Stalk cell formation in monolayers from isolated prestalk and prespore cells of *Dictyostelium discoideum*: evidence for two populations of prestalk cells

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Summary

Cells from the pseudoplasmodial stage of *Dictyostelium discoideum* differentiation were dispersed and separated on Percoll gradients into prestalk and prespore cells. The requirements for stalk cell formation in low-density monolayers from the two cell types were determined. The isolated prespore cells required both the Differentiation Inducing Factor (DIF) and cyclic AMP for stalk cell formation. In contrast, only part of the isolated prestalk cell population required both cyclic AMP and DIF, the remainder requiring DIF alone, suggesting the possibility that there were two populations of prestalk cells, one independent of cyclic AMP and one dependent on cyclic AMP for stalk cell formation. The finding that part of the prestalk cell population required only a brief incubation in the presence of DIF to induce stalk cell formation, whilst the remainder required a consider-ably longer incubation in the presence of both DIF and cyclic AMP was consistent with this idea. In addition, stalk cell formation from cyclic-AMP-dependent prestalk cells was relatively more sensitive to caffeine inhibition than stalk cell formation from cyclic-AMP-independent prestalk cells. The latter cells were enriched in the most anterior portion of the migrating pseudoplasmodium, indicating that there is spatial segregation of the two prestalk cell populations.

The conversion of prespore cells to stalk cells took longer and was more sensitive to caffeine when compared to stalk cell formation from cyclic-AMP-dependent prestalk cells.

Key words: development, differentiation, monolayers, prestalk, prespore, morphogen.

Introduction

Upon starvation, the amoebae of *Dictyostelium discoideum* aggregate to form a multicellular mass which eventually elongates into a migrating pseudoplasmodium. Partially differentiated prestalk and prespore cells are clearly discernible in a well-defined anterior–posterior pattern within the pseudoplasmodium. These cells differentiate into the stalk and spore cells of the terminal fruiting body after a precisely orchestrated sequence of morphogenetic steps (Loomis, 1975). Terminal differentiation can occur without morphogenesis if amoebae of strain V12-M2 are plated in submerged monolayers at low cell density in the presence of cyclic AMP and a low molecular weight, stalk cell Differentiation Inducing Factor (DIF) (Town *et al.* 1976; Town & Stanford,

1979). The chemical structure of the major active DIF species, DIF-1, has recently been elucidated (Morris *et al.* 1987).

The acquisition of cyclic AMP and DIF independence during *in vivo* differentiation was analysed by determining the requirements of dispersed cells for stalk cell formation in low-density monolayers (Sobolewski & Weeks, 1988). Independence from cyclic AMP was first observed at 12 h of differentiation, a time that corresponded to the transition between the tipped aggregate and first finger stages. The number of cyclic-AMP-independent cells increased as differentiation progressed, indicating that there was considerable heterogeneity for the cyclic AMP requirement within the differentiating cell population (Sobolewski & Weeks, 1988). In contrast, cells did not become independent of DIF until late culmi-

nation, suggesting that DIF was not only required for prestalk cell formation (Kopachik *et al.* 1983; Williams *et al.* 1987; Sobolewski & Weeks, 1988), but also necessary for the conversion of prestalk cells to stalk cells.

These results suggested that isolated prestalk and prespore cells would also require DIF for stalk cell differentiation, but might be distinguished by their requirements for cyclic AMP. In this study, we have tested this possibility by assessing the requirements for stalk cell formation from isolated prestalk and prespore cells.

Materials and methods

Organism and culture conditions

Dictyostelium discoideum, strain V12-M2, was grown in association with *Enterobacter aerogenes* on rich nutrient plates until the bacterial lawn had visibly cleared (Sobolewski *et al.* 1983). The cells were harvested and washed in Bonner's salts (10 mM-NaCl, 10 mM-KCl, 2 mM-CaCl₂ (Bonner, 1947) and then allowed to differentiate on non-nutrient agar (2 %) containing 5 % Bonner's salts until the migrating pseudoplasmodial stage of development (16–18 h). For the pseudoplasmodia dissection experiments, the amoebae were incubated in the presence of neutral red, prior to differentiation (MacWilliams & Bonner, 1979).

