Glutathione initiates the development of Dictyostelium discoideum through the regulation of YakA

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1. Introduction

Glutathione (γ-L-glutamyl-L-cysteinylglycine) is the most abundant intracellular non-protein thiol. It is a tripeptide found in almost all organisms. Under physiological conditions, most cellular constituents are reduced. In cells, glutathione is maintained in the reduced form (GSH) by the action of glutathione reductase and NAD(P)H. Because the oxidized form (GSSG) is efficiently reduced, the intracellular ratio of GSH to GSSG is high in most eukaryotic cells [1]. GSH plays important roles in diverse cellular functions; for example, GSH is involved in antioxidant defenses [2,3] and functions in the regulation of intracellular redox status, signal transduction, cell proliferation and death, immune responses [4–9], detoxification, and prostaglandin metabolism [10–12].

GSH is synthesized sequentially from glutamate, cysteine, and glycine by two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS) and glutathione synthase (GSS). GCS catalyzes the rate-limiting step in GSH synthesis and is subject to feedback inhibition by GSH [4]. Depletion of GSH by disrupting the gene which encodes GCS leads to GSH auxotrophy [13–17], cell cycle arrest [18,19], and apoptosis [20,17] in some microorganisms, plants, and mammals. Further, in plants and mammals, GCS-null mutations cause early embryonic lethality and failure of multi-organ differentiation [21–24]. These reports show that GSH plays critical roles in cell growth and development.

The life cycle of Dictyostelium discoideum comprises two phases. During the vegetative phase, cells grow as solitary amoebae and feed on bacteria in the soil. When nutrients are exhausted, cells stop growing and aggregate to develop into a multicellular structure with only two main cell types, the spore and stalk [25,26]. Coordinated cell-type differentiation and morphogenesis lead to a final fruiting body that allows the dispersal of spores. The study of the transition from proliferation to differentiation has increased our understanding of general developmental mechanisms, because the complex signaling networks that control Dictyostelium development are conserved in other organisms.

The transition from growth to differentiation is regulated by complex molecular mechanisms that control the activities of numerous regulators. Upon starvation, the expression of vegetative genes is reduced, whereas genes required for development are induced. Several genes regulating the transition from growth to development are present in Dictyostelium [27]. For example, members of the discoidin I gene family are among the first to be activated by starvation responses. During growth, cells secrete prestarvation factor (PSF) and estimate their density relative to the concentration of nutrients. When PSF reaches an appropriate concentration, it induces the expression of dscA, which

Abstract

Reduced glutathione (GSH) is an essential metabolite that performs multiple indispensable roles during the development of Dictyostelium. We show here that disruption of the gene (gcsA) encoding γ-glutamylcysteine synthetase, an essential enzyme in GSH biosynthesis, inhibited aggregation, and that this developmental defect was rescued by exogenous GSH, but not by other thiols or antioxidants. In GSH-depleted gcsA−/− cells, the expression of a growth-stage-specific gene (cprD) was not inhibited, and we did not detect the expression of genes that encode proteins required for early development (cAMP receptor, carA/cAR1; adenylyl cyclase, acaA/ACA; and the catalytic subunit of protein kinase A, placA/PCA-C). The defects in gcsA−/− cells were not restored by cAMP stimulation or by cAR1 expression. Further, the expression of yakA, which initiates development and induces the expression of PKA-C, ACA, and CAR1, was regulated by the intracellular concentration of GSH. Constitutive expression of YakA in gcsA−/− cells (YakAOE/gcsA−) rescued the defects in developmental initiation and the expression of early developmental genes in the absence of GSH. Taken together, these findings suggest that GSH plays an essential role in the transition from growth to development by modulating the expression of the genes encoding YakA as well as components that act downstream in the YakA signaling pathway.

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Abbreviations:
GSH, reduced glutathione; GSSG, oxidized glutathione; PSF, prestarvation factor; CMF, conditioned medium factor; cAR1, cAMP receptor; ACA, adenylyl cyclase; PKA, protein kinase A; DTT, dithiothreitol; NAC, N-acetylcysteine; mBBr, monobromobimane

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encodes the discoidin I alpha chain, and prepares cells for developmental initiation. Discoidin accumulates continuously during early development until its transcription is inhibited by extracellular cAMP at the end of the aggregation phase. Thus, the expression of discoidin is an excellent indicator of the cell state in the developmental life cycle. The expression of dia2 (differentiation-associated protein) indicates the transition from growth to development. According to Chae et al. [28] and Hirata et al. [29], dia2 transcripts accumulate exclusively in differentiating cells.

