



Shortening of the cell cycle and developmental interruption in a *Dictyostelium discoideum* cell line due to RNAi-silenced expression of allantoicase

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ABSTRACT. The signaling molecules NH_3 (unprotonated volatile ammonia), as well as cyclic adenosine monophosphate and differentiation-inducing factor, play important roles in the multicellular development of the slime mould *Dictyostelium discoideum*. One of the downstream metabolic products catalyzed by allantoicase (allC) is ammonia. We observed the role of allC by RNAi-mediated manipulation of its expression. The allC gene of *D. discoideum* was silenced by RNAi. We found significant downregulation of allC mRNA and protein expression levels. Recombinant allC RNAi mutant cell lines had a shortened cell cycle, a reduction in cell size relative to wild-type cells and interrupted development. We conclude that the normal functions of allC include retarding cell division until a specific cell size is reached and coordinating the progression of development.

Key words: Allantoicase; Ammonia; *Dictyostelium discoideum*; Transformant

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* has a unique life cycle consisting of a unicellular growth phase and a multicellular developmental phase. When food sources such as bacteria are available, *Dictyostelium* amoeboid cells proliferate by cytokinesis. Starvation triggers cells to undergo developmental processes during which up to 10^5 cells display chemotaxis toward cyclic adenosine monophosphate (cAMP) and form multicellular aggregates. On top of the aggregates, a small projection is formed, and this process is called tip formation. Cells located at the anterior of aggregates differentiate into prestalk cells, precursors of stalk cells, while the rest of the aggregates become prespore cells, precursor of spores. The tipped aggregates form elongated multicellular structures called slugs. Slugs migrate and eventually culminate to form a fruiting body consisting of a mass of spores supported by a stalk (Strmecki et al., 2005).

A number of diffusible molecules regulate the development of *Dictyostelium*, including cAMP, differentiation-inducing factor, adenosine, and ammonia (Mahadeo and Parent, 2006). Ammonia has been shown to affect many developmental events in *Dictyostelium*. For example, in the presence of ammonia, the production and secretion of cAMP are inhibited, resulting in the impairment of chemotaxis toward cAMP and subsequent tip formation during early development (Schindler and Sussman, 1979; Gee et al., 1994). During later stages of development, ammonia acts against differentiation-inducing factor, suppressing differentiation into prestalk cells, and promoting differentiation into prespore cells (Gross et al., 1983; So and Weeks, 1992). In addition, ammonia plays an important role in the choice between the formation of a migrating slug and culmination. High concentrations of ammonia keep slugs migrating and block the initiation of culmination (Schindler and Sussman, 1977). The exhaustion of the ammonia supply triggers culmination at least in part by activating protein kinase A through the DhkC signaling pathway (Singleton et al., 1998). In fruiting bodies, extremely high concentration of ammonium phosphate in sori maintain spore dormancy through the activation of the sporulation-specific adenylyl cyclase ACG (Cotter et al., 1999).

Allantoicase (allC) is one of enzymes participating in purine catabolism. It catalyzes the chemical reaction: allantoate + $H_2O \rightleftharpoons$ (S)-ureidoglycolate + urea. This enzyme belongs to the family of hydrolases, those acting on carbon-nitrogen bonds other than peptide bonds, specifically in linear amidines. The systematic name of this enzyme class is allantoate amidinohydrolase (EC 3.5.3.4). Urease (EC 3.5.1.5) catalyzes the conversion of the urea molecule to two ammonia molecules and one carbon dioxide molecule.

In 2002, RNA interference-mediated gene silencing was established in *Dictyostelium* (Martens et al., 2002). RNAi-mediated silencing of gene expression is induced by double-stranded RNA and its processing to 23 mers, which cause the degradation of endogenous target mRNAs (Hannon, 2002).

It is shown here that a hairpin RNA construction targeted against allC effectively reduces the cellular allC concentration. Recombinant cell lines showed a shorter cell cycle, a reduction of the cellular size and an interruption of development.

