POSTER PRESENTATIONS

1. **Jyotika Rajawat,** Hina Mir, Iqbal Vohra and Rasheedunnisa Begum, Dual Role of PARP in *Dictyostelium discoideum* development and oxidative stress induced paraptotic cell death. Poster presented at international symposium *at AICBC* Dec 2009, University of Hyderabad
2. **Jyotika Rajawat**, Hina Mir, Rakesh Gupta, Shalmali Pradhan, Iqbal Vohra and Begum R, Differential role of Poly (ADP) ribose polymerase in paraptotic and necrotic cell death in *D. discoideum.* Poster presented at Two day National Symposium on Apoptosis and Cancer held on 28-29th December, 2007 at MSU Baroda **(Bagged Best Poster Award).**
3. **Jyotika Rajawat**, Hina Mir, Rakesh Gupta, Shalmali Pradhan, Iqbal Vohra and Begum R, Differential role of Poly (ADP) ribose polymerase in paraptotic and necrotic cell death in *D. discoideum.* Poster presentation at International symposium of developmental biology held on 18-19th October, 2007 at Holiday Inn, Agra.
4. **Jyotika Rajawat**, Hina Mir and Begum R, Role of Poly (ADP) ribose polymerase in *D. discoideum* cell death and development. Poster presentation at International symposium of developmental biology held on 25-27th November, 2006 at Agharkar Research Institute, Pune.
5. Iqbal Vohra, Hina A Rehman, **Jyotika Rajawat** and Begum R. UV-C induced changes in cAMP signaling during *Dictyostelium discoideum* development. Poster presented at Two day TIFR seminar on modern biology "Facets and prospects" *seminar*, held on 10-11th Oct 2005 at MSU Baroda.

ORAL PRESENTATION

Has been awarded **First Prize** in oral presentation at Two day National Symposium on Apoptosis and Cancer held on 28-29th December, 2007 at MSU Baroda.

the FEBS Journal

Effect of oxidative stress and involvement of poly(ADP-ribose) polymerase (PARP) in *Dictyostelium discoideum* development

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Keywords
benzamide; development; *Dictyostelium discoideum*; oxidative stress; PARP

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Dictyostelium discoideum, a unicellular eukaryote, exhibits multicellularity upon nutrient starvation and is a good model system for developmental studies, and for the study of various signal transduction pathways. Reactive oxygen species at low doses act as signaling molecules; however, at high doses they are known to cause DNA damage that results in the activation of poly(ADP-ribose) polymerase (PARP). We have earlier reported the high resistance of the unicellular stage of *D. discoideum* to oxidative stress, and we now show the response of this organism to oxidative stress and the role of PARP during development. We used hydroxylamine (HA) to induce *in situ* generation of H_2O_2 and monitored the effect of benzamide, a PARP inhibitor, on oxidative stress-induced changes in *D. discoideum* development. Interestingly, oxidative stress resulted in PARP activation within 5 min that was inhibited by benzamide. Oxidative stress-induced delay in developmental pattern was also partially restored by benzamide. We studied the long-term effects of PARP inhibition under oxidative stress, and our results demonstrated that spores formed under HA stress exhibited significant delay in germination in comparison to benzamide-pretreated HA-stressed cells. However, second-generation cells showed normal development, signifying that PARP inhibition has no deleterious effect on *D. discoideum* development under oxidative stress.

Dictyostelium discoideum, a unicellular eukaryote, exhibits multicellularity and thus provides a simple but excellent model system for the study of various signal transduction pathways [1], the findings of which can later be confirmed with complex eukaryotic systems. *D. discoideum* in the unicellular stage is known to be highly resistant to DNA-damaging agents and oxidative stress [2,3]. However, the response of *D. discoideum* development to oxidative stress is not clearly understood. Recent studies showed that superoxide plays a vital role in the aggregation process of *D. discoideum* cells [4], as inhibition of superoxide-dependent signaling events affects the transition from the unicellular to the multicellular phase. During development, *D. discoideum* cells produce nitric oxide, which is also postulated to act as a signaling molecule [5].

Reactive oxygen species (ROS) nevertheless also have deleterious effects and are known to cause DNA damage [6], which in turn results in the activation of poly(ADP-ribose) polymerase (PARP). This catalyzes the transfer of ADP-ribose moieties to acceptor proteins by utilizing NAD^+ as the substrate, and helps in DNA repair [7,8]. PARP also monitors the status of DNA before entry into mitosis [9,10], and hence has been implicated in checkpoint control. Cells are

Abbreviations
FITC, fluorescein isothiocyanate; HA, hydroxylamine; LD, lethal dose; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PBA, phosphate-buffered agar; ROS, reactive oxygen species; SB, Sorenson's buffer.

arrested at different phases of the cell cycle, depending upon the extent of PARP activation [11] under stress conditions. Thus, in higher eukaryotic cells, PARP contributes to cell homeostasis under mild stress conditions, and conversely, during conditions of moderate/severe cellular stress, PARP overactivation leads to cell death, which results in several disease conditions [12]. Pharmacological inhibition of PARP during moderate/severe cellular stress is beneficial [13,14]; however, the consequences of such inhibition for genomic integrity are not yet understood. *D. discoideum* is reported to have nine potential PARP genes [15], unlike another unicellular eukaryote, *Saccharomyces cerevisiae* [16]. Hence, we selected *D. discoideum* as a model system to study the role of PARP in its development under oxidative stress conditions.

We have studied the dose-dependent effect of hydroxylamine (HA) (for *in situ* H_2O_2 generation) on *D. discoideum* development and also the role of PARP in oxidative stress-induced effects on development. Our present study is the first report on the activation of PARP under oxidative stress in *D. discoideum*, and our results suggest that *D. discoideum* is an excellent model system with which to investigate the long-term effects of PARP inhibitors for two successive generations.

Results

Dose-dependent effect of oxidative stress on *D. discoideum* cell death

Cell death was induced by treating *D. discoideum* cells for 1 h with different concentrations (1.0, 2.5 and 4.0 mM) of HA, a known catalase inhibitor [17], in order to promote *in situ* generation of H_2O_2. HA-induced cell death was measured after 24 h by the Trypan blue exclusion method. The percentage of cells undergoing cell death was found to increase from 15% to 90% as the concentration of HA was increased from 1.0 mM to 4.0 mM, and 50% cell death was seen at 2.5 mM HA (Fig. 1).

D. discoideum growth under oxidative stress

To monitor the effect of HA on the *D. discoideum* cell cycle, a growth curve was obtained. The growth curve showed a dose-dependent increase in the lag phase from 36 h to 60 h, 72 h and 96 h at lethal dose (LD)15 (1 mM), LD50 (2.5 mM) and LD90 (4 mM), respectively. Furthermore, the log phase was shortened to 48 h, 48 h and 36 h at LD15, LD50 and LD90, followed by faster attainment of stationary phase

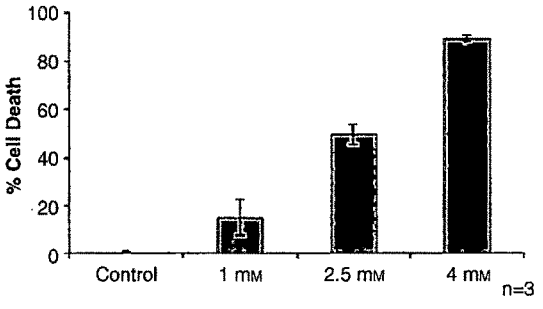

Fig. 1. Dose-dependent effect of HA on *D. discoideum* cell death determined by the Trypan blue exclusion method. Cells were treated with different doses of HA, and cell death was assessed by the Trypan blue method after 24 h. HA at 1 mM caused 15% cell death, and hence this dose was considered to be LD15; a 2.5 mM dose was found to be LD50, as 50% of cells were dead; 4 mM HA was found to be LD90, as this dose caused 90% cell death.

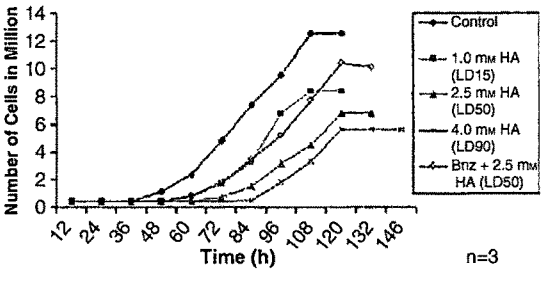

Fig. 2. Effect of PARP inhibition during oxidative stress-induced growth changes in *D. discoideum*. Under oxidative stress, the growth curve showed a dose-dependent increase in the lag phase. The log phase was shortened, and this was followed by faster attainment of stationary phase. Benzamide-pretreated cells showed a reduction in the lag phase from 72 to 60 h at LD50, followed by a longer log phase. Results are means of three independent experiments performed in duplicate.

(Fig. 2), suggesting that HA caused cell cycle arrest leading to an increased lag phase.

D. discoideum development under oxidative stress

To study the effect of oxidative stress on differentiation, developmental studies were performed. The dose-dependent effect of HA on *D. discoideum* development was studied by exposing the cells to different concentrations of HA (1.0, 2.5 and 4.0 mM) for 1 h and then allowing them to develop. As can be seen from Table 1 and Fig. 3A, development was delayed in a dose-dependent manner at the loose aggregation stage by 2 h and 12 h at LD15 and LD50 of HA,

Table 1. Developmental stages of *D. discoideum* at different time intervals. Cells (2.5×10^6) were treated with 2.5 mM and 4 mM HA for 1 h, plated on non-nutrient agar, and observed at different time points. Also shown is the effect of PARP inhibition by benzamide during oxidative stress on *D. discoideum* development. LA, loose aggregate; TA, tight aggregate; SF, slug formation; FBF, fruiting body formation; CD, cell death; FB, fuiting body; –, no development until after 1 week.