The activation of key components of the cAMP signaling system, such as the major cAMP receptor (cAR1) and the aggregation-stage adenylyl cyclase A (ACA), is one of the earliest responses to starvation in Dictyostelium [30–32]. When nutrients are depleted, the cells stop growing and activate starvation responses by secreting a glycoprotein called conditioned medium factor (CMF). Secreted CMF is required to activate cAMP signaling and to initiate aggregation [33–35]. Certain starved cells secrete cAMP, which stimulates neighboring cells to migrate toward cAMP until an aggregate is formed. Further, cAR1 recognizes secreted cAMP and induces the production of additional cAMP by activating ACA [36].

Protein kinase A is a central mediator of development that regulates the levels of expression of genes that respond to cAMP signaling. The expression of pkaC encoding protein kinase A (PKA)-C (catalytic subunit of PKA) is activated at the onset of development. Increased pkaC expression parallels the activity of PKA and triggers the expression of ACA and cAR1 [37–40]. The disruption of PKA-C leads to developmental failure [41], while overexpression of PKA-C or the disruption of PKA-R, the regulatory subunit of PKA, induces rapid development [42–44].

The YakA signaling pathway comprises YakA, PuFA, and PKA (cAMP-dependent protein kinase A) and is activated early when cells are starved. YakA is a member of the dual-specificity tyrosine-related kinase (DYRK) family of serine/threonine kinases and a homologue of yeast Yak1p growth-regulating protein kinase [45]. During growth, yakA mRNA accumulates and reaches a maximum level at the time of starvation. YakA induces developmental processes such as growth arrest, downregulates the expression of genes encoding vegetative functions, and upregulates the expression of PKA-C, ACA, and cAR1 [45,46]. The initiation of development requires the cooperative and tight regulation of the expression of these genes. Further research will be required to understand fully the mechanisms underlying the transition from growth to development.

We previously reported that intracellular GSH plays critical roles during the growth and development of Dictyostelium [18,47]. For example, the growth of a γ-glutamylcysteine synthetase null-mutant (gcsA−) is arrested by methylglyoxal accumulation [19]. The developmental status of gcsA− cells is determined by the concentration of intracellular GSH [18,47]. Although GSH deficiency affects the development in Dictyostelium, animals, and plants [23,24], the precise mechanism of action of GSH remains unclear.

2. Materials and methods

2.1. Cell culture and induction of development

*Dictyostelium discoideum* wild-type strain KA3x and KA3x mutants were grown axenically with shaking at 22 °C in liquid medium (HL5) containing 100 μg/ml of streptomycin and 100 units/ml of penicillin. Every cell culture was harvested during exponential growth. To deplete completely intracellular GSH in gcsA− cells [18], cells were grown exponentially in HL5 media with 1 mM GSH and were then reinoculated at a density of 2 × 10^6 cells/ml in media containing 0.5 mM GSH. cAMP-dependent cells were washed twice with non-nutrient KK2 buffer and then plated at a density of 2.5–3 × 10^6 cells/cm² on a nitrocellulose filter or on 1.5% KK2 agar plates. To induce development in suspension, washed cells were developed in KK2 buffer at a density of 1 × 10^7 cells/ml and shaken at 150 rpm at 22 °C. During the development of GSH-depleted gcsA− cells, 1 mM GSH was added to KK2 buffer or was not added. Stimulation with cAMP was performed by adding 3 × 10^−8 M cAMP every 6 min after 2 h [48].