MATERIAL AND METHODS

Growth and development of *D. discoideum*

D. discoideum KAx3 strain was grown at 22°C in axenic medium HL5. Development

was induced by counting, harvesting, washing cells twice in ice-cold phosphate buffer (1.2 mM NaH₂PO₄, 0.4 mM Na₂HPO₄, pH 6.4), then washing cells once in ice-cold PDF buffer (9.2 mM K₂HPO₄, 11.8 mM KH₂PO₄, 20.1 mM KCl, 5.3 mM MgCl₂, pH 6.4) and spreading 500 µL PDF buffer including 1 x 10⁸ cells on 2% agar plate (40 mm in diameter). The agar plates were incubated at 22°C in a humid chamber. The developmental processes of wild-type and allC RNAi mutant cells were observed with OLYMPUS BX51TF microscope using bright field and photographed with a CoolSNAP-ProCF digital camera.

Transformation of *D. discoideum*

D. discoideum KAx3 cells were grown in axenic medium HL5 to a density of 1-2 x 10⁶ cells/mL, harvested cells by centrifugation at 500 g for 5 min at 4°C, washed twice with ice-cold H-50 buffer (19.98 mM HEPES, 50.03 mM KCl, 9.93 mM NaCl, 1 mM MgSO₄, 5 mM NaHCO₃, 1.3 mM NaH₂PO₄, pH 7.0), resuspended in H-50 buffer at 1 x 10⁸ cells/mL, and 100 µL of this suspension containing 10 µg plasmid DNA in a volume of approximately 10 µL was electroporated with Bio-Rad MicroPulser™ electroporation apparatus. Transformed cells were grown on suitable selective media by plating approximately 3 x 10⁵ cells per G100 plate with 0.5 mL HKB and clonal populations were obtained (Gaudet et al., 2007). Transformants were cultured in HL5 medium.

Construction of plasmids

cDNA was obtained by reverse transcription of total RNA from vegetative *D. discoideum*. Construction of the RNAi-encoding plasmid pAct15-Gal-LS for allC was done in three steps. First, a longer fragment of 642 bp amplified with primers 5'-ggaattcCATATGTATGTATCTGAGTGTGCCG-3' and 5'-ccatcgatTGAACGACCTGGCATGATG-3', and a shorter fragment of 397 bp amplified with primers 5'-gaagatcTATGTATCTGAGTGTGCCG-3' and 5'-ccatcgatGAGCCAAGATCGGATTTG-3' were cloned into vector pMD18-T. The underlined lower case letters are the restriction endonuclease recognition site, and the other lower case letters are the restriction site protection base. Second, pAct15-Gal-L was generated by ligating the longer fragment in reverse orientation into pAct15-Gal after digestion with *Nde*I and *Cla*I. Finally, the shorter fragment was subcloned in sense orientation into *Bgl*III/*Cla*I-digested pAct15-Gal-L to obtain pAct15-Gal-LS (Figure 1).

Real-time qRT-PCR analysis

KAx3 and allC RNAi mutant cells were grown at 22°C in axenic medium HL5. Exponentially growing cells were harvested. Total RNA were extracted according to TaKaRa RNAiso™ Plus specifications. Real-time qRT-PCR was conducted using the CFX96™ Real-Time System (Bio-Rad, USA). Gene-specific primers were designed based upon the GenBank (AY894718.1) mRNA of 561 bp, sense primer: 5'-GAATCACTAATGTCCAATCC-3', anti-sense primer: 5'-AAATCCGATCTTGGCTC-3'. The specificity of the amplified products was evaluated through the analysis of the dissociation curves generated by the equipment. allC relative expression levels between wild-type and allC RNAi mutant cells were calculated by the formula: $Ratio_{(test/calibrator)} = E^{C_T(calibrator) - C_T(test)}$. Real-time qPCR amplifications were carried

out in a final volume of 25 μL , which contained 12.5 μL 2X SYBR Premix Ex TaqTM (TaKaRa), 1 μL diluted template including 200 ng cDNA, 11.0 μL PCR-grade water, and 0.25 μL of each primer. PCR conditions were as follows: predenaturation at 95°C for 30 s, 39 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 20 s, and elongation at 72°C for 20 s; resultant data were analyzed using the CFX ManagerTM software (version 1.0).

