAx3 and GCS-null cells. (A) *Dictyostelium* cells were developed on black filter under overhead light. GCS-null cells cultured with 0.2 mM GSH formed mound, but almost aggregates were arrested at this stage. The insets indicate magnification of representative cells. (B) When GCS-null cells cultured with 0.2 mM GSH were developed on black filter, some aggregates formed abnormal terminal structure. (C) *Dictyostelium* cells were developed on agar under dark condition. GCS-null cells cultured with 0.2 mM GSH formed bent stalk-like structures. (D) GCS-null cells cultured with 1 mM GSH formed glassy sorus. (E) GCS-null cells cultured with 0.2 mM GSH were developed on black filter containing thiol compounds.

cultured with. GCS-null cells starved for 1 day could not form any spore and those precultured with more than 0.2 mM GSH could form spores. However, most of spores originated from GCS-null cells were not viable regardless of the supplemented GSH concentration, with which GCS-null cells had been precultured. It indicates that GSH could be involved in transformation to spore and/or in spore germination.

Table 2
Spore formation efficiency and viability

Cell type	Spore formation[a] (%)	Viability[b] (%)
KAx3	100	100
gcsA-null (0 mM GSH)	0	0
gcsA-null (0.2 mM GSH)	1.8 ± 0.85	0
gcsA-null (0.5 mM GSH)	38 ± 11	0
gcsA-null (1 mM GSH)	52 ± 7.2	2.8 ± 1.4
gcsA-null (2 mM GSH)	59 ± 9.5	0
BSC9 (gcsA$^-$[*Act15/gcsA*])	19 ± 5.7	91 ± 18
BSC10 (gcsA$^-$[*Act15/gcsA*])	38 ± 11	93 ± 11

The percentages of spore formation efficiency and viability were determined as described in Materials and methods. Results are the average of separate experiments ± the standard deviation.

[a] The spore number of the KAx3 sample was set at 100%.

[b] The ratio of germinated spores to the total number of spores. The viability of the KAx3 sample was set at 100%.

In GCS-null cells precultured with 0.2 mM GSH, prestalk-specific gene is induced but prespore-specific genes and spore-specific gene are not detected

To gain more information on the development of GCS-null cells, we examined the expression of developmentally regulated genes by Northern blot (Fig. 6A). *csaA* is used as a marker for aggregation stage gene expression and *lagC* is used as a marker for post-aggregative gene expression. In GCS-null cells precultured with 0.2 mM GSH, *csaA* was induced 4 h later than in KAx3 cells, and did not decrease up to 20 h. *lagC* of GCS-null cells was induced similar to that of KAx3 cells, but remained high until 24 h. These extended expressions of *csaA* and *lagC* corresponded to the developmental arrest of GCS-null cells at the mound stage. Prestalk-specific gene *ecmA* was induced 8 h later in GCS-null cells than in KAx3 cells and the *ecmA* expression level of GCS-null cells was similar to that of KAx3 cells. However, expressions of the prespore-specific gene *cotC* and *pspA* were not detected in GCS-null cells. In addition, spore-specific gene *spiA* expression was not detected in GCS-null cells. Although spore-specific *spiA* expression was not detected by Northern blot analysis, it may be possible that *cotC* was expressed at very low