2.2. cAR1 and YakA expression constructs

Full-length carA (1.3 kb) was amplified using polymerase chain reaction (PCR) with genomic (g)DNA as the template. The PCR-primer sequences were as follows: forward 5′-GGATCCTGGAATGAGGTATGACGAC-3′ and reverse 5′-CTCGAGATGTCCTGACAGAATG-3′. A cAR1 fragment (1.3 kb) was amplified by cloning the full-length gDNA carA amplicon into the Exp4(+) vector containing a constitutively active Act15 promoter [49]. The constructs were introduced into KA3x or gcsA− cells using electroporation [50], and transformants were selected and maintained in medium containing 10 μg/ml of G418. To express YakA, full-length yakA (4.3 kb) was amplified into two fragments by using PCR with complementary (c)DNA as the template; these fragments were then ligated to each other. The primer sequences of yakA fragment 1 (1 to 2621) for PCR were as follows: forward, 5′-CAATAGGATCCATGGGTCTTTTAGATG-3′; reverse, 5′-GGATCCTGGAATGAGGTATGACGAC-3′. The primer sequences of yakA fragment 2 (2622 to 4377) were as follows: forward 5′-CAATGGAGAAATGATGCACAC-3′ and reverse 5′-CTCGAGATGTCCTGACAGAATG-3′. yakA was constitutively expressed in KA3x or gcsA− cells as described above for cAR1.

2.3. RNA extraction and northern blot analysis

Total RNA was isolated using TRizol reagent (Invitrogen) and solubilized in formamide. The RNA (20 μg) sample was separated by electrophoresis through a 1% agarose gel containing 0.22 M formaldehyde and then transferred to a nylon membrane (GE Healthcare). The specific probes were labeled with [α-32P]-dATP [51]. Hybridization was performed using various probes dissolved in Rapid-Hyb buffer (GE Healthcare) according to the manufacturer’s instructions. The blots were incubated in Rapid-hyb buffer without the probe for 1 h and then probe was added for 2 h. The blot was washed twice with SSC buffer (0.1% SDS, 0.3 M NaCl, 30 mM trisodium citrate) for 10 min at 65 °C.

2.4. Real-time RT-PCR

Each RNA sample (50 ng/μl) was reverse-transcribed into cDNA using a Superscript III Reverse Transcriptase Kit (Promega). Real-time PCR was performed in a 20-μl volume in the wells of 96-well reaction plates (Bioplates). Each PCR assay was performed using SYBR Premix Ex Taq (TaKaRa), and nrnA served as an endogenous control. Fluorescence was detected using an Applied Biosystems 7500 Real-Time PCR system. The reactions for each gene at each time point were performed in triplicate, and cycle threshold values generated from the reactions were averaged [52]. The cycle threshold values of each gene were normalized to the endogenous controls and calibrated to an average expression level for the gene analyzed.

2.5. PKA assay

PKA activity assays were performed as described previously [53] using the SignalTECT PKA Activity System (Promega). Samples were prepared from cells developed in non-nutrient KK2 buffer with cAMP pulses. Cell extracts containing 10 μg of protein were prepared as specified by the manufacturer and were used in reactions with 10 μM cAMP.
and in the presence or absence of 20 μM of the PKA-specific inhibitor PKI [43]. PKA activity is defined as the amount of the phosphorylated substrate, kemptide (nmol/min/mg of protein), in the absence of PKI minus the amount of phosphorylated substrate in the presence of PKI.

2.6. GSH concentration

To determine the intracellular GSH concentration, cell extracts were reacted with monobromobimane (mBBr) to form derivatives and then analyzed using a modification of a method described by Newton and Falhey [54]. Cells were extracted with 50% aqueous acetone containing 50 mM Hepes (pH 8.0), 2 mM EDTA, and 2 mM mBBr. After incubation at 60 °C for 15 min, the samples were acidified with 5 μl of 5 M methanesulfonic acid. Cell debris was removed from the crude extract by centrifugation at 12,000 rpm for 15 min, and the resulting supernatant was analyzed using HPLC. Control samples were treated with 5 mM N-ethylmaleimide (NEM) and incubated for 10 min before derivatization to prevent labeling of thiol groups with mBBr. The concentrations of oxidized and reduced GSH were determined using 2 mM dithiothreitol (DTT), which reduces GSSG to GSH. Samples (10 μl) were passed through a ZORBAX SB-C18 column. HPLC was performed using a Waters system equipped with a Hewlett-Packard 1050 series fluorescence detector. The mBBr-derived thiol compounds were detected using excitation and emission at 370 and 480 nm, respectively. The mobile phase consisted of buffer A (methanol, HPLC grade) and buffer B (0.1% trifluoroacetic acid). The proportion of buffer A in the continuous gradient was as follows: 15% at 0–2 min, 25% at 30 min, 100% at 34 min, 15% at 37 min, and 15% at 40 min. If necessary, samples were co-injected with GSH standards.