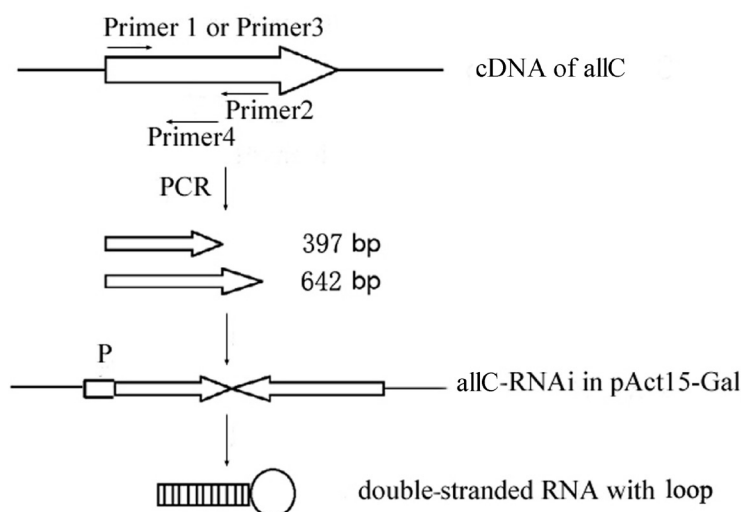


Figure 1. RNAi construct directed against allantoicase (allC). cDNA was obtained by reverse transcription of total RNA. The long and short fragment were amplified with primers 1 and 2 or primers 3 and 4, respectively. They were then fused in opposite orientation and cloned in pAct15-Gal.

SDS polyacrylamide gel electrophoresis and Western blotting

Extracts obtained from 1×10^6 cells were separated by 12% polyacrylamide/0.1% SDS gels, transferred to nitrocellulose membranes, incubated with 1:2000 diluted rabbit antiserum against allC at room temperature for 2 h, subsequently incubated with 1:100 diluted AP-conjugated goat anti-rabbit IgG(H+L) at room temperature for 1 h. Developer containing BCIP and NBT was added to membranes and incubated in the dark until a suitable band intensity was reached.

Flow cytometry

Cells (1×10^6) were fixed in 75% ice-cold ethanol at -20°C for a night, washed twice in wash buffer (PBS containing 1% BSA), resuspended in PBS/Triton solution (PBS containing 0.25% Triton X-100) for 5 min, washed once in wash buffer, incubated with 1:1000 rabbit antiserum against allC at room temperature for 30 min, washed thrice in wash buffer, incubated with 1:100 diluted FITC-conjugated goat anti-rabbit IgG(H+L) at room temperature for 10 min, washed thrice in wash buffer, resuspended in 500 μL PBS, and subsequently analyzed by the BD FACSCalibur flow cytometer. Negative controls were performed with PBS instead of 1:1000 rabbit antiserum against allC.

Cellular measurement and statistics

Wild-type and allC RNAi mutant cells were observed with LEICA DM4000B microscope using bright field and photographed with a LEICA DFC 500 digital camera. Cellular cross-sectional area were measured with Leica Application Suite V3.3.0 and Image J. Statistical analyses were carried out with Office Excel 2003.

Growth curve and determination of cell cycle

Wild-type cells (1.75×10^7) and 1.75×10^7 allC RNAi mutant cells grown respectively at 22°C in 100 mL axenic medium HL5 were harvested once every 3 h, and counted with blood cell counting chamber. Subsequently, growth curves were made with Office Excel 2003.

Given two measurements of a quantity during exponential phase, q_1 at time t_1 and q_2 at time t_2 , the cell cycle was calculated as:

$$T_d = (t_2 - t_1) * \frac{\log(2)}{\log\left(\frac{q_2}{q_1}\right)} \quad (\text{Equation 1})$$

RESULTS

Downregulation of allC mRNA expression

To verify if allC mRNA levels in allC RNAi mutant decreased, we used real-time qRT-PCR. The amplification efficiency was 101%. E value was 2.01. The mean of C_T value for KAx3 wild-type and allC RNAi mutant cells was 23.23 and 36.26, respectively. According to the formula: $Ratio_{(test/calibrator)} = E^{C_T(calibrator) - C_T(test)}$, the relative quantification was carried out. The mean of allC mRNA relative quantity in KAx3 wild type was 1, and the standard deviation was 0.1. The mean of allC mRNA relative quantity in allC RNAi mutant was 0.00012, and the standard deviation was 0.00005. Expression level of allC mRNA in allC RNAi mutant was reduced evidently, when compared to KAx3 wild type.