	LA (h)	TA (h)	SF (h)	FBF (h)	% CD	% FB
HA (mM)						
0.0	6	12	18	24	1	100
1.0	8	14	20	26	15	100
2.5	18	24	30	36	50	30
4.0	–	–	–	–	90	–
HA (mM) + 1 mM benzamide						
0.0	6	12	18	24	1	100
1.0	6	12	18	24	5	100
2.5	12	17	23	29	20	70
4.0	18	24	30	36	40	20

respectively, as compared to control cells. At 18 h of development, 40% loose aggregates were seen in 2.5 mM HA as compared to controls. The percentage involvement of cells was slightly increased with time. Nevertheless, cells treated with LD90 of HA showed no development until after 1 week, suggesting that development was arrested. HA-treated *D. discoideum* cells exhibited dose-dependent decreases in the number and size of fruiting bodies as compared to control cells (Fig. 3B).

Oxidative stress induces PARP activation

PARP activity in *D. discoideum* was assayed at various time points (5, 10, 20 and 60 min and 4 h) after HA stress. PARP activity was increased initially, and significant peak PARP activity was seen at 5 min after exposure of the cells to 2.5 mM HA (Fig. 4A,B). No difference in fluorescence intensities was observed at time points after 10 min.

PARP inhibition by benzamide

To address the role of PARP under oxidative stress, PARP inhibition studies were performed. Peak PARP activity, which was observed after 5 min of 2.5 mM HA exposure, was significantly inhibited by 1 mM benzamide (Fig. 4A,B), confirming PARP activation in *D. discoideum* under oxidative stress.

PARP inhibition during oxidative stress-induced growth changes in *D. discoideum*

PARP inhibition conferred protection against 2.5 mM HA-induced delay in growth. The lag phase in benzamide-pretreated cells was reduced from 60 h to 50 h, and was followed by a longer log phase (Fig. 2).

Role of PARP during *D. discoideum* development

The role of PARP in *D. discoideum* development was investigated by its inhibition with benzamide.

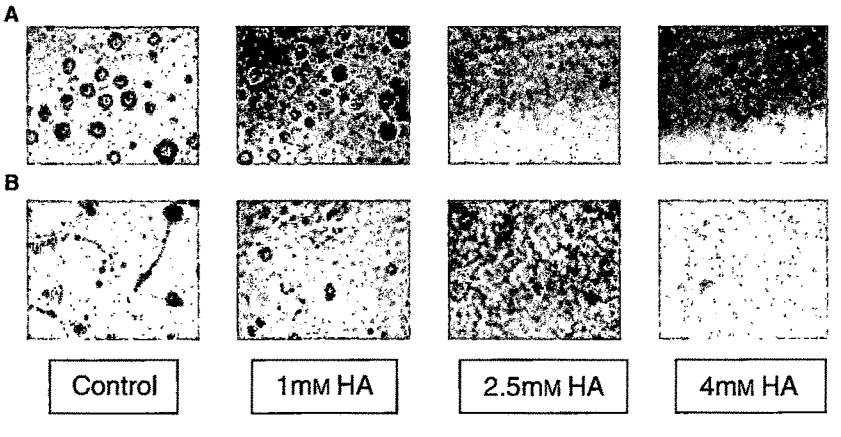

Fig. 3. Development of *D. discoideum* cells at 12 and 24 h under oxidative stress. (A) Developmental phenotypes of control and 1 mM HA-treated *D. discoideum* cells at 12 h. Cells after HA treatment were starved on nutrient-free agar medium and photographed at 4× magnification. (B) Developmental stages of control cells, and 2.5 mM and 4 mM HA-treated cells, at 24 h. Scale bar, 10 μm. Results are means of three independent experiments performed in duplicate.

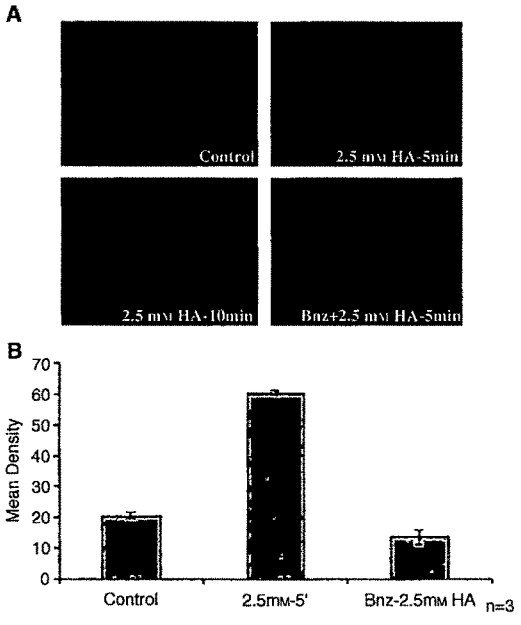

Fig. 4. Fluorescence images for PARP assay under 2.5 mM HA stress at varying time intervals. (A) Cells after treatment with HA were fixed and incubated with antibody to PAR, and were then treated with FITC-conjugated secondary antibody to assess PARP activity. PAR immunoreactivity was barely detectable in controls, whereas peak activity was seen at 5 min after 2.5 mM HA stress, and was reduced to basal level by 10 min. Benzamide significantly inhibited peak PARP activation. (B) Representation of the results for PARP activation in the form of a histogram; a significant increase in PARP activity was seen at 5 min. $P < 0.001$.

Benzamide (1.0, 2.0 and 3.0 mM) did not show any effect on development. However, benzamide at 4 mM caused a 3–4 h delay in the tight aggregate-to-slug transition (Table 2). Interestingly, *D. discoideum* cells treated with 3.0 and 4.0 mM benzamide showed abnormal fruiting bodies with larger fruits.

PARP involvement during oxidative stress-induced developmental changes in *D. discoideum*

To determine the role of PARP in oxidative stress-induced developmental changes, *D. discoideum* cells were exposed to benzamide (1 mM for 24 h) prior to HA (LD15, LD50 and LD90) treatment, and allowed to develop; the results are shown in Table 1. Benzamide-pretreated cells, upon exposure to a high dose of HA (2.5 mM), exhibited development, and the delay at the loose aggregation stage was reduced from 18 h to 12 h (Table 1). The percentage of loose aggregates formed was also increased, whereas in the case of LD90, delayed development could be observed in the presence of benzamide, as compared to developmental arrest of 4 mM HA-treated cells. The fruiting bodies formed were very small, with poor stalks and small fruits, and the fruits were few in number (Fig. 5).

Table 2. Effect of the PARP inhibitor benzamide on *D. discoideum* development. LA, loose aggregate; TA, tight aggregate; SF, slug formation; FBF, fruiting body formation; CD, cell death; FB, fruiting body.

Benzamide (mM)	LA (h)	TA (h)	SF (h)	FBF (h)	% CD	% FB
0.0	6	12	18	24	1	100
1.0	6	12	18	24	2	100
2.0	6	12	18	24	2	100
3.0	6	12	18	24	4	95
4.0	6	12	22	28	10	95

PARP inhibition restored spore germination that was delayed due to oxidative stress

To investigate the germination efficiency of spores and the fate of the germinated amoebae, spore revival was attempted. Control and benzamide-treated spores germinated within 108–120 h, whereas the spores formed under 2.5 mM HA stress showed a significant delay, i.e. ~56 h ($P < 0.001$) in germination. There was a partial rescue of the developmental delay, i.e. ~32 h ($P < 0.012$) in the presence of benzamide. Spores formed from benzamide-pretreated and 4 mM HA-treated cells germinated after 60 h as compared to controls (Fig. 6). To avoid ambiguity in the number of fruiting bodies added to each flask, fruiting bodies were picked up from at least four different areas and it was ensured that a single fruiting body was inoculated per milliliter of medium. Our results were also confirmed by microscopically counting the number of cells germinated from each spore, and this was found to be the same for each dose.

For spore revival when log phase had been reached (2.5×10^6 cells/mL), the cells were plated on phosphate-buffered agar (PBA) plates for development, and cells treated with 2.5 mM and 4 mM HA exhibited normal development (data not shown).

Discussion

Among the eukaryotic organisms, the cellular slime mold *D. discoideum* is an excellent model system for studying cell death and developmental aspects [18]. The ability of living cells to cope with various stresses is very crucial for maintaining their correct development. ROS at lower concentrations have physiological

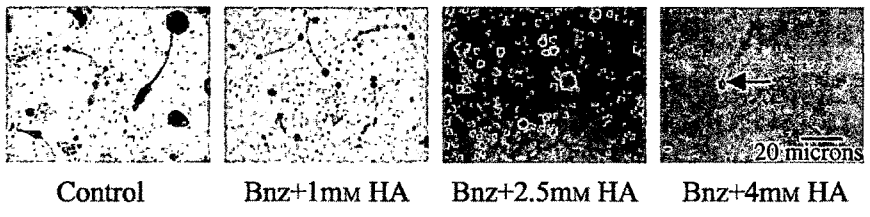

Fig. 5. Effect of PARP inhibition during oxidative stress-induced developmental changes in *D. discoideum*. Cells were preincubated with 1 mM benzamide for 24 h, treated with HA, washed, and plated at a density of 2×10^5 cells·cm^{-2}. Benzamide pretreatment restored the development that was delayed by 2.5 mM HA, and rescued the developmental arrest of 4 mM HA-treated cells. The arrow indicates the fruiting body. Fruiting body formation at different time intervals in the development of HA-treated cells pre-exposed to benzamide is shown. The fruiting body was small in comparison to that of controls. Scale bar, 20 μm. Data are means of three independent experiments performed in duplicate.