3. Results

3.1. The development of gcsA cells depends on the concentration of GSH

We demonstrated previously that GSH is essential for the normal development of Dictyostelium [18]. The gcsA cells exhibit different developmental phenotypes as exogenously added GSH concentration. In the present study, to determine how GSH regulates the development of Dictyostelium, we analyzed cell morphology when GSH was completely depleted. The GSH-depleted gcsA cells did not develop on non-nutrient KK2 agar plates (Fig. 1A and Supplementary Fig. S1A, B). However, they formed fruiting bodies in the presence of 1 mM GSH, and the efficiency of fruiting body formation and the viability of spores were much lower than those of wild-type KAx3 cells as described by Kim et al. [18]. These results indicate that GSH regulates development, particularly its initiation.

3.2. GSH is required for the transition from growth to development in response to starvation

For detailed analysis, cells were developed in suspension, because this is an effective method for observing early developmental processes, especially cell aggregation. We found that gcsA cells did not form aggregates in the absence of added GSH and remained as single cells. However, gcsA cells formed aggregates in the presence of GSH, although they were small compared with KAx3 cells (Fig. 1B).

We examined whether exogenously added pulses of cAMP rescued the developmental defect in gcsA cells. Issall et al. [55] reported that some aggregate-deficient mutants form aggregates and induce the expression of CAMP response genes when they are periodically stimulated with exogenous cAMP. Our present results show that the gcsA cells cultured in the absence of GSH remained as single cells when pulses of cAMP were administered (Fig. 1C, D). These results demonstrate that GSH regulates the initiation of development in Dictyostelium.

3.3. GSH is not a cellular redox regulator during Dictyostelium development

GSH acts as an important antioxidant via its potent reducing potential. We determined whether other thiol-containing compounds or reducing agents rescued the developmental defect in gcsA cells in the absence of GSH (Fig. 2A). We found that GSH-depleted gcsA cells failed to form aggregates regardless of the concentration of thiol-containing molecules such as DTT, N-acetylcysteine (NAC), and the antioxidant ascorbic acid. These results suggest that GSH plays indispensable roles independent of its redox properties in the initiation of Dictyostelium development.

3.4. GSH modulates activation of the cAMP signaling pathway

We determined the expression pattern of genes required to induce development (Fig. 2B). KAx3 and gcsA cells were developed in suspension, and the expression of cprD and cAMP pathway-related genes such as carA and acaA was monitored during development. The cprD gene expresses a cysteine protease during growth but not during development [29]. The regulation of the expression of carA and acaA is required to initiate development [30,31,42,43]. Northern blot analysis revealed that the expression level of cprD decreased after 4 h of development and that the expression of carA and acaA was induced in KAx3 cells. In contrast, the level of cprD expression remained high during development in gcsA cells, and the expression levels of carA and acaA decreased significantly during the entire time in the absence of added GSH. When 1 mM GSH was added to gcsA cells, the expression pattern of cprD, carA, and acaA was similar to that of KAx3 cells, although it was slightly delayed.

The intracellular concentration of GSH was also measured in gcsA and KAx3 cells during suspension development. In gcsA cells treated with exogenous GSH (1 mM), the intracellular GSH level after commencement of starvation was approximately 10% of that in KAx3 cells and increased to approximately 40% at 10 h after development started; however, it was difficult to detect in gcsA cells without the addition of GSH (Fig. 2C and Supplementary Fig. S1C). The lower intracellular GSH level in gcsA cells compared with the levels in KAx3 cells in the presence of the added 1 mM GSH may explain delayed developmental processes and gene expression, as shown in Figs. 1 and 2B. These results indicate that GSH induces development through activating the expression of early developmental genes, particularly those involved in cAMP signaling.

3.5. The cAMP receptor does not rescue the developmental defects in gcsA cells

We thought that developmental defects in gcsA cells could be explained by multiple events. The first possibility is that cAMP was not synthesized in gcsA cells because the expression of acaA was not detected in the absence of GSH (Fig. 2B). As our results, in the absence of GSH, gcsA cells did not develop even though exogenous cAMP was added periodically (Fig. 1C, D) and failed to induce the expression of carA and acaA (data not shown). Therefore, a deficiency in CAMP production did not cause the developmental defect in gcsA cells.