Downregulation of allC protein expression

Western blots with specific antibodies against *D. discoideum* allC revealed that allC of the mutant was reduced to barely detectable amounts (Figure 2). The result showed the evident downregulation of allC protein expression in allC RNAi mutant compared with wild type.

To further verify if allC levels in allC RNAi mutant decreased, twenty thousand cells of wild-type and allC RNAi mutant were detected with flow cytometer. There were 19,328 allC-positive cells and 625 allC-negative cells in wild type, 1031 allC-positive cells and 18,936 allC-negative cells in allC RNAi mutant. The percentage of allC-positive cells in wild type was 96.64% (Figure 3B), and the percentage of allC-negative cells in allC RNAi mutant was 94.68% (Figure 3D). The result was in substantial agreement with one of Western blotting.

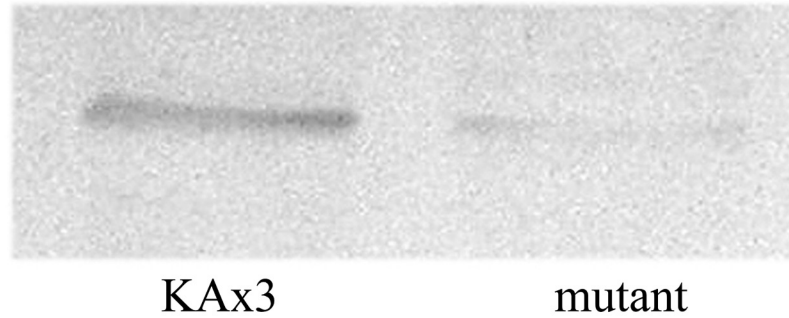


Figure 2. Expression of allC in wild-type and allantoicase (allC) RNAi mutant cells. Extracts obtained from 1×10^6 cells were separated by 12% polyacrylamide/0.1% SDS gels, blotted onto nitrocellulose, and blots were probed with 1:2000-diluted allC-specific antibody.