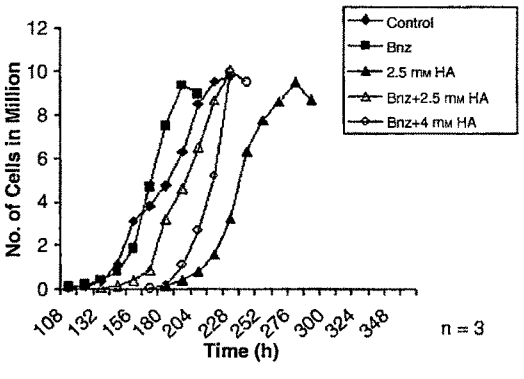

Fig. 6. Effect of PARP inhibition on the fate of spores that were developed under oxidative stress. Spores of control cells germinated within 108 h, whereas spores formed under oxidative (2.5 mM HA) stress exhibited a 56 h delay in germination, which was partially rescued by benzamide pretreatment. Spores formed from cells that were pre-exposed to benzamide and HA-stressed (2.5 and 4 mM HA) germinated earlier than cells treated only with 2.5 mM HA; 4 mM HA-treated cells showed no development and hence no spores. Data are means of three independent experiments performed in duplicate.

functions and serve as second messengers in different signal transduction pathways [19]; however, ROS at higher concentrations cause DNA damage [20] among other cytotoxic effects. PARP is known to play an important role under oxidative stress [21]; however, there is no report on the role of PARP in *D. discoideum* development. We have investigated the role of PARP in *D. discoideum* development by inhibiting its activity with the known PARP inhibitor benzamide, and studied its effects on development and oxidative stress-induced development. Our results suggest that 2.5 mM HA delayed development due to cell cycle arrest, whereas 4 mM HA caused 90% cell death, meaning that cell density was not sufficient for aggregation, leading to complete developmental arrest. Our results show that *D. discoideum* exhibits basal PARP activity (Fig. 4A), and its inhibition by benzamide (1–3 mM) did not affect development. However, benzamide (4 mM)-treated *D. discoideum* cells were unable to differentiate properly (Table 2) and exhibited delayed development, especially at the differentiation stage of prestalk and prespore formation. These results suggest that lower doses of benzamide have no deleterious effects on *D. discoideum* development.

HA-induced oxidative stress activates PARP within 5 min (Fig. 4A,B), and its role during oxidative stress is further confirmed by the use of low concentrations of benzamide. Preincubation of cells with benzamide prevented the peak activity observed during oxidative stress (Fig. 4A,B). Under oxidative stress, partial inhibition of PARP activity led to altered growth, suggesting that oxidative stress could be leading to cell cycle arrest [22] and that PARP inhibition possibly overcomes this arrest. PARP inhibition also rescued the oxidative stress-induced delay in development (Table 1), although the fruiting body was smaller than in controls (Fig. 5). Thus, our results suggest not only the presence of PARP in *D. discoideum*, but also its overactivation under moderate to severe oxidative stress. Our present study is the first report on the role of PARP in *D. discoideum* development.

PARP inhibitors are powerful cell-protective agents that block cell death in response to oxidative stress and hence are used as therapeutic molecules to control oxidative stress-related diseases [12]. However, the consequences of the blockade of cell death by PARP inhibitors for long-term cell survival are not entirely clear. In this context, we have studied the effect of PARP inhibition under oxidative stress on two generations by reviving the spores and monitoring growth and doubling time. It was found that in normal cells, PARP inhibition (1 mM benzamide) has no effect on spore germination. However, when cells were exposed to oxidative stress (2.5 mM HA) and allowed to develop, the spores remained dormant for longer time

as compared to control spores, as the spores took more time (56 h) to germinate as compared to control spores. Conversely, when cells were exposed to oxidative stress (2.5 mM and 4 mM HA) with PARP inhibition and allowed to develop, the spores showed faster germination (32 h and 60 h) as compared to cells exposed to oxidative stress alone (2.5 mM HA), as seen in Fig. 6. Interestingly, the amoebae thus formed due to spore germination (2.5 and 4 mM HA with and without PARP inhibition) exhibited normal development (data not shown), suggesting that second-generation cells had overcome the effect of oxidative stress. Thus, our results demonstrate that partial PARP inhibition under mild or severe oxidative stress did not affect repair of the damage incurred due to oxidative stress, as the amoebae formed upon spore germination exhibited normal growth and development for two successive generations. Our data support the idea that PARP inhibition is beneficial under oxidative stress and that PARP inhibitors are potential therapeutic molecules for the control of oxidative stress-related diseases. This study also opens the possibility for identifying the genes involved in *D. discoideum* spore dormancy under stress conditions.

Experimental procedures

Materials

Hydroxylamine, benzamide and anti-mouse IgG (whole molecule) fluorescein isothiocyanate (FITC) conjugate developed in rabbit were obtained from Sigma Aldrich (St Louis, MO), and mouse mAb (10H) to poly(ADP-ribose) (PAR) (Ab-1) was obtained from Calbiochem (San Diego, CA, USA).

Cell culturing

D. discoideum cells (Ax-2 strain) were grown in suspension in HL5 medium with shaking at 150 r.p.m. and 22 °C. Developmental studies were carried out on non-nutrient agar plates. All the experiments were carried out with *D. discoideum* cells at mid-log phase with a cell density of 2.5×10^6 cells·mL^{-1}. Amoebae were washed with 1 × Sorenson's buffer (SB) (17 mM potassium phosphate, pH 6.4) by centrifugation at 300 *g* for 5 min, and spread on phosphate-buffered agar (PBA) plates at a density of 2.5×10^5 cells·cm^{-2}. The plates were allowed to develop at 22 °C.

Dose-dependent effect of HA on *D. discoideum* cell death

Cells (2.5×10^6) were harvested by centrifugation at 300 *g* for 5 min at 4 °C, resuspended in HL5 medium, exposed to different doses (1.0, 2.5 and 4.0 mM) of HA, and shaken at 150 r.p.m. at 22 °C for growth [23]. Cell death was checked by a Trypan blue exclusion method after 24 h.

Effect of HA on *D. discoideum* growth

Cells (0.5×10^6) were harvested by centrifugation at 300 *g* for 5 min at 4 °C, resuspended in 4 mL of HL5 medium so that the cells entered lag phase, and then exposed to different concentrations (1.0, 2.5 and 4.0 mM) of HA for 1 h. The cells were washed with 1 × SB two or three times, and finally suspended in HL5 medium (pH 6.5) and shaken at 150 r.p.m. and 22 °C for growth. The cells were counted using a hemocytometer every 12 h up to 132 h (6 days) [23].

Effect of HA on *D. discoideum* development

Cells (2.5×10^6) were harvested and processed as described above for HA treatment (1.0, 2.5 and 4.0 mM), and the cells were then resuspended in 100 μL of 1 × SB and spread on non-nutrient agar plate (PBA plates). The plates were kept at 22 °C, and different stages of development were observed. Grids 1 mm square were made on a 35 mm plate, and then fruiting bodies in five such squares of different regions were counted under a microscope. Approximately 40 fruiting bodies were counted in the experiment.

PARP activation under HA stress

Cells treated with different doses of HA were centrifuged and washed once with NaCl/Pi, fixed in 70% chilled methanol for 10 min at −20 °C, washed with blocking solution (1.5% BSA with 0.05% Tween-20 in NaCl/P_i), and then incubated for 1 h with antibody to PAR raised in mouse at a concentration of 0.5 μg·mL^{-1} [24]. Cells were washed two or three times with blocking solution, and incubated for 1 h with FITC-conjugated anti-mouse IgG as secondary antibody, used at a dilution of 1 : 200. Cells were washed two or three times with NaCl/P_i, and fluorescence was observed at 60× magnification using a Nikon (Tokyo, Japan) fluorescence microscope with a charge-coupled device camera; results are shown for 2.5 mM HA only. Data were analyzed by IMAGE PROPLUS software to calculate the mean density of fluorescence from different fields, and ~50 cells were examined for each dose.

PARP inhibition by benzamide

A culture in log phase with a cell count of 1.0×10^6 cells was incubated with 1 mM benzamide, a PARP inhibitor [25], for 24 h. Cells were then treated with 2.5 mM HA and observed for PARP activation as for the PARP assay.

Effect of benzamide on HA-induced changes to *D. discoideum* growth

Cells (0.5×10^6) were treated with the 1 mM benzamide for 24 h, and then the cells were exposed to HA (2.5 mM) for 1 h. Cells were washed and resuspended in 4 mL of sterile HL5, and growth was monitored for 6 days.

Dose-dependent effect of benzamide on *D. discoideum* development

Cells (1.0×10^6) were harvested, resuspended in HL5 medium, and exposed to different concentrations (1.0, 2.0, 3.0 and 4.0 mM) of benzamide for 24 h at 22 °C. After 24 h of incubation, the cells were washed three times with 1 × SB and processed for development.

Effect of benzamide on oxidative stress-induced changes to *D. discoideum* development

Cells (1.0×10^6) were harvested, resuspended in HL5 medium, and exposed to 1 mM benzamide for 24 h at 22 °C. After 24 h of incubation, cells were treated with different concentrations of HA (2.5 and 4.0 mM) for 1 h. The cells were then centrifuged at 300 *g*, washed two or three times with 1 × SB, plated on PBA plates, and monitored for development.