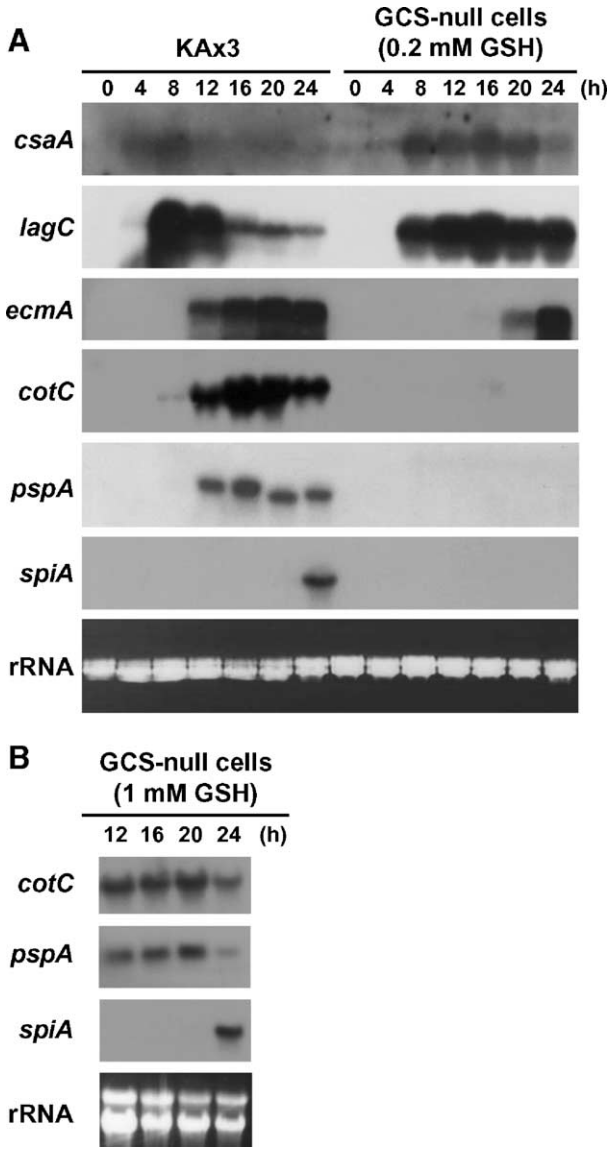

Fig. 6. Developmental gene expression in KAx3 and GCS-null strain. Each strain was developed on agar plates and total RNA was extracted at the indicated times. Total RNA was blotted onto a nylon membrane, and probed with radiolabeled fragments of the indicated genes. rRNA was used as the loading control. *csaA*, aggregation-specific gene; *lagC*, post-aggregative genes; *ecmA*, prestalk-specific; *cotC* and *pspA*, prespore-specific; *spiA*, spore-specific. (A) KAx3 and GCS-null cells precultured with 0.2 mM GSH. (B) GCS-null cells precultured with 1 mM GSH.

level since GCS-null strain precultured 0.2 mM GSH formed visible spores (Table 2).

On the contrary, prespore-specific and spore-specific gene expressions of GCS-null mutant precultured with 1 mM GSH were similar to those of KAx3 strain (Fig. 6B). These results indicate that GCS-null cells precultured with 0.2 mM GSH could differentiate into prestalk cells, but not differentiate into prespore cells. It suggests that morphological and developmental defects of the GCS-null cells precultured with 0.2 mM GSH may be caused by the defect of differentiation to prespore cells and GSH may be essential for differentiation into prespore cells.

Expression of GCS from the constitutive actin promoter in GCS-null cells can partially rescue the developmental defect of GCS-null cells

GCS-null cells were transformed with the construct expressing *gcsA* under the control of the constitutive actin15 promoter. Transformants were selected in HL5 medium containing G418 and blasticidin and 10 clones (BSC1–10) were obtained. Among BSC clones (*gcsA*$^{-}$[*Act15*/*gcsA*]), GSH concentrations in BSC9 and BSC10 were 86% and 168% of that in parental KAx3, respectively (Table 1). BSC9 and BSC10 could grow without GSH and form fruiting bodies with viable spores. However, more than half of aggregates were arrested at tight-mound stage. As a result, spore formation efficiency of them was lower than that of KAx3 cells but most of spores of BSC9 and BSC10 were viable (Table 2). It suggests that proper regulation of *gcsA* is required for *D. discoideum* development.

Dictyostelium amoebae lacking GCS form prestalks rather than prespores in chimeras with parental KAx3