A second possibility is that there were problems in cAMP recognition in gcsA cells. The CAMP receptor is required to induce development. Binding of cAMP to cAR1 is required for the activation of several second-messenger pathways, including G-protein-independent stimulation of calcium uptake and G-protein dependent stimulation of adenyllyl and guanylyl cyclases [56–61]. We reasoned that the failure of gcsA cells to develop might be attributed to the absence of cAR1, because carA was not expressed in GSH-depleted gcsA cells. Thus, we determined whether constitutive expression of cAR1 in gcsA cells reversed the defect in gcsA cells. However, gcsA cells expressing cAR1 (cAR1<sup>Ch</sup>/gcsA) did not develop in the absence of added GSH (Fig. 3A).
This inability to respond to cAMP does not explain the aggregate-less phenotype of gcsA cells.

To determine whether the developmental defect in cAR1OE/gcsA cells was due to a deficiency in a downstream component of cAR1, the expression levels of acaA and pkaC were determined. We found that expression of acaA and pkaC mRNAs was induced by the constitutive expression of cAR1 in gcsA cells (Fig. 3B). In other words, gcsA cells did not form aggregates in the absence of GSH when cAR1 was expressed, although cAMP signaling was activated at a functional level. These results suggest that GSH may interact with an upstream component of the cAMP signaling pathway to regulate the transition from growth to development.

3.6. GSH regulates the expression of yakA

Activation of the YakA signaling pathway is the earliest regulatory event before cAMP signaling occurs. YakA inhibits the expression of vegetative-state-specific gene expression, in particular to reduce pufA expression, which inhibits translation of pkuC mRNA [46] and induces an increase in the expression of aggregation-state-specific genes such as...
as carA and acaA [45]. To investigate the relationship between GSH and YakA, we determined the expression of yakA in gcsA− cells and found that the level of yakA expression in the gcsA− cells remained very low during the development in suspension without the addition of GSH, which was similar to findings for yakA− cells (Fig. 4A). The expression of yakA was induced by adding 1 mM GSH to gcsA− cells; however, the levels were slightly lower than those in KAx3 cells. These findings indicate that the expression of yakA may be regulated by the concentration of intracellular GSH.

To confirm these results, we analyzed yakA expression levels in KAx3 cells after adding GSH (Supplementary Fig. S2A). As the concentration of GSH increased, the expression of yakA increased proportionally and reached a peak more rapidly compared to that with the control. Aggregation started slightly faster in proportion to the concentration of GSH (Supplementary Fig. S2B). The same events occurred in KAx3 cells constitutively expressing GCS (GCSOE/KAx3) (Supplementary Fig. S3). The intracellular GSH content of GCSOE/KAx3 cells significantly increased (data not shown). The expression of yakA significantly increased, and the formation of aggregates occurred at a faster rate than in KAx3 cells.

We also determined the expression of downstream regulators of the YakA signaling cascade, such as pufA and pkaC, and found that the expression of pufA increased and that of pkaC slightly decreased in GSH-depleted gcsA− cells (Fig. 4B and Supplementary Fig. S4A). The activity of PKA was also determined, because the expression of pufA, which inhibits PKA-C translation, increased significantly in gcsA− cells. PKA activity was much lower in gcsA− cells without the addition of GSH than in KAx3 cells (Fig. 4C). In contrast, the expression of pufA, and pkaC and the activity of PKA recovered to similar levels compared with KAx3 cells when 1 mM GSH was added (Supplementary Fig. S4A and Fig. 4C).

yakA− cells showed remarkably similar patterns of gene expression and PKA activity compared with gcsA− cells (Fig. 4B). The expression of
carA, acaA, and pkaC decreased significantly. In contrast, the expression of pufA increased significantly in GSH-depleted gcsA- and yakA- cells compared with KAx3 cells. These results show that in gcsA- cells, yakA and its downstream regulators such as acaA and carA were expressed at low levels in the absence of added GSH. Taken together, these results suggest that intracellular GSH regulates the expression of yakA and is required for the transition from growth to development.