Effect of benzamide on the fate of spores formed under HA stress

Spores formed after treatment with 2.5 and 4 mM HA in the presence and absence of benzamide were picked from different areas with the help of a sterilized nichrome loop, and added to 5 mL of HL5 medium. Flasks were continuously shaken at 150 r.p.m. and 22 °C. After germination, the cells were counted every 12 h using a hemocytometer.

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Review Article

Signaling molecules involved in the transition of growth to development of *Dictyostelium discoideum*

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The social amoeba *Dictyostelium discoideum*, a powerful paradigm provides clear insights into the regulation of growth and development. In addition to possessing complex individual cellular functions like a unicellular eukaryote, *D. discoideum* cells face the challenge of multicellular development. *D. discoideum* undergoes a relatively simple differentiation process mainly by cAMP mediated pathway. Despite this relative simplicity, the regulatory signaling pathways are as complex as those seen in metazoan development. However, the introduction of restriction-enzyme-mediated integration (REMI) technique to produce developmental gene knockouts has provided novel insights into the discovery of signaling molecules and their role in *D. discoideum* development. Cell cycle phase is an important aspect for differentiation of *D. discoideum*, as cells must reach a specific stage to enter into developmental phase and specific cell cycle regulators are involved in arresting growth phase genes and inducing the developmental genes. In this review, we present an overview of the signaling molecules involved in the regulation of growth to differentiation transition (GDT), molecular mechanism of early developmental events leading to generation of cAMP signal and components of cAMP relay system that operate in this paradigm.

Keywords: *Dictyostelium discoideum*, GDT, Signal transduction, cAMP

Introduction

D. discoideum often referred as 'slime mould' or 'social amoeba', is one of the simplest studied eukaryotes that possesses true multicellularity[1]. *D. discoideum* amoebae grow and divide asexually while feeding on bacteria or a defined medium. The most common stress that *D. discoideum* encounters is nutrient depletion and responds to it by shutting down growth and cell division and initiating a developmental program. Its development shows much of the complexity seen in a metazoan. One fundamental difference is that metazoans develop from a single cell, the zygote by a combination of cell division, growth and differentiation but *D. discoideum* development, requires no growth and multicellularity is achieved by aggregation of many unicellular amoebae. Developmental fate of the cells is determined by the cell cycle phase amongst other various factors[2-5].

Initiating events of *D. discoideum* development include sensing starvation and cell density, which in turn results in the isolated cells acquiring the ability to aggregate. The mechanism of density sensing by starved cells ensures that aggregation occurs only when there are sufficient number of starved cells to form aggregates and subsequent structures of appropriate size for optimized spore dispersal[6-8]. There are reviews discussing the molecular aspects of late stages of *D. discoideum* development, but not on the early events of development. Recent investigations have revealed the involvement of several components in regulating the initiation of development[9-12], however little information exists on how the cells exactly sense starvation and in particular amino acid deprivation. This review discusses about the signaling molecules involved in the early development of *D. discoideum* with an attempt to address the molecular events associated in sensing amino acid starvation.

Pre-starvation facto (PSF)

Throughout the vegetative growth, *D. discoideum* amoebae secrete an autocrine factor known as pre-starvation factor (PSF). It is a 68 kDa glycoprotein that is secreted while cells are in growth phase and accumulates as an indicator of the ratio of the cell density to the food supply[7,13]. The PSF response is inhibited by the bacteria used as a food supply, however when the bacterial population drops, the PSF inhibition is relieved, and PSF induces genes such as discoidin-I and cyclic nucleotide phosphodiesterase (PDE) that trigger the developmental process[14] (Fig. 1). Discoidin-I is a soluble lectin synthesized by

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aggregating cells which helps in adhesion of the cells to substratum[15] and its expression acts as a marker for growth to differentiation transition. Nevertheless, once the nutrients are depleted, PSF production declines and a second cell density sensing pathway mediated by conditioned medium factor (Fig. 2) gets activated[7,8,12].

Conditioned medium factor (CMF)

Cells coordinate their development so that aggregation occurs only when the density of starved cells is sufficiently high (about ~10^5 cells/cm^2). The starved cells differentiate when present at high density, whereas cells at low density generally do not differentiate. CMF, a 80 kDa glycoprotein is sequestered in vegetative cells but it is secreted upon starvation regardless of the cell density[7]. Accumulation of CMF into the medium is also affected by *p*H, light, cAMP pulses or cell cycle phase at the time of starvation, but CMF itself is the major factor that potentiates its own accumulation. Observed cell density necessary for differentiation matches with the diffusion calculation predicted cell density. The behavior of cells at different cell densities and the accumulation rate, diffusion coefficient, and activation threshold of CMF suggest that it serves as a part of cell density sensing system allowing *D. discoideum* cells to coordinate the onset of cAMP pulse mediated aggregation[16]. CMF initiates signal transduction pathway (Fig. 2) that amplifies at the cAMP level[8].

CMF increases the frequency of pseudopod formation and hence is important for chemotaxis. In the presence of high levels of CMF, the cAMP pulse causes the cell to chemotax towards cAMP, relay cAMP signal and express specific genes. CMF is necessary for the cAMP induced Ca^{2+} influx, activation of adenylyl cyclase (ACA) and guanylyl cyclase (GCA) (Fig. 3). Binding of cAMP to cAR1 causes a transient influx of Ca^{2+} and activates an associated heterotrimeric G protein. CMF among

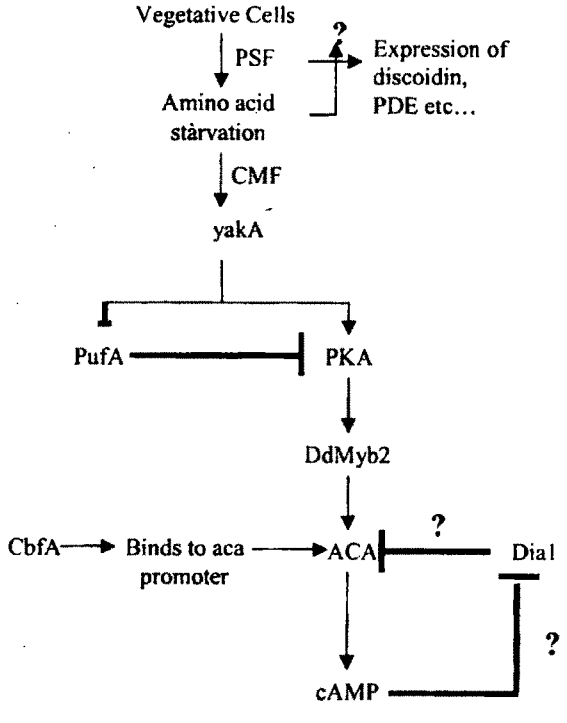

Fig. 1—A schematic description of the pathway controlling onset of *D. discoideum* development. PSF after sensing amino acid starvation leads to Yak A activation, releasing negative control of Puf A on *pka-C* mRNA translation which consequently results in increased production of cAMP by adenylyl cyclase. Question marks indicate the uncharacterized aspects of *D. discoideum* development.

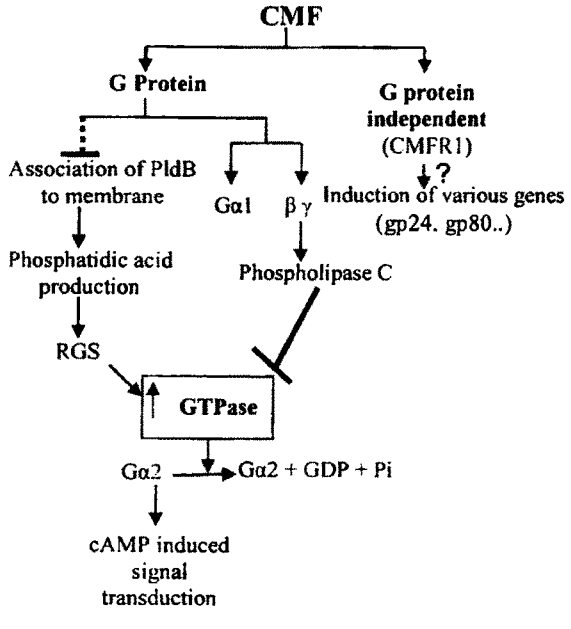

Fig. 2—Two hypothesized signal transduction pathways induced by CMF to mediate the cAMP induced chemotaxis in *Dictyostelium discoideum* cells. CMF has both G protein dependent and independent actions by which it affects cAMP signal transduction and gene expression, respectively. CMF either affects the GTPase by regulating the RGS activity through phospholipase D or by modulating phospholipase C activity through G beta-gama. The question mark indicates the uncharacterized aspect/s and the dotted line indicates an unknown mechanism.

others regulates CRAC (cytosol regulator of adenylyl cyclase), which in turn assists the Gβγ subunit to transiently activate ACA either by modulating its activity or by changing its subcellular localization, while the Gα2 subunit activates GCA. Thus CMF mediates activation of guanylyl cyclase and adenylyl cyclase via the cAMP receptor 1 (Fig. 3). Regulation of guanylyl and adenylyl cyclases is independent, as mutants lacking adenylyl cyclase have normal cAMP stimulated guanylyl cyclase activation, and *vice versa*[8].

CMF signal transduction also involves G protein coupled receptor as GTPγS partially inhibits the binding of CMF to membranes (Fig. 2). Gα1 null cells fail to show this GTPγS induced inhibition or CMF regulation of cAMP signal transduction, which indicates that a putative CMF receptor interacts with Gα1[17].