In order to find out whether parental KAx3 cells can rescue the developmental defect of GCS-null cells, GCS-null cells precultured with 0.2 mM GSH and KAx3 cells were mixed in various ratios and allowed to develop on black filter (Fig. 7A). When KAx3 cells and GCS-null cells were mixed in a ratio of 1:3, chimeras could form culminant after 20 h and finally form fruiting bodies but fruiting bodies of chimeras had smaller spore masses than those of KAx3. When KAx3 cells and GCS-null cells were mixed in a ratio of 1:9, chimeras could form fruiting bodies in spite of small proportion of KAx3 cells. However, spore masses of 1:9 chimera were smaller than those of 1:3 chimera. As the fraction of the KAx3 cells in the chimera decreases, spore masses of chimera fruiting bodies decrease, suggesting that spores of chimera are derived from KAx3 cells. To confirm the result that spores from the chimeric fruiting bodies were derived from KAx3 strain, KAx3 cells and GCS-null cells expressing GFP were mixed in various ratios and developed. Spores from the chimeric fruiting bodies showed no GFP signal and their amoeba could grow well without GSH, supporting that all spores were derived from KAx3 (data not shown). To examine localization of KAx3 cells in chimeras of KAx3 and GCS-null cells, KAx3 cells expressing GFP were mixed with GCS-null cells precultured with 0.2 mM GSH in ratios of 1:3 and 1:9 and allowed to develop (Fig. 7B). When chimeras formed slugs, KAx3 cells were concentrated in the posterior prespore region of the slugs, and when fruiting bodies formed, KAx3 cells were localized to spore region (Fig. 7C). Next, we examined whether GCS-null cells precultured with 0.2 mM GSH had prestalk tendency during development. GCS-null cells expressing GFP were mixed with KAx3 cells in a ratio of 1:3 and allowed to develop (Fig. 7D). Localization of GCS-null cells precultured with 0.2 mM GSH was anterior prestalk

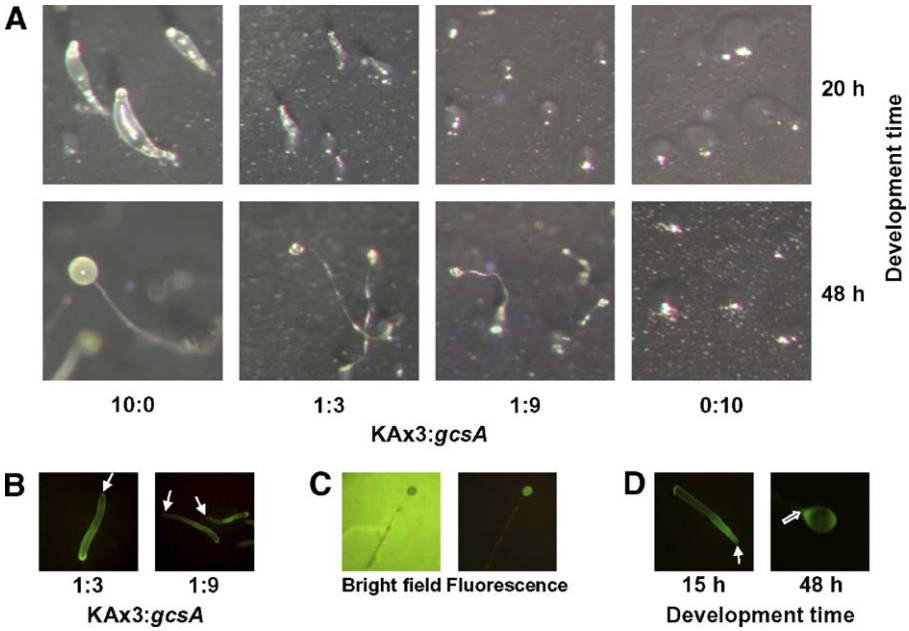

Fig. 7. Development of chimeras of KAx3 and GCS-null cells and localization of KAx3 cells and GCS-null cells in chimeras. (A) GCS-null cells cultured with 0.2 mM GSH and KAx3 cells were mixed at the indicated ratios and allowed to develop on black filter. (B) Localization of KAx3 cells expressing GFP in chimeric slug of GCS-null and KAx3 cells. GCS-null cells cultured with 0.2 mM GSH and KAx3 cells were mixed at indicated ratios and allowed to develop on agar plate. Arrowheads point to the anterior region of slugs. (C) Localization of KAx3 cells expressing GFP in chimeric fruiting body of GCS-null and KAx3 cells. GCS-null cells cultured with 0.2 mM GSH and KAx3 cells were mixed in a 1:3 ratio and allowed to develop on agar plate. (D) Localization of GCS-null cells expressing GFP in chimeras of GCS-null and KAx3 cells. GCS-null cells cultured with 0.2 mM GSH and KAx3 cells were mixed at a ratio of 1:3 and allowed to develop on agar plate. Open arrowhead indicates the lower cup of sorus.

region in chimeric slugs. In addition, when fruiting body was formed, GCS-null cells were localized to upper cup and lower cup region of spore head. It suggests that GCS-null cells precultured with 0.2 mM GSH have prestalk tendency. Taken together, these results suggest that since KAx3 cells in chimera differentiate into prespore cells, chimera can form fruiting bodies with spore mass and developmental phenotype of GCS-null cells is due to cell autonomous manner rather than intercellular signaling.