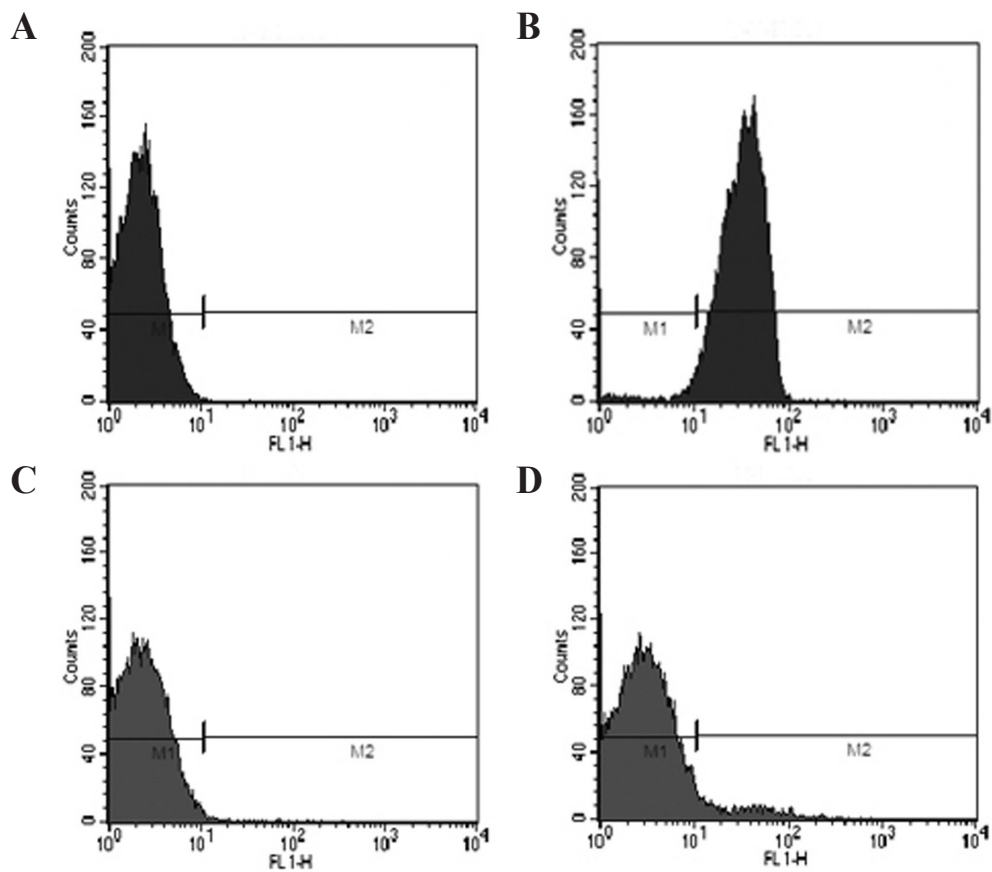


Figure 3. Expression of allC in wild-type and allantoicase (allC) RNAi mutant cells with flow cytometry. **A** and **C** show negative controls of wild-type and allC RNAi mutant cells, respectively. **B** and **D** are results of expression of allC in wild-type and allC RNAi mutant cells with flow cytometry, respectively.

Growth comparison between wild-type and allC RNAi mutant cells

The cell cycle of allC RNAi mutant was 5.5 h; however, wild type was 16.53 h. The cell cycle of allC RNAi mutant shortened evidently, which was about one third as long as wild type. The equations of growth curve about wild-type and allC RNAi mutant cells were $y = 0.437\ln(x) + 4.9685$ ($R^2 = 0.9356$), and $y = 0.0007x^3 - 0.0424x^2 + 0.7924x + 4.0509$ ($R^2 = 0.9763$), respectively. x is time of growth, and y is logarithm of cellular number per milliliter of culture. The growth curve of allC RNAi mutant had a typical exponential phase at which there was a steep rise compared with wild type (Figure 4). This indicated that the growth speed of allC RNAi mutant is very quicker than wild type. At stationary phase, cells of allC RNAi mutant reached a density about 7×10^8 cells/mL; however, the density of wild type was about 5×10^6 cells/mL. After cell counting lasted 144 h, wild-type and allC RNAi mutant cells were still at the stationary phase.

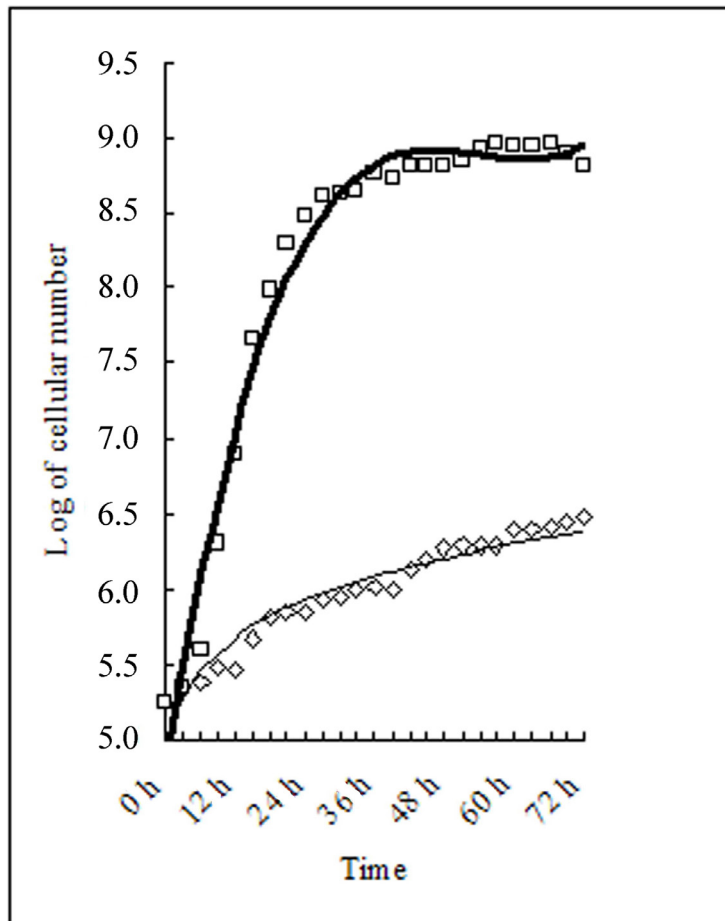


Figure 4. Growth curves of wild-type (slender line) and allantoicase RNAi mutant cells (thick line). Abscissa: time of growth; ordinate: logarithm of cellular number per milliliter of culture.