CMF affects cAMP signal transduction by regulating the Gα2-GTP conformation. The CMF leads to Gα1/βγ dissociation and thereby activates phospholipase C (PLC) (Fig. 2). Activated PLC inhibits the Gα2-GTPase and hence stabilizes the cAMP activated Gα2-GTP, ultimately promoting the cAMP signal transduction process[17,18]. Another hypothesis for the regulation of the GTPase activity by CMF involves RGS (Regulator of G protein signaling) protein and PldB (a phospholipase D). RGS proteins act as GTPase activating proteins for heterotrimeric G proteins and are regulated by phosphatidic acid (PA) produced by PldB (Fig. 2).

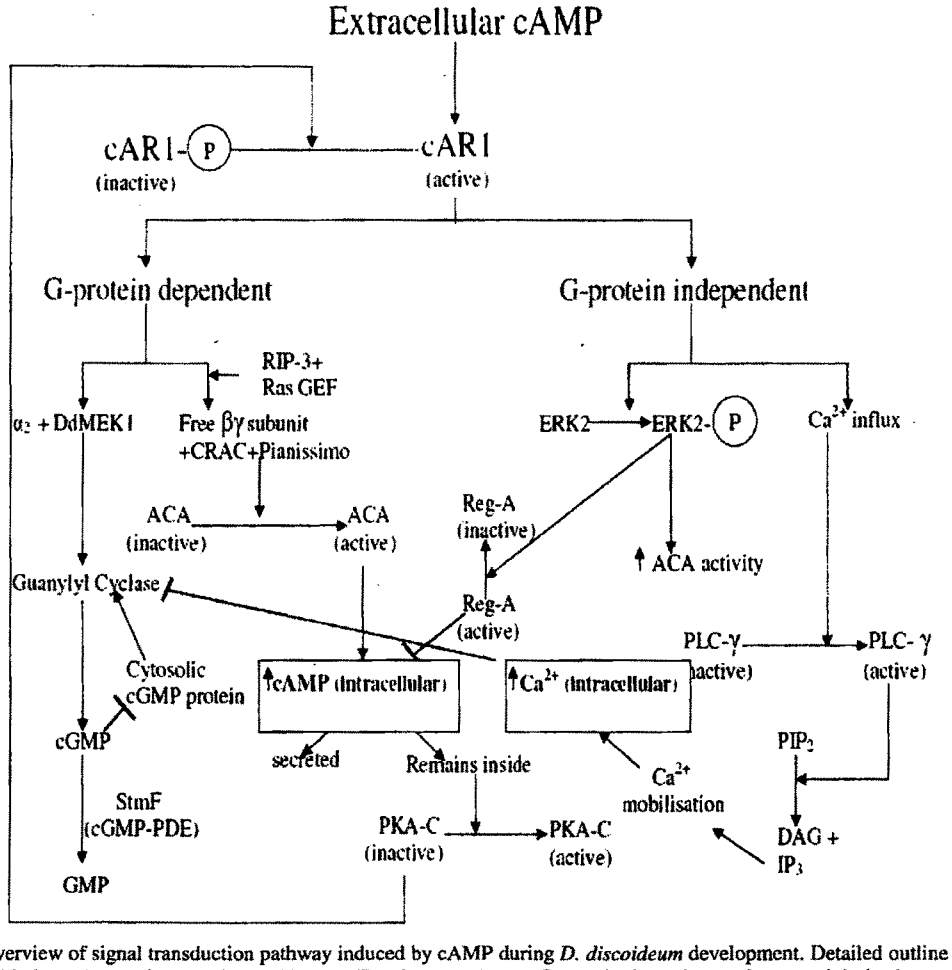

Fig. 3—An overview of signal transduction pathway induced by cAMP during *D. discoideum* development. Detailed outline of G-protein dependent and independent pathways triggered by cAMP pulses are shown. G-protein dependent pathway mainly leads to an increase in the intracellular cAMP, whereas G-protein independent pathway results in increased intracellular Ca^{2+} levels. However, the two cascades do cross talk at some points and the effects may not be mutually exclusive.

PldB is found to be indispensable for the proper aggregation as cells lacking *pldB* aggregate at even low cell density bypassing the need for CMF, while *pldB* overexpressing cells do not aggregate at high cell density, and neither do such cells respond to exogenous addition of CMF. PldB has a PH domain (phospholipid binding domain), which is responsible for translocating proteins bearing the PH domain to the plasma membrane during chemotaxis. PldB (through localization provided by its PH domain) could be involved in the localized regulation of Gα2, the G protein mediated cAMP chemotaxis. This localized control of G protein activity could influence the polarization of chemotaxing cell. It is also hypothesized that CMF could decrease the PldB association with the membrane, leading to decreased PA production. This would consequently decrease RGS activity and thereby allowing increased signaling through Gα2 (Fig.2). Also *pldB* null cells show higher levels of *cAR1* expression earlier than the wild type cells, which indicates that PldB plays an important role in the timing of development[19].

CMF also has a role in the induction of pre-stalk and prespore gene expression along with cAMP in a G protein-independent manner. A 50 kDa membrane protein, CMFR1, binds to CMF during affinity purification and may be responsible for these effects of CMF at the gene level (Fig. 2). Heterologous expression of *cmfr1* leads to increased CMF binding while disruption of *cmfr1* in *D. discoideum* cells leads to about 50% reduction in CMF binding and all of its associated G-independent signal transduction[20]. This limited effect of *cmfr1* disruption exclusively on G-protein independent functions suggests that CMF has more than one receptor[21]. CMFR1 upregulates cAMP binding and is involved in the regulation of gene expression in growth to differentiation transition. Among others gp24 and gp80 are under CMFR1 control (Fig. 2); gp24 is important for the initial formation of filopodia mediated intercellular contacts and aggregation, whereas gp80 is involved in regulating cellular streaming. Thus, CMF is required for proper aggregation of *D. discoideum* cells under starvation (Fig. 2).

Myb2

DdMyb2, a transcription factor contains three Myb repeats, a DNA-binding helix-turn-helix motif, a potential nuclear localization signal, two potential PKA-C phosphorylation sites, and glutamine-, proline- and acidic amino acid-rich regions of Myb-related transcription factors. *DdMyb2* null cells show undetectable levels of adenylyl cyclase *(ACA)* transcript and no cAMP production. Ectopic expression of *aca* rescues differentiation and morphogenesis of *DdMyb2* null mutants suggesting that development in *D. discoideum* starts by starvation-mediated DdMyb2 activation. Protein kinase A (PKA-C) might be involved in starvation-mediated DdMyb2 activation and thus regulating its translocation to the nucleus, which then binds to the upstream region of *aca* gene and induces adenylyl cyclase expression (Fig. 1). The adenylyl cyclase A thus formed produces the very first few molecules of extracellular cAMP that induces chemotaxis and aggregation in neighboring cells. Intercellular signaling by secreted cAMP then induces expression of other genes required for further stages of development (Fig. 3). Thus DdMyb2 that mediates the initial induction of adenylyl cyclase seems to play a central role in the growth to development transition in *D. discoideum*[22].

Several genes have been found to regulate the growth to differentiation transition (GDT) in *Dictyostelium discoideum*. Positive regulators of GDT are *yakA, pka, dia2, amiA* and *amiB* while negative regulators are *pufA, dia1 and gdt2.*

YakA

To begin differentiation cells must reach a specific point in G2 phase of the cell cycle and specific cell cycle regulators would facilitate the exit from cell cycle and mediate transition from growth to development. One such factor is YakA. YakA is a cytosolic protein kinase which phosphorylates itself as well as myelin basic protein. *yakA* is expressed at low levels in all cells during growth phase, and peaked at the onset of starvation and then decreases but is present throughout the development. *yakA* null cells have faster and smaller cell cycle compared to wild type, suggesting a role for YakA in cell cycle regulation and coordination between growth and cell cycle progression[11]. Pronounced defect in actin polymerization and cGMP accumulation in *yakA* null cells and the phenotypic similarity between *yakA*⁻ and *gβ*⁻ cells suggests that YakA has a role in G-protein mediated signaling pathways. *yakA* overexpression caused a growth arrest in *gβ*⁻ cells and cAMP receptors are still coupled to G-proteins in the *yakA* mutant suggesting that YakA operates downstream to Gβ[23]. Studies with temperature sensitive *yakA* mutants suggest that YakA is not only required during onset but throughout the development.

The conditioned medium containing PSF induces the accumulation of yakA mRNA in wild type cells (Fig. 1), suggesting that *yakA* expression may be controlled by PSF. PSF signaling is independent of *yakA* as discoidin-I was expressed normally in *yakA* null cells. The regulation of YakA by PSF might provide a way for the cells to coordinate nutrient availability with cell division (Fig. 1).

YakA has a dual role in starvation sensing- growth arrest and induction of PKA-C. YakA controls cell division during growth by ensuring that cells are of proper size before they divide and also regulates the interval between two cell divisions. Overexpression of *yakA* arrests the growth of *Dictyostelium* cells in G2 phase[11]. Induction of *yakA* upon starvation leads to a decrease in vegetative phase gene expression such as *cprD* and induces the expression of *pka, aca* and *cAR1*. Induction of *yakA* consequently relieves PufA mediated translational block on pkaC mRNA (Fig.1). During the first 4-6 h of development there is about 5 fold increase in *pkaC* mRNA, PKA-C protein and its YakA mediated activation[9].