Lowering GSH in D. discoideum increases the percentage of G1 phase cells

There have been reports that choice of cell type differentiation is influenced by cell-cycle phase of growing cells in *Dictyostelium* (Araki et al., 1994; Weijer et al., 1984a; Zimmerman and Weijer, 1993; Wood et al., 1996). At starvation, the cells in M, S, and early G2 phases of cell cycle differentiate into prestalk cells while the cells in late G2 phase differentiate into prespore cells. Since GSH depletion leads to cell-cycle arrest at G1 phase (Russo et al., 1995; Spector et al., 2001; Vernoux et al., 2000), extreme low GSH concentration in GCS-null cells may increase the proportion of G1 phase of cell cycle. It is possible that increased G1 phase cells and decreased G2 phase cells in GCS-null mutant influence ratio of prestalk cells to prespore cells and significant increase of the ratio might cause developmental defect. Therefore, to investigate the possibility that GCS-null cells exhibit prestalk tendency due to extended G1 phase, the cell cycle of GCS-null cells was examined by flow cytometry (Fig. 8). Because G1 phase is not detected in *Dictyostelium* axenic strain (Ohmori and Maeda, 1987; Weijer et al., 1984b), most of KAx3 cells had a DNA content greater than 2N (Fig. 8A).

To distinguish G1 phase and G2 phase in *D. discoideum*, we used hydroxyurea-treated KAx3 cells as a G1 control. Hydroxyurea, a potent inhibitor of the enzyme ribonucleotide reductase, is routinely used to induce cell-cycle arrest at the G1/S phase boundary (Borel et al., 2002) and inhibits *Dictyostelium* growth in the concentration range of 2 to 4 mM (Deering and Michrina, 1982). 2 mM Hydroxyurea treatment for 20 h shifted most KAx3 cells to the G1 phase (Fig. 8B). FACS profile of GCS-null cells precultured with 0.2 mM GSH showed G1 phase peak and G1 phase cells were 24.8% (Fig. 8C). In GCS-null cells precultured with 0.5 mM GSH, G1 phase cells were 12.8% (data not shown). In addition, GCS-null cells deprived of GSH for 12 h contained a larger portion of G1 phase cells (41.9%, Fig. 8D) than GCS-null cells precultured with 0.2 mM GSH. As GSH concentration in GCS-null cells was lower, the percentage of the mutant cells in G1 phase increased and that in G2 phase decreased.

Since these decreases in cellular DNA content may be caused by the loss of the mitochondrial DNA, we estimated mitochondrial DNA level by Southern blot analysis with the mitochondrial DNA-specific probe *rps4* (Chida et al., 2004). Total cellular DNA was prepared from the same