3.7 YakA expression rescues the developmental defects in gcsA- cells

We next determined whether constitutive expression of YakA restored the defects in gcsA- cells. YakA was continuously expressed in KAx3 (YakAOE/KAx3) and gcsA- (YakAOE/gcsA-) cells. In suspension development, YakAOE/gcsA- cells formed aggregates, which were similar to those of KAx3 cells regardless of the addition of GSH (Fig. 5A and Supplementary Fig. S3B).

YakA expression also influences the expression of early developmental genes. The expression of carA and acaA was significantly increased by the constitutive expression of YakA in gcsA- cells, although GSH was not added (Fig. 5B). The expression of carA, acaA, and pkaC mRNAs was significantly higher in YakAOE/KAx3 cells than in yakA- cells. Further, the levels of pufA expression decreased in YakAOE/gcsA- cells without the addition of GSH (Fig. 5B). These results demonstrate that the developmental defects in GSH-depleted gcsA- cells are due to the decreased expression of yakA. Thus, YakA expression rescues the expression of early developmental genes and eventually leads to proper development.

3.8 YakA modulates the concentration of glutathione

To define the relationship between YakA and GSH, we determined the intracellular concentration of GSH and total glutathione, which is the sum of the GSH and GSSG levels (Fig. 6). When cells were starved in the absence of exogenously added GSH (gcsA- cells: 0 mM GSH; YakAOE/gcsA- cells: 0 mM GSH), intracellular GSH and total glutathione during development were undetectable. When 1 mM GSH was added (gcsA- cells: 1 mM GSH; YakAOE/gcsA- cells: 1 mM GSH) the intracellular concentration of GSH and total glutathione content increased significantly (Fig. 6A). And there were not significant differences between gcsA- and YakAOE/gcsA- cells. In yakA- cells, the concentration of GSH and the total glutathione content were 40% lower than those in KAx3 cells. In YakAOE/KAx3 cells, the concentration of GSH decreased slightly, but the total glutathione level was higher than that in KAx3 cells (Fig. 6B, C). The calculated concentration of GSSG was too low for detection, because the GSSG is efficiently reduced. These results show that the intracellular concentration of GSH was affected by YakA expression in KAx3 but not in gcsA- cells. Moreover, YakAOE/gcsA- cells developed (Fig. 5A), although they contained intracellular GSH levels similar to those of gcsA- cells (see Discussion).
3.9. The expression level of gcsA is controlled in YakA mutant cells

Because the intracellular concentration of GSH seemed to be regulated by YakA in KAx3 cells, we examined the expression levels of gcsA in yakA and YakAOE/KAx3 cells. The expression level of gcsA was difficult to detect in KAx3 cells at 0 h when cells were exposed to developmental conditions, gradually increased as cells formed aggregates, and reached a maximum at 10 h. The level of gcsA mRNA was high in yakA cells when development started (0 h) (Fig. 7A). Further, the induced expression of gcsA mRNA was consistently maintained in yakA cells throughout aggregation (Fig. 7B, C). The patterns of gcsA expression in KAx3 cells and YakAOE/KAx3 cells were similar, indicating that the expression of gcsA was transcriptionally regulated in YakA mutants.

4. Discussion

In the present study, we investigated the roles of GSH in Dictyostelium development by using gcsA-mutant cells defective in the synthesis of GSH. We have previously shown that GSH serves important roles in normal growth and differentiation [18,19,47]; however, the mechanism responsible for GSH function is not known. Our findings showed that intracellular GSH regulates the transition from growth to development by modulating the expression of yakA and downstream regulators, which are essential for initiating Dictyostelium development.

Because GSH is a major antioxidant in cells, we reasoned that its absence might cause oxidative stress. Supplementation with exogenous thiol-containing compounds is used for replenishing GSH in cells, and exogenously added DTT and NAC restore intracellular thiols depleted by various oxidants and thus protect the cell against oxidation [14,21,62]. We found that GSH, but not exogenous DTT or NAC, rescued the developmental defect in the GSH-depleted gcsA cells (Fig. 2A). These results suggest that GSH plays indispensable roles in Dictyostelium development that cannot be compensated for by antioxidants.