PKA-C controls the timing of early developmental events by regulating expression of the key cAMP signaling proteins such as cAR1 and ACA through Myb2[22,24,25] (Fig. 1). It is possible that PKA-C or cAMP signaling is required for YakA mediated response during development but neither of them is required for YakA induced growth arrest[11]. PufA is the key effector of YakA starvation response pathway leading to multicellular development.

PufA

PufA is a translational regulator belonging to Puf protein family. PufA binds to the PufA regulatory elements (PREs) present in the 3′ end of *pkaC* mRNA and regulates its translation directly. There is an inverse relationship between PKA-C protein and pufA or pka-c PRE complex (Fig. 1). *pufA* null cells rescue developmental defects in yakA null cells and have no obvious cell cycle defect. *pufA* null mutation does not alter the fast growth phenotype of *yakA* mutants nor change the growth rate of wild type cells but *yakA* null and *pufA* null double mutants showed accelerated development during the initial hours of starvation compared to wild type cells. Thus inactivation of PufA restores developmental gene induction but not the growth phase gene repression in *yakA* null cells. Inactivation of *pufA* rescues early developmental stages of *yakA* null cells but exhibit arrestation at culmination phase[9]. This reflects additional function for PufA during later stages of development, which is yet to be studied. Thus, the possible mechanism is that YakA shuts off *pufA* expression at the onset of development, therefore YakA is the master regulator switch between vegetative and developmental gene expression. Inhibition of PufA by YakA relieves the negative control on PKA-C thereby causing an increase in cAMP production via adenylyl cyclase and thus leads to development.

Gdt2

Gdt2 is a serine/threonine protein kinase and it is expressed in vegetative cells and also throughout development at the same level but with a slight peak at the time of aggregation. *gdt2* null mutants develop prematurely and in such cells discoidin can be detected at a density of 1×10^5 cells. As these cells can sense folate, it is more likely that *gdt2* null mutants have an impaired mechanism in sensing amino acid/s. *gdt2* null mutants have no effect on PKA activation, suggesting that Gdt2 is downstream to PKA[26]. Gdt2 is involved in the control of growth to differentiation transition via an unknown pathway.

Dia1

Cells in any phase of the cell cycle can start differentiating from PS (Putative Shift) point. Several genes are expressed in response to initial differentiation from PS point. One such gene is *dia1*, which is expressed at 2h after starvation, reaching a peak at 4h followed by a rapid decrease in its levels. *dia1* is adjacent to *impA* on chromosome 4, with their start codons separated by intergenic region of 654 bp. It is an example of bidirectional regulation where intergenic region regulates the expression of these two genes during growth and development. These two genes are inversely expressed before and after GDT. There are three regions within this 654 bp sequence, which play significant role in the regulation of *dia1* and *imp* gene. A pair of 7-bp direct repeats in the 92 bp region proximal to *impA* is essential for expression of both the genes. Middle region is essential for repression of *dia1* expression in growing cells. This region contains a sequence, which is recognized by a repressor and blocks transcription of *dia1* but not of *impA* during growth. This repressor disappears following a shift from nutrient to starving medium[27]. Expression of *dia1* is transient and is seen only during initial stage of development. *dia1* overexpression

suppresses the progression of differentiation and such cells showed delay in aggregation (and some of them could not even aggregate), but the fruiting body formed by such cells was found to be normal. Antisense mediated gene inactivation of *dia1* has shown to enhance the progression of differentiation. Such cells become aggregation competent and formed aggregates within 5.5 h compared to 8 h in control cells. *dia1* expression affects genes involved in cAMP signaling and its overexpression reduces the expression of cAR1 upto 2-4 h, while *aca* mRNA was expressed weakly after 4-6h of starvation. Underexpression of *dia1* exhibited the precocious expression of cAR1 and *aca*. Thus *dia1* plays an inhibitory role during early development by reducing the expression of cAR1 and *aca* genes of cAMP relay system (Fig. 1). DIA1 protein seems to be negatively coupled with cAR1 and ACA associated events but exact mechanism is yet to be elucidated. Developmental defect of *dia1* overexpressing cells were nullified by mixing these cells with the wild type cells. cAMP pulses also restored the delayed aggregation of *dia1* overexpressing cells, suggesting that cAMP secreted from wild type differentiating cells would remove the inhibitory effect of DIA and allow *dia1* overexpressing cells to develop normally. Thus, cAMP most likely acts as a suppressor of the DIA1 function[28] (Fig. 1). DIA2 and DIA3 are also required for proper expression of early genes such as cAR1 and *aca*[29].

AmiA and AmiB

AmiA and AmiB positively regulate the GDT possibly via regulation of adenylyl cyclase expression[28]. AmiB is also necessary for aggregation as *amiB* null cells failed to repress the vegetative gene *cprD* (a cysteine proteinase) during growth, suggesting that they cannot sense starvation[10]. *amiB* null cells exhibited changes in the distribution of actin, Apr and myosin II resulting in defective locomotion possibly due to altered cytoskeletal regulation[30]. Genetic studies suggested that amiA is involved in bridging communication between cAMP receptor and adenylyl cyclase[31]. Interestingly, *amiA* null cells also showed partial disruption in cell division[32]. The exact mechanism of action of AmiA and AmiB in the control of growth to differentiation transition is yet to be studied.

CbfA

CbfA (C-module DNA binding factor) interacts *in vitro* with a regulatory element in retrotransposon TRE5-A. It is a transcription factor which binds to AT-rich target sites in *Dictyostelium* genome and regulates the expression of its target genes. CbfA is not required for pre-starvation response as yakA pathway functions normally in *cbfA*^{am} amber mutant cells. It seems to act downstream of the yakA pathway (Fig. 1) and controls transcription of *acaA* and other genes directly or indirectly. CbfA depleted cells were unable to aggregate and *cbfA*^{am} cells failed to activate cAMP induced genes in early development. *cbfA*^{am} cells when supplied with cAMP pulse, causes induction of *acaA* and showed further development. Sensing of cell density and starvation are independent of CbfA[33], however, CbfA binds to the *acaA* promoter to provide a basal transcription activity that is required for induction of *acaA* expression after the onset of *D. discoideum* development[34].

Protein kinase (PKA)

PKA, a cAMP dependent protein kinase plays multiple roles during *D. discoideum* development and it is the central component in signal transduction pathway. It phosphorylates a variety of proteins and thereby affects their activity. Inactive form of PKA consists of catalytic subunit (PKA-C) associated with regulatory subunit (PKA-R). Exponentially growing *D. discoideum* cells consist of both the subunits, as PKA is not required for growth. There is a five-fold increase in catalytic subunit levels in the first 6 h of development and is maintained till culmination[9]. The signal transduction pathway that initiates from cell surface binding of cAMP to accumulation of mRNA appears to act through PKA (Fig. 1) since *acaA* is not expressed in *pkaC*-null cells. PKA plays a central role in timing of the burst of adenylyl cyclase activity. PKA regulates this adenylyl cyclase activity by phosphorylating adenylyl cyclase or any of its coupling components. PKA is regulated by *regA* which encodes a phosphodiesterase that can reduce the cAMP available to PKA-R and hence free form of PKA-R will associate with PKA-C resulting in an inactive form[35] (Fig. 3).

cAMP

In the social amoeba *D. discoideum*, cAMP via PKA controls almost all the major life cycle transitions including growth to development transition. Aggregation of *D. discoideum* amoebae into multicellular structures is organized by cyclic AMP (cAMP), which acts as a chemo-attractant, as a

second messenger, and as a morphogen. Once the cAMP accumulates in sufficient amount it triggers the cascade of events (Fig. 3).

Components of the cAMP relay system

During starvation, *D. discoideum* amoebae become responsive to cAMP, which is released in a pulsatile fashion and governs the process of aggregation. The cells achieve competence to relay cAMP signals within a period of 6 h of starvation. Initially at lower concentration of cAMP, the receptors undergo excitation leading to a cascade of processes, but as the cAMP concentration rises extracellularly, the receptors become desensitized due to the modification/sequestration/internalization/degradation of receptors/uncoupling of receptors and target proteins, etc. Desensitization can be reversed if the cAMP signal remains absent for a fixed period of time and this reversal is facilitated by the removal of cAMP by extracellular phosphodiesterase (ePDE)[36] (Fig. 3). Rapidly developing (rde) mutants of *Dictyostelium discoideum*, in which cells precociously differentiated into stalk and spore cells without normal morphogenesis, were investigated genetically and biochemically. Genetic complementation tests demonstrated that the rde mutants could be classified into at least two groups (groups A and C). Measurements of cell-associated and extracellular phosphodiesterase activities and intracellular and total cAMP levels revealed that cAMP metabolism in both groups are significantly altered during development. Group A mutants showed precocious and excessive production of phosphodiesterase and cAMP during the entire course of development; intracellular cAMP levels in group C mutants were extremely low, and spore and stalk cell differentiation occurred without an apparent increase in these levels[37].

cAMP receptors

Serpentine G-protein-coupled cAMP receptors are the key components in detection and relay of the extracellular cAMP waves that control chemotactic cell movement during *D. discoideum* development. During development the cells sequentially express four closely related cAMP receptors of decreasing affinity (cAR1-cAR4). Of these cAR1 and cAR3 are high affinity receptors expressed before and during aggregation, respectively[38,39], whereas cAR2 and cAR4 are low affinity receptors expressed after aggregation in pre-stalk cells[40,41].

cAR1 is the first one to be expressed during early aggregation and its expression continues in the later stages of development in all cells. It is necessary for aggregation because cells lacking cAR1 fail to aggregate. cAR1 is involved in activation of ACA, GCA and ERK2 with nanomolar concentrations of cAMP[42-44] during aggregation. Expression of aggregative genes by cAMP pulses is mediated by cAR1[45]. Desensitization of cAR1 occurs due to prolonged stimulation of the receptors by micromolar concentration of cAMP, by internalization and degradation of the receptor[46]. The half time of this process is 15-30 min and once the cAMP stimulus is removed, it takes several hours for the receptor to reaccumulate. cAR3 shows the highest affinity for cAMP and it is expressed during aggregation. However, deletion of *cAR3* has no obvious phenotype[47]. In the slug, the expression of *cAR3* becomes confined to the pre-spore cells. Expression of *cAR2* starts at the mound stage in the cells forming prestalk zone. cAR4 is expressed in a pre-stalk specific manner at the slug stage. Deletion of cAR4 leads to defects during culmination[41]. This activation of ACA, GCA and ERK2 may also be brought about by other cARs (other than cAR1) during other developmental stages. cAMP induces an increase in the intracellular Ca^{2+} levels directly by increasing the Ca^{2+} influx[48,49] and indirectly by stimulating phospholipase C (PLC). The influx of Ca^{2+} can be induced by all four receptors[50]. cAMP signals can activate PLC in null mutants of cAR1/cAR3 in aggregative cells which do not show cAR2 and cAR4 expression. This suggests the possibility of Gα2 associated fifth cAR.