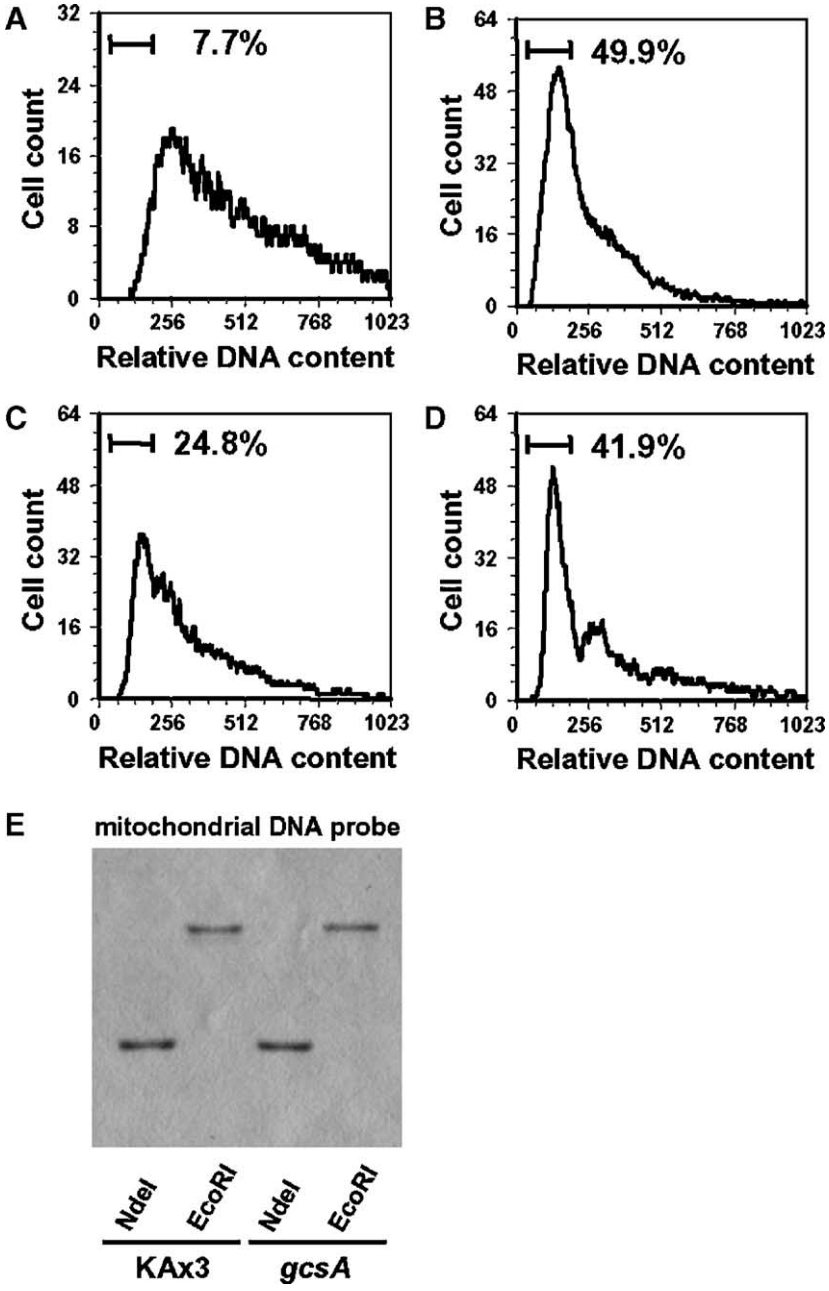

Fig. 8. Cell-cycle phases in GCS-null cells. (A–D) FACS profiles of KAx3 and GCS-null cells. The DNA content of exponentially growing cells was measured by flow cytometry after propidium iodide staining. The bar indicates the range of cells in G1 phase (N). The percentages of cells in G1 phase for KAx3 and GCS-null strain were determined by flow cytometry and were the average of separate experiments. GCS-null cells show extended G1 phase. (A) KAx3 cells, (B) KAx3 cells incubated with 2 mM hydroxyurea for 20 h, (C) GCS-null cells cultured with 0.2 mM GSH, (D) GCS-null cells deprived of GSH for 12 h. (E) Mitochondrial DNA level of KAx3 and GCS-null cells. Total DNA from KAx3 cells and GCS-null cells deprived of GSH for 12 h was digested with the indicated restriction enzymes and electrophoresed. After the size-fractionated DNA fragments were transferred to nylon membranes, they were hybridized with the DIG-labeled mitochondrial DNA-specific probe *rps4*.

number of KAx3 cells and GCS-null cells deprived of GSH for 12 h, digested with restriction enzymes and compared after Southern blotting (Fig. 8E). This result showed that the mitochondrial DNA level of GCS-null strain was similar to that of KAx3 strain and therefore the decrease in cellular DNA content was caused by shift of the GCS-null cells from G2 into the G1 phase. These results indicate that lowering GSH in *D. discoideum* increases the proportion of G1 phase cells and as a result of extended G1 phase, GCS-null cells may show prestalk tendency.