The transition from growth to development is regulated by a complex series of signals designed to ensure that aggregation occurs under optimal conditions. To understand the roles played by GSH in this process, we determined the levels of expression of genes required to initiate Dictyostelium development. The expression of these genes was not precisely controlled in gcsA cells in the absence of GSH. For example, the expression of a vegetative-stage specific serine proteinase (cprD) did not decrease (Fig. 2), and the expression of dscl and din2, which act as markers for the transition from growth to development [27],

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**Fig. 4.** Expression of yakA and activity of PKA as a function of intracellular GSH concentrations. A) The expression levels of yakA in KAx3, gcsA and yakA cells determined using real time RT-PCR. All expression data were normalized by dividing the amount of target gene by the amount of rnlA used as a control. The values represent mean ± S.E.M. of three independent experiments. B) Northern blot analysis of the expression of genes known to be regulated in yakA and gcsA cells. carA, cAMP receptor; acaA, adenyl cyclase A; pufA, member of the pumilio protein family, which is a target of YakA regulation. C) The activity of PKA in KAx3, gcsA, and yakA cells. PKA activity was measured using the SignaTECT PKA Activity System. The values represent mean ± standard error of the mean (S.E.M.) of two independent experiments.
decreased in the GSH-depleted gcs\textsuperscript{A} cells (Supplementary Fig. S4B). Thus, gcs\textsuperscript{A} cells arrest at the growth stage even in the presence of a developmental signal.

The early events in multicellular development in Dictyostelium, in particular the role of the cAMP signaling pathway [63] that controls multicellular aggregate formation, have been studied extensively. The initiation of development requires sophisticated regulation of the expression of ACA and cAR\textsubscript{1} [30–36]. Our present analyses demonstrated that cAR\textsubscript{1} and ACA were not expressed at physiologically appropriate levels in GSH-depleted gcs\textsuperscript{A} cells. Moreover, the expression of car\textsubscript{A} and aca\textsubscript{A} was not enhanced in the absence of GSH in gcs\textsuperscript{A} cells (Fig. 2B), suggesting a defect in the activation of cAMP-dependent signaling.

Administering pulses of exogenous cAMP rescues the expression of car\textsubscript{A} and other components of cAMP signaling in some aggregation-defective mutants such as gcs\textsuperscript{2} and Ddmyb\textsuperscript{Z} [64–66]. However, in the present study, pulsed addition of exogenous cAMP did not rescue the expression of car\textsubscript{A} and aca\textsubscript{A} or the developmental defect in gcs\textsuperscript{A} cells in the absence of GSH (Fig. 1 and 2B). Therefore, we suspected that gcs\textsuperscript{A} cells might not respond to cAMP because of their lack of extracellular cAMP recognition. However, cAR\textsubscript{1} expression also failed to produce aggregates, although car\textsubscript{A} and aca\textsubscript{A} were expressed in gcs\textsuperscript{A} cells in the absence of GSH (Fig. 3). These data demonstrate that the synthesis and recognition of cAMP are not the main cause of the developmental defect in gcs\textsuperscript{A} cells. Thus, GSH may be required at a step upstream of cAMP signaling. Taken together, our
Fig. 6. Intracellular concentration of glutathione during development. A) The intracellular GSH levels in KAx3, gcsA− cells and YakA-expressing cells (YakAOE/KAx3 and YakAOE/gcsA−) were determined as described in Materials and methods in terms of nmol/cell wet weight. Cells were harvested at 0 h. The concentration of GSSG was calculated using the value of the concentration of total glutathione minus the concentration of reduced glutathione. B, C) Relative percentage values of reduced and total glutathione. GSH and total glutathione (=GSH + GSSG) were calculated relative to the values of KAx3 cells. The values represent mean ± S.E.M. of three independent experiments.

Fig. 7. The expression of gcsA according to the YakA expression level. A) The expression of gcsA was analyzed in KAx3, gcsA−, and yakA− cells at 0 h and 10 h after development in suspension. The gcsA− cells with no exogenous addition of GSH are designated gcsA−/−. gcsA− cells with exogenous addition of 1 mM GSH are designated gcsA−/+; B) The expression of gcsA in yakA− cells during suspension development. C) Effects of YakA expression in KAx3 cells on the expression of gcsA. Analysis of gcsA expression in KAx3, YakAOE/KAx3, and yakA− cells developed in suspension.
findings strongly suggest that intracellular GSH plays essential roles in the regulation of the transition from growth to development in Dictyostelium.