Comparison of cellular size between wild-type and allC RNAi mutant cells

Microscopic examination revealed that the exponential phase cells of allC RNAi mutant were much smaller than wild type (Figure 5). The statistical results of cross-sectional area of wild type and allC RNAi mutant cells were 111.88 ± 37.38 and 7.25 ± 2.84 (means \pm SD) respectively, whose means were about one fifteenth in cross-sectional area of wild type. There was a significant difference in the cellular cross-sectional area between wild-type and allC RNAi mutant cells with *t*-test.

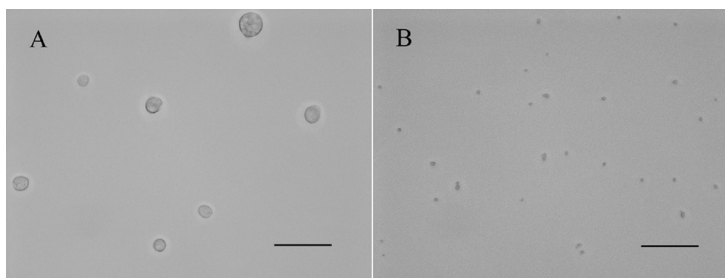


Figure 5. Cells of wild type were shown in **A** (bar = 50 μ m), and allantoicase RNAi mutant cells are shown in **B** during exponential phase (bar = 50 μ m).

Developmental interruption of allC RNAi mutant in comparison to wild type

After the vegetative cells of wild type were induced by starvation (Figure 6A), cells entered a 24-h development cycle. During this developmental process, many cells aggregated to form a mound (Figure 6B), then formed a migrating slug (Figure 6C), and eventually developed into a mature fruiting body consisting of differentiated stalk and spore cells (Figure 6D). However, after vegetative cells were induced by starvation (Figure 6E) lasting 48 h, development of allC RNAi mutant was still at the phase of aggregation (Figure 6F). This showed that the development of allC RNAi mutant was interrupted at the phase of aggregation, suggesting that allC coordinates the progression of development.

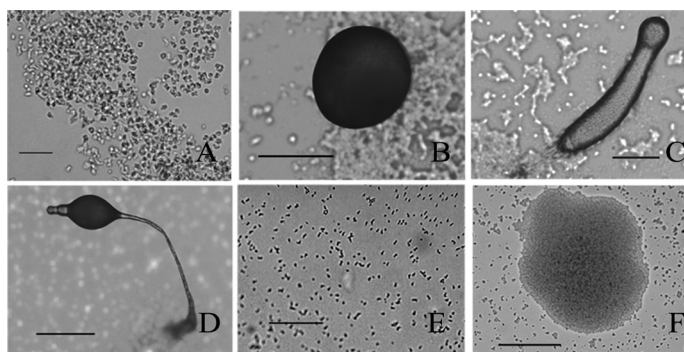


Figure 6. Process of development in wild-type and allantoicase (allC) RNAi mutant cells. Development of wild type: **A.** Vegetative cells; **B.** mound; **C.** slug; **D.** fruiting body. Development of allC RNAi mutant: **E.** Vegetative cells; **F.** aggregation. The development of allC RNAi mutant was interrupted at the phase of aggregation. Bar: A = 100 μ m; B = 200 μ m; C = 300 μ m; D = 200 μ m; E = 100 μ m; F = 200 μ m.

DISCUSSION

We identified that allC in *Dictyostelium* is essential for the proper control of cell growth and development.

Since wild-type cells have a normal range of sizes during growth, it is likely that cell size is tightly controlled. allC is required to maintain the appropriate cell size, presumably by affecting the regulation that links cell growth to the cell cycle. Most cell growth occurs in G₂ during *Dictyostelium*, so size regulation would be expected to involve a coordination between the growth of a cell in G₂ and its entry into mitosis (Souza et al., 1998). The smaller size of allC RNAi mutant cells, and their shorter cell cycle time, suggest that this coordination is altered in allC RNAi mutant cells. According to this idea allC RNAi mutant cells would consistently undergo mitosis prior to attaining normal size, resulting in a smaller steady-state cell size. Besides catalyzing the hydrolysis of allantoate, another function of allC is to prevent cell division until a specific cell size is reached.