Discussion

GSH is essential in eukaryotes and plays important roles in many cellular processes including development. How-

ever, little is known about the role of GSH in *D. discoideum* development.

We cloned *gcsA* gene encoding γ-glutamylcysteine synthetase from *D. discoideum* and generated the *gcsA* disruptant by homologous recombination. In mammal, GCS is a heterodimer that consists of heavy subunit and light subunit. GCS heavy subunit has all of the catalytic activity and GCS light subunit has a role in regulating glutathione synthesis. However, GCS from invertebrates except *D. melanogaster* does not have functional regulatory subunit (Fraser et al., 2002). There is no candidate gene in *D. discoideum* database with high similarity to GCS light subunit. Although *D. discoideum* GCS shows high sequence similarity to *D. melanogaster* GCS catalytic subunit and human GCS catalytic subunit, it may function as a monomer like other invertebrate.

Northern blot analysis showed that transcript level of GCS increased during aggregation. In addition, transcript level and activity of GR increased during aggregation (our unpublished data). Therefore, the increase of GSH concentration at aggregation stage (8 h) results from the increase of GR and GCS. After aggregation stage, the level of GSH decreased. This decrease of GSH may be, at least in part, due to increase of glutathione *S*-transferase (GST). Northern blot analysis and microarray data showed that transcript of *gstA* increased at 12 h and remained high during development (Iranfar et al., 2001). Therefore, it is likely that in spite of increase of *gcsA* at culmination stage, GSH level decreases after aggregation stage. These results suggest that GSH is important to aggregation stage in *D. discodieum* development. GSH concentration changes during development were reported in slime mold *P. polycephalum* and dimorphic fungi *C. albicans* (Allen et al., 1985; Thomas et al., 1991). GCS expression during development showed spatial patterning as well as temporal patterning. GCS expression was selectively induced in prespore region, suggesting that GSH is required for sporulation.

GCS-null cells showed GSH auxotrophy like other eukaryotes, but other thiol compounds except GSH and GSSG could not rescue the defective growth of *D. discoideum* GCS-null mutant. Although 1 mM NAC delayed arrest of growth, finally GCS-null cells died. In yeast and mammals except *C. albicans*, GSH can be replaced by other thiol compounds such as NAC and DTT. It suggests that not the redox property of GSH but GSH itself is related to GSH auxotrophy in *D. discoideum*. The enzymes specific for GSH such as glutaredoxin may be essential for growth of *D. discoideum*.

Development of GCS-null cells was dependent on GSH concentration that GCS-null cells were cultured with and other thiol compounds could not recover development of GCS-null cells. GCS-null cells precultured with 0.2 mM GSH showed developmental arrest and GCS-null cells precultured with more than 1 mM GSH could not form a viable spore. That *gcsA* was expressed in prespore cells predominantly and transcript of *gcsA* increased at culmination stage is consistent with the result that GCS-null cells cannot form a viable spore and GSH may play an important role in spore maturation. In addition, expression of *gcsA* from actin15 promoter, which resulted in constitutive expression of *gcsA*, partially rescued the development of GCS-null cells, suggesting that delicate regulation of GSH in temporal and spatial expression is necessary for proper development.

It has been known that reactive oxygen species (ROS) influences *D. discoideum* development (Bloomfield and Pears, 2003; Garcia et al., 2000, 2003). *D. discoideum* has two catalase genes that are differentially regulated and prespore-specific catalase B-null strain displays a delay in development, and scavenging superoxide inhibits formation of aggregates. In addition, oxidative injury by GSH depletion leads to growth inhibition in plant and animal cell (May and Leaver, 1993; Arrick et al., 1982). Therefore, ROS level may increase and increased ROS may influence development in GCS-null strain. However, thiol compounds such as NAC (ROS inhibitor) could not rescue the development of GCS-null cells and only GSH could compensate for developmental defect of GCS-null cells. Although GSSG could rescue the growth defect of GCS-null cells, it could not completely rescue their developmental defect. It suggests that although redox status has an effect on development of *D. discoideum*, glutathione itself is indispensable for development of *D. discoideum*.