Next, we studied the YakA signaling pathway, which functions upstream of the cAMP signaling pathway. Previous reports have shown that YakA is necessary for the transition from growth to development in Dictyostelium and that the expression of YakA is required for the turning off growth-phase genes and for the induction of differentiation-associated genes [45,46]. Moreover, yakA and GSH-depleted gcsA−/− cells show similar developmental phenotypes and do not express carA and acaA at detectable levels. The level of yakA mRNA decreased significantly in GSH-depleted gcsA−/− cells (Fig. 4A). Further, the expression of yakA was modulated by the concentration of GSH added exogenously in KAx3 and gcsA−/− cells (Fig. 4A and Supplementary Fig. S2A). Therefore, we believe that our findings provide compelling evidence to support the conclusion that GSH regulates the initiation of development by inducing yakA expression.

The role of GSH in regulating the expression of yakA was supported by the results acquired from monitoring other components of YakA pathway. As expected, similar to yakA cells, GSH-depleted gcsA−/− cells showed increased pufA expression and decreases in pkac expression and PKA activity (Fig. 4B, C). Thus, the depletion of GSH blocks development because the YakA signaling system is not activated in the absence of GSH.

To confirm the relationship between GSH and YakA, YakA was constitutively expressed in KAx3 and gcsA−/− cells (YakA+KAx3 and YakA+/gcsA−/−). GSH-depleted gcsA−/− cells developed and formed aggregates when YakA was expressed (Fig. 5A). Moreover, we detected increases in the expression of carA, acaA, and pkac, which encode downstream components of the YakA pathway (Fig. 5B). These data imply that intracellular GSH induces the expression of yakA in response to a starvation signal to initiate developmental processes. Taken together, these findings suggest that intracellular GSH regulates the transition from growth to development by modulating YakA expression and downstream signaling.

The intracellular concentration of GSH in yakA−/− cells decreased by approximately 40% compared with KAx3, although gcsA was constitutively expressed during aggregation (Figs. 6 and 7). According to Bloomfield and Pears [67], a significant amount of superoxide is generated in response to CMF during the transition to the multicellular phase of development. Further, Taminato et al. [68] reported that yakA−/− cells are hypersensitive to oxidative and nitrosative stress. Therefore, we postulate that the hypersensitive reaction to oxidative stress may cause the reduction in the intracellular GSH level in yakA−/− cells. Further, yakA−/− cells may consume more GSH than KAx3 cells to protect against oxidative stress. Increased gcsA expression could be explained by the reduced intracellular GSH level in yakA−/− cells. The decreased intracellular GSH level may induce gcsA expression through feedback regulation. The intracellular content of GSH was reduced by 90% in gcsA−/− cells treated with exogenous GSH (1 mM) compared with KAx3 cells when development commenced; however, at 10 h after development started, the concentration rose to 40% of that in KAx3 cells (Fig. 2C and Supplementary Fig. S1C). In growing KAx3 cells, GSH-depleted gcsA−/− cells decreased by approximately 40% compared with KAx3, although yakA was expressed (Fig. 8A). Moreover, we detected increases in the expression of yakA, which encode downstream components of the YakA pathway (Fig. 5B). These data imply that intracellular GSH induces the expression of yakA in response to a starvation signal to initiate developmental processes. Taken together, these findings suggest that intracellular GSH regulates the transition from growth to development by modulating YakA expression and downstream signaling.

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We propose a model of developmental initiation in which GSH regulates the expression of YakA and other components of the YakA signaling pathway (Fig. 8). GSH plays an essential role in the transition from growth to development by regulating YakA and signal transduction when development initiates. We expect that further studies designed to determine the molecular mechanisms that govern the regulation of gene transcription by GSH may provide insights into general mechanisms underlying the initiation of cell development.

![Fig. 8. Proposed model of the signaling pathway regulated by the intracellular concentration of GSH during the transition from growth to development. Intracellular GSH regulates the transition from development to growth by inducing the expression of yakA, which controls the synthesis of PuF, PkaC, cAR1, and ACA. The transcription level of gcsA is also controlled by feedback regulation of the intracellular synthesis of GSH.](http://dx.doi.org/10.1016/j.bbamcr.2013.12.014)

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