Starvation results in an extensive modification of the pattern of gene expression within *Dictyostelium* cells. Upon starvation, the expression of some vegetative genes is reduced, while genes essential for development become induced. Among the earliest genes to be induced after starvation are the ones required to modulate cell motility and allow aggregation of the cells. Aggregation is mediated by chemotaxis to cAMP, so many of these genes' products are required for producing and sensing cAMP, including an adenylyl cyclase, a cell surface cAMP receptor and the cAMP-dependent protein kinase. The cAMP produced by some cells in the population stimulates nearby cells to migrate in the direction of the signal until an aggregate is formed (Van Haastert, 1995). The cells in the absence of allC aggregated indicated that allantoicase appears to have a negligible effect on the expression of the earliest genes to be induced after starvation and required to modulate cell motility, while have a significant effect on the expression of the following genes essential for development, so the development of allC RNAi mutant was interrupted at the phase of aggregation.

Souza et al. (1998) reported that YakaA-null cells are smaller and their cell cycle is accelerated relative to wild-type cells. When starved, YakaA-null cells cannot develop. Hence, we think that Yaka is correlated with allC in the regulation of growth and development in *D. discoideum*. The Yaka mechanism of coordination between growth and development has been demonstrated by Souza et al. (1998). However, the allC mechanism is not clear and it is necessary to further study.

Ammonia was proposed to be a signaling molecule influencing the multicellular development of *D. discoideum* quite a long time ago (Gross et al., 1983). NH₃ is produced by protein and nucleic acid catabolism. Ammonia metabolism seems to be regulated by the action of both NADH-dependent and NADPH-dependent glutamate dehydrogenases (NADH-GDH, NADPH-GDH). NADH-GDH probably degrades glutamate to α -oxoglutarate and ammonia during vegetative growth and during prestalk to stalk maturation. In the process of prespore to spore differentiation either NADH-GDH or NADPH-GDH catalyzes the reverse reaction and generates glutamate from ammonia and α -oxoglutarate (Cotter et al., 1992). Additionally, the NH₃ of spores could be removed by the action of glutamine synthetase catalyzing the biosynthesis of glutamine from ammonia and glutamate (NH₃ + glutamate + ATP → glutamine + ADP + Pi). Presumably, an important consequence of these enzymatic reactions is the establishment of an ammonia gradient. Some experiments indicate that glutamine synthetase could be the key enzyme regulating gradient formation (Dunbar and Wheldrake, 1995).

Undoubtedly, RNAi-silenced expression of allantoicase resulted in a decrease of NH_3 that is the downstream metabolic product catalyzed by allantoicase. This could further reduce the whole levels of intracellular $\text{NH}_3/\text{NH}_4^+$ to evoke pleiotropic cellular responses, such as a decrease of cell cycle, a reduction of cellular size and an interruption of development. If so, our research confirmed the following views. Small signaling molecules including NH_3 possess the ability to create dynamic concentration gradients and gradients of pH. In this way, they can influence not only one or a few target molecules but also the properties of cells or distinct cellular compartment, thus evoking pleiotropic cellular responses (Palková and Váchová, 2003).

In the presence of high levels of intracellular ammonia, the production and secretion of cAMP are inhibited, resulting in the impairment of chemotaxis toward cAMP (Schindler and Sussman, 1979). Development of allC RNAi mutant was still at the phase of aggregation after lasting 48 h showed the existence of chemotaxis. This could indicate the reduction of whole levels of intracellular NH_3 .

During vertebrate evolution, the purine catabolism pathway was differently truncated, with the possible advantage of preserving water. Consequently, the activity of the enzymes in this pathway as well as its end products varies from species to species. As far as concerns allantoicase, one of the enzymes in this pathway, most likely appeared very early during evolution, but its activity appears to have been lost during vertebrate evolution (Keilin, 1959), as it is present in most fish and amphibians but not in amniotes (Urich, 1994). So the comparative research on wild-type and allC RNAi mutant cells could offer certain help to elucidate the mechanism for loss allantoicase during animal evolution.

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