Adenylyl cyclases (AC)

Genes encoding three distinct adenylyl cyclases have been characterized and are shown to be expressed at different stages of *D. discoideum* development[51,52]. Adenylyl cyclase A (ACA), a G-protein-coupled adenylyl cyclase is one of the first genes to be expressed upon starvation. It produces extracellular cAMP, which is the signaling molecule required for the chemotaxis and aggregation of neighbouring cells. The osmosensory adenylyl cyclase, *acgA* is expressed only during germination of spores[51,53,54], while adenylyl cyclase R (ACR) produces internal cAMP necessary for terminal differentiation of spores[55]. ACG is required for the maintenance of spore dormancy and is known to have intrinsic osmosensing property.

ACA is activated when the G-protein coupled surface receptor CAR1 binds extracellular cAMP (Fig. 3). However, during culmination, ACR activity is tenfold higher than ACA activity. Studies showed that ACR activity is essential for morphogenesis as well as the maturation of spores[53,56,57]. Thus, *acrA* cannot fulfil the role of *acaA* in production of extracellular cAMP necessary for chemotaxis while *acaA* cannot fulfil the role of *acrA* in production of internal cAMP necessary for terminal differentiation of spores. The discovery of ACR helped to resolve many controversial observations on the roles of extra- and intra-cellular cAMP in the regulation of gene expression. ACR seems to be independent of G-proteins[52], and capable of activating PKA-C in the absence of ACA.

Developmental studies with *acaA* null cells showed that adenylyl cyclase is essential for the chemotactic response. The behavioral defects of *acaA* null cells were interestingly similar to those of null mutants of *regA*, which encodes the intracellular phosphodiesterase that hydrolyzes cAMP and, hence, functions opposite to adenylyl cyclase A (ACA). Thus ACA and RegA are components of a receptor-regulated intracellular circuit that controls protein kinase A activity (Fig. 3) and the suppression of lateral pseudopods in the front of a natural wave depends on a complete circuit. Hence, deletion of any component of the circuit (i.e., RegA or ACA) would result in the same chemotactic defect[58].

Guanylyl cyclases (GCAs)

The cAR1 activated Gα2 subunit leads to guanylyl cyclase activation[50,59]. Activated guanylyl cyclase causes an increase in cGMP concentration, which in turn leads to pseudopod extension via myosin phosphorylation[60,61] (Fig. 3). Null mutants of *cAR1* and *Gα2* do not show cAMP induced guanylyl cyclase activation. Activation of guanylyl cyclase by cAR1 requires Gα2 subunit and MAP Kinase-DdMEK1[62]. In addition to cAMP signal, activation of guanylyl cyclase requires binding to a cytosolic cGMP binding protein. When the concentration of cGMP is low this protein is free to bind to the enzyme and activate it, while at high concentration of cGMP it binds to cGMP thereby preventing the guanylyl cyclase activation. Increase in the concentration of cGMP due to activation of guanylyl cyclase is transient because cGMP is rapidly degraded by cGMP-phosphodiesterase (PDE), which is activated by cGMP itself. Also the activity of guanylyl cyclase is inhibited by Ca^{2+}at nanomolar range, which is easily achieved by Ca^{2+} influx and Ca^{2+} mobilization (Fig. 3). Adaptation at the receptor level also keeps cGMP concentration in check. There are also evidences indicating the presence of two different MAP kinase cascades involved in aggregation, one consists of ERK2, mediating the cAMP effects, and the other contains DdMEK1, essential for the cAMP mediated activation of guanylyl cyclase.

Phospholipase C (PLC)

The activated Gα2 subunit also activates PLC γ that catalyses the conversion of phosphatidylinositol-(4,5)-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), the latter product in turn leads to Ca^{2+} mobilisation causing an increase in intracellular Ca^{2+} concentration. However, a novel metabolic pathway independent of PLC has also been demonstrated[63]. Ca^{2+} is required for PLC activity, which could be provided either directly by inducing Ca^{2+} influx or indirectly by Ca^{2+} mobilization brought about by IP3 (Positive Feedback Effect) (Fig. 3). Contribution of Ca^{2+} requirement to the cAMP mediated regulation of phospholipase C activity is not clearly understood. IP3 can also be generated by breakdown of IP5[64,65] suggesting that IP3 signaling is important for aggregation, but evidence to the contrary exists, that IP3 dependent calcium signaling may not be required during aggregation[66]. The interaction between cAMP and CMF signaling involves IP3 and PLC activity (Fig. 3). CMF binding to its receptor activates PLC, while PLC inhibits the GTPase activity of Gα2, prolonging the lifetime of the response[17] (Fig. 2).

Gα2 null mutants do not show PLC activation, but *cAR1/cAR3* double null mutants show PLC activation in aggregation stage indicating the presence of fifth cAR and thus cAR5-Gα2 combination is important for the activation of PLC. 3'-deoxy-3'-aminoadenosine 3':5'-monophosphate (3'NH-cAMP) is a partial antagonist of cAMP which can only inhibit PLC. This inhibitory effect of 3'NH-cAMP is lost in cAR1 and Gα1 null mutants[64] suggesting that cAR1- Gα1 combination is involved in adaptation of PLC. Recently it has been identified that PLC also plays an essential function in germination of spores. Under adverse environmental conditions PLC activity is inhibited and so reduced IP3 levels prevent germination of spores. Inhibition of spore germination by high osmolarity is probably a dual control of ACG and PLC[67].

MAP kinase ERK

Two MAP-kinase genes *erk A* and *erk B*, encoding ERK1 and ERK2, respectively, have been identified. ERK1 is required for vegetative growth and during multicellular development[68], while ERK2 is essential for cell aggregation. ERK2 regulates the receptor mediated adenylyl cyclase activation however, it is not yet clear whether this activation is direct or indirect. ERK2 mediates the coupling of extracellular stimuli such as cAMP and folic acid to adenylyl cyclases through two pathways (Fig. 3). In aggregating cells exogenous cAMP leads to rapid and transient activation of ERK2[50,51], in the presence of cAR1, this activation is also observed in Gα 4 null mutants indicating the presence of novel receptor mediated pathway for ERK2 activation[69]. Activation of ERK2 is under negative regulation of Ras signaling pathway. PKA-C and CRAC are involved in adaptation of ERK2[49,70]. *erkB* null mutants show aggregation defect which is suppressed by *pka-c* overexpression, implying that PKA lies downstream of ERK2 mediated responses[68] (Fig. 3). Apart from these classical signaling molecules reactive oxygen species are also involved in the *D. discoideum* development. Interestingly, oxidative stress and UV-C irradiation were found to affect the development of *D. discoideum*[71-73]. These aspects of *D.discoideum* are also very intriguing however, the details are beyond the scope of this review.

Counting Factor

In sporulating organism like *Dictyostelium discoideum* a balance is maintained for differentiating as many spores as possible and supporting the spores by stalks that are sufficiently tall and strong to elevate the spore mass above the substratum and to assist the dispersion of spores to an environment that is richer in food. In response to cAMP pulse *D. discoideum* cells form fairly uniform aggregates at a sufficient density, suggesting that a mechanism exists to restrict aggregate size within an optimal range. Individual cells would be able to sense the number of cells in a group by secreting and sensing a diffusible factor known as counting factor[13,74,75], which is required for proper function of a cell counting mechanism that regulates organism size. Counting factor is a large complex of >450 kDa of at least five polypeptides, with molecular masses of about 60, 50, 45, 40, and 30 kDa, and its oversecretion leads to the formation of smaller fruiting bodies.

Computer simulations indicate that a stream stays intact if the cell-cell adhesion is high and the random cell motility forces are relatively low[76]. If the adhesion forces are less than the random motility forces, the cells will instead begin to disperse, disrupting the integrity of the stream. Thus the size of the groups depends inversely on the extent and length of time the adhesion forces are less than the motility forces[77,78]. cAMP stimulated cGMP pulse is repressed by CF (Fig. 4). Cells oversecreting CF have attenuated cGMP pulse whereas cells with mutated countin have increased cGMP compared to wild type cells. These results were further supported by the addition of anticountin-Ab to wild type cells which led to increased cGMP, while addition of recombinant countin yielded decreased cGMP levels. CF is able to bring back the group size to normal in streamer F cells (mutants forming abnormal fruiting bodies due to lack of cGMP PDE) and *countin* null cells show high GCA activity. Hence this repression of cGMP levels by CF is mediated by modification at the GCA activity level rather than any changes in the cGMP PDE activity (Fig. 4).