In GCS-null cells precultured with 0.2 mM GSH, extended expressions of aggregation-specific gene and post-aggregative gene corresponded to developmental arrest at mound stage. Considering GSH increase at mound stage, GSH may be important for the aggregation stage and low GSH concentration may cause these results. Although prestalk-specific gene *ecmA* expression was delayed in GCS-null cells, the level was similar to that of KAx3 cells. On the contrary, prespore-specific genes and spore-specific gene expression were not detected in GCS-null cells. These results coincide with the spatial patterning of *gcsA* expression. It suggests that GCS-null cells cannot differentiate into prespore cells and GSH is essential for differentiation to prespore cell.

Chimera experiments that GCS-null cells and KAx3 cells were mixed and were allowed to develop showed that even 10% KAx3 cells could make chimeric organism form fruiting bodies with spore masses and KAx3 cells localized to posterior prespore region. In addition, all spores from the chimeric fruiting body were derived from the KAx3 cells and GCS-null cells localized to anterior prestalk region. These results suggest that GCS-null cells preincubated with 0.2 mM GSH have prestalk tendency and tend to differentiate into prestalk cells. Therefore, it is possible that chimeric organism might form spores as KAx3 cells differentiated into prespore cells that GCS-null cells could not differentiate to.

D. discoideum cells in M, S, or early G2 phase at the time of starvation tend to differentiate into prestalk cells

while cells in late G2 phase tend to differentiate into prespore cells (Wang et al., 1988; Weijer et al., 1984a, Wood et al., 1996). Also, it has been known that Ca^{2+} and cytosolic pH could act as downstream signal to determine cell fate (Azhar et al., 2001; Gross et al., 1983; Maeda and Maeda, 1974; Saran et al., 1994).

The existence of G1 phase in axenic *Dictyostelium* is debatable and it is not known whether cells in G1 phase differentiate into prestalk cell or prespore cell. However, bacterially grown NC4 has a G1 phase and amoebae in the G1 phase contain relatively high Ca^{2+} levels like amoebae in S and early G2 phases (Azhar et al., 2001). In addition, when GCS-null cells were deprived of GSH for 2 days, more than 80% of the total cells were in G1 phase. When these GCS-null cells in G1 phase were plated on agar plate containing 0.2 to 1 mM GSH, they were arrested at mound or finger stage and could not form a spore mass at the end of development (data not shown). Therefore, it is postulated that cells in G1 phase may also tend to differentiate into prestalk cells. To prove this proposition, we compared DNA content of mutant strain with that of parental strain. First of all, FACS analysis (Fig. 8) showed that G1 phase of axenic *Dictyostelium* was observed during growth. This result clearly demonstrates the existence of G1 phase in axenic *Dictyostelium* and suggests that GSH is required in entry into S phase or to traverse G1 phase. Depletion of GSH in human cell line induces p21, an inhibitor of cyclin-dependent kinase activities that are necessary for progression of G1 phase into S phase (Russo et al., 1995). In *Arabidopsis*, an adequate level of GSH is required for G1-to-S phase transition by modulating cyclin activity (Vernoux et al., 2000). Therefore, it is likely that disruption GCS in *Dictyostelium* leads to a cell division block during the G1 phase by regulating cyclin activity. This cell-cycle block by mutation offers a good experimental tool to study cell cycle by synchronizing *Dictyostelium* cells. Based on the result that GCS-null cells exhibit prestalk tendency and arrest in G1 phase, it is suggested that increase of G1 phase cells may influence initial cell type differentiation and prevent development beyond mound stage.

It was reported that initial cell type choice was independent of cell-cycle phase at starvation in *rtoA* mutant (Wood et al., 1996). *rtoA* mutant stalls in development at the tight-mound stage, with ~90% of the aggregates never progressing further. The developmental phenotype and prestalk tendency of *rtoA* mutant are similar to those of GCS-null mutant. Ratio of prestalk cells is important to proper development and significant increase of the ratio might cause developmental defect.

Our results indicate that GSH is indispensable to the growth of *D. discoideum* and is related to cell type differentiation. In addition, precise regulation of GSH may be required for proper development of *D. discoideum*. Further investigations of GCS-null cells will lead to better understanding of roles of GSH in *D. discoideum* development.

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