CF potentiates the cAMP-stimulated cAMP pulse without affecting the kinetics of the cAMP receptor, cAMP-induced GTP binding to membranes, the subsequent GTP hydrolysis, the GTPγS inhibition of cAMP binding, or the binding of the cytosolic regulator of adenylyl cyclase (CRAC) to membranes[76]. The binding of CRAC to membranes is due to cAMP activating a phosphatidylinositol 3-kinase, which creates phosphatidylinositol 3,4,5-

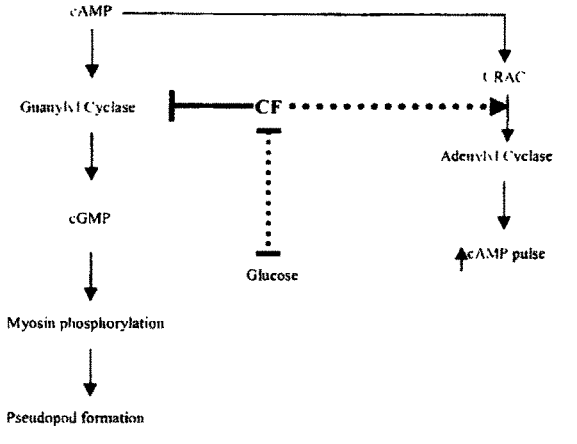

Fig. 4—A schematic representation of the counting factor (CF) induced regulation of chemotaxis and pseudopod formation during *D. discoideum* development. CF and glucose have opposing effects. Dotted lines indicate unknown mechanism of action of CF.

trisphosphate and phosphatidylinositol 3,4-bisphosphate on the inner surface of the plasma membrane; a pleckstrin homology domain on CRAC then binds to these lipids[79,80]. CF is not regulating group size by regulating the cAMP receptor or its activation of G proteins. It appears that CF regulates cAMP signal transduction at a step downstream of the cAMP receptor and G protein activation[81] (Fig. 4).

One of the component polypeptides of CF was purified and termed countin. Disrupting the expression of *countin* essentially abolishes CF activity, indicating that either countin is a key component of CF that directly affects cells or countin is simply a necessary part of the CF. Optimal concentration of recombinant countin did not cause an increase in group number or a significant decrease in adhesion as purified CF, suggesting that other components of CF are needed for a maximal change in group number. Purified CF potentiates the cAMP stimulated cAMP pulse within 60s while 60s exposure of cells to countin can decrease myosin polymerization and an increase in actin polymerization, myosin phosphorylation, and GTPγS stimulated activity of adenylyl cyclase. This suggests that countin, like CF, stimulates a rapid signal transduction pathway that has a direct effect on actin polymerization and a modulating effect on the cAMP receptor to adenylyl cyclase pathway (Fig. 4). *countin* null cells have a considerably higher cell-cell adhesion than parental cells. Also recombinant countin modulates the GTPγS stimulated activity of adenylyl cyclase without affecting the basal or Mn^{2+}-stimulated activities. CF possibly affects the cAMP-stimulated cAMP pulse at a step between the binding of CRAC to membranes and adenylyl cyclase[82] (Fig. 4).

Disrupting the expression of *cf50,* another component of CF, has essentially the same effect as disruption of *countin* with respect to group size, adhesion and motility, but unlike the effect of disrupting *countin*, disrupting *cf50* affects the initial cell-type choice. However, recombinant CF50 does not seem to increase group number to the extent that CF can. Thus neither CF50 nor countin is the sole effector molecule in the CF complex, but both the molecules can independently affect group size, as when the medium of countin null cells are immunodepleted with antibodies against CF50 or vice versa the cells form larger fruiting bodies compared to the single null cells.

Countin also shows its effect on cAMP pulse. One minute treatment of wild type cells with countin increases ACA activity by GTPγS and thereby increases cAMP pulse while *Erk2* is repressed. Effect of CF50 is found to be exactly opposite on these two factors. CF50 deletion reduces the percentage of CP2 positive (prestalk) cells and increases the percentage of SP70 positive (prespore) cells. Such changes in differentiation are not seen with countin deletion. Altogether these facts implicate that though countin and CF50 have negative effect on the group size, they have different and unique effects on the initial cellular differentiation and cGMP pulse and Erk2 activation. This implicates that countin and CF50 may activate two different signal transduction pathways, which have some different/unique effects, converging at some downstream point to give common effect on the group size[82,83].

Relation of CF and glucose has been unravelled to certain extent and this further elucidates the mechanism of group size regulation. Like CF, glucose affects stream breakup rather than altering territory size or mound breakup. Glucose partially negates the effects of countin and CF50 addition. Glucose affects two main downstream targets of CF, cell-cell adhesion and motility. Either increasing the glucose levels or decreasing CF would increase gp24 levels, adhesion, and myosin polymerization and decrease actin polymerization and motility. CF increases the cAMP induced cAMP pulse while addition of glucose decreases the cAMP pulse size. Also *countin* null cells have a large and prolonged cGMP pulse, whereas exposure of cells to high glucose results in prolonged pulse. All these facts together suggest that glucose per se or one of its metabolites may affect CF signal transduction pathway and the difference in cGMP pulse response in countin null cells and cells exposed to glucose points out that CF affects a pathway in addition to the one involving glucose[84]. Recently, CF has been shown to affect the activity of microsome associated glucose-6-phosphatase enzyme[85]. However, the importance of glucose-6-phosphatase and CF interaction in the regulation of the organism size is not yet clear.

CF45, a component of CF is expressed in vegetative and early developing cells and cells lacking CF45 form huge groups. Like *countin* null and *cf50* null cells, *cf45* null cells have high glucose levels, high cell-cell adhesion and low motility. Exogenous CF45 rescues the huge group size in *cf45* null cells to

some extent; however, the group size is not comparable to the wild type cells. High extracellular levels of countin causes *cf45* null cells to form small groups. *countin* null and *cf50* null cells oversecrete CF45 than wild type yet, form larger groups. Thus CF45 functions as a part of CF complex but not as the sole factor to determine the group size and also that the three proteins, countin, CF45 and CF50, affect each other's secretion or stability and they seem to have overlapping as well as exclusive functions[86].

CF60, fourth component of the ~450 kDa CF complex, has been recently identified[87]. After secretion of CF complex (counting factor) by the starving cells, CF60 dissociates from the complex in the absence of CF50. Its activity is dependent upon CF50 while independent of countin. Decreased expression of CF60 also led to formation of large groups while overexpression resulted in very small groups[87].

Recently, an autocrine proliferation repressor, AprA has been identified in *Dictyostelium* system. This 60 kDa protein has similarity to bacterial proteins of unknown function. It serves as a part of about 150 kDa complex. AprA has been reported to slow down the proliferation and thereby cell cycle and coordinate cytokinesis with mitosis. *aprA* null cells form larger fruiting bodies. The correlation between its effect on growth and on the formation of larger fruiting bodies is yet to be characterized. Similarity in phenotypes between *yakA* null cells and *aprA* null cells poses an interesting question regarding the association of these proteins in the AprA induced signal transduction pathway leading to regulation of size during *Dictyostelium* development [88].

Conclusion

Relatively simpler life cycle of *D. discoideum* makes it a good model organism for studying cellular movement, chemotaxis, cell-cell interaction, cellular differentiation and cell death. These processes are involved during multicellular development. cAMP is the key molecule responsible for the signaling pathways in *D. discoideum*. Binding of cAMP to its receptor results in certain short term responses, which could be G-protein dependent or G-protein independent processes. G-protein independent responses are due to the Ca^{2+} influx and phosphorylation of cAR1[57,58] while the other cAMP responses are mediated by hetero-trimeric G-protein. Long-term responses of cAMP include expression of certain genes which are consistent for cell specificity such as prestalk specific *rasD* gene. Most of the genes are induced by nanomolar pulses of cAMP while certain genes require mM levels. Changing levels of cAMP pulses are involved in regulating cAMP induced developmental gene expression[89]. Regulation of intracellular cAMP levels is also involved in pathways required for the pulses of ACA activation during aggregation. Activation and adaptation of ACA is normally mediated by cAR1. Evidence suggests that besides cAR1, three components such as DdMyb2, AmiB and PKA-C, function in the same or related pathways to regulate *aca* expression and many such molecules during transition from growth to differentiation are yet to be identified.

Though many aspects of the signal transduction pathways have been elucidated, yet there are some unanswered questions, which might be addressed well with the completed *Dictyostelium* genome sequence. The genome sequence reveals that *Dictyostelium* is complex, highly evolved and contains coding sequences for approximately 12,500 proteins[90] and as many as 20% of all predicted proteins in the *D. discoideum* genome are arranged in a number of large gene families that are involved in processes such as motility and signaling. *D. discoideum* cells are also accessible for imaging, and the use of tags such as green-fluorescent protein (GFP) fused to proteins of interest or certain fluorescent dyes (like DAPI)[91] allow to visualize their location during chemotaxis and to know the fate of cells during development under various stress conditions. Interestingly *D. discoideum* is known to exhibit caspase independent form of programmed cell death after differentiating into pre-stalk cells. Besides various developmental studies *D. discoideum* is also a good model organism to study evolutionary aspects of cell death under oxidative stress[92]. Such cell death mechanism would throw light on the evolutionary changes in programmed cell death.

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