Separation of prestalk and prespore cells on Percoll gradients

The separation of prestalk and prespore cells was performed using a modification of a previously described protocol (Ratner & Borth, 1983). Pseudoplasmodia were harvested, disrupted by mixing vigorously in 20–30 ml phosphate buffer (20 mM-K₂HPO₄/KH₂PO₄, pH 6·0) and centrifuged at 1000 g for 5 min. The pellets (~4×10⁸ cells) were resuspended in 15 ml phosphate buffer, vortexed vigorously, filtered through a fine nylon sieve (mesh size 20 μm) to remove slime sheath and recentrifuged. The cells were resuspended in 1·0 ml of 5 mM-MES, pH 6·2, 0·06 % β-mercaptoethanol and 1 mg ml⁻¹ Sigma Type XIV protease and incubated at room temperature for 10 min with constant trituration through a 23-gauge needle. Samples (0·3 ml), containing approximately 10⁸ cells, were layered onto the surface of an ice-cold discontinuous Percoll gradient and centrifuged in a Beckman SW 41 rotor at 12 000 revs min⁻¹ for 5 min. The discontinuous gradients contained 2·5 ml 45 % Percoll in the bottom layer; 2·5 ml of 30 % Percoll in the middle layer and 2·5 ml of 15 % Percoll in the top layer, each diluted in 20 mM-MES, pH 7·0, 20 mM-EDTA, pH 7·0. Cells containing the prestalk-specific isozyme, acid phosphatase II, were recovered as a band at the interface between the top and middle layers of the gradient, whereas cells containing the prespore-specific enzyme UDP-galactosyl:mucopolysaccharide transferase were recovered between the middle and bottom layers (data not shown). Cells were collected with a Pasteur pipettete, diluted with ice-cold Bonner's salts and centrifuged at

1000 g for 5 min. Cell pellets were washed once in Bonner's salts by resuspension and recentrifugation.

Separation of prestalk and prespore cells by dissecting migrating pseudoplasmodia

Using a microdissecting knife, approximately 100 migrating pseudoplasmodia were dissected into three portions: a segment comprising the anterior 10 %; an adjacent segment comprising the next 10 % and, finally, a large segment comprising the remaining 80 % of the pseudoplasmodium. The first two segments comprised all the neutral red staining, prestalk cell region of the pseudoplasmodium (MacWilliams & Bonner, 1979). The cut segments were maintained on ice in 5 mM-MES, pH 6·2 until the dissection was complete, usually about 30 min. The suspending solution was adjusted to 0·06 % β-mercaptoethanol and 1 mg ml⁻¹ Sigma Type IV protease and the segments were triturated continuously over a 10 min period to disrupt the cell masses, while maintaining the suspension on ice. The cells were centrifuged at 700 g for 5 min and washed twice in Bonner's salts. The cell pellets were again resuspended in Bonner's salts solution and the isolated cells were plated in low-density monolayers as described below.

Differentiation in vitro in low-density monolayers

Pseudoplasmodial cells isolated either by Percoll gradient or dissection were diluted to a density of 10⁴ cells ml⁻¹ in a buffered Bonner's salts solution (BBS) that comprised Bonner's salts; 5 mM-MES, pH 6·2; 100 μg ml⁻¹ streptomycin sulphate and 5 μg ml⁻¹ butylated hydroxytoluene (BHT). 2 ml samples of this suspension were added to 5 cm Nunc tissue culture plates to give a cell monolayer density of 10³ cells cm⁻² and incubated at 22°C in the presence or absence of appropriate concentrations of cyclic AMP and DIF. In some experiments, the supernatants were removed at the indicated times and replaced with 2·0 ml BBS. Stalk cell formation was assessed by phase-contrast microscopy, 48 h after the commencement of the experiment, as described previously (Sobolewski *et al.* 1983), and all determinations were performed in triplicate. In some experiments, stalk cell formation was determined microscopically after shorter incubation times to give a measure of the rate of stalk cell formation. DIF was prepared by extracting cellular exudates with heptane as described previously (Sobolewski *et al.* 1983), and as such contains a mixture of the various active species of DIF (Kay *et al.* 1983).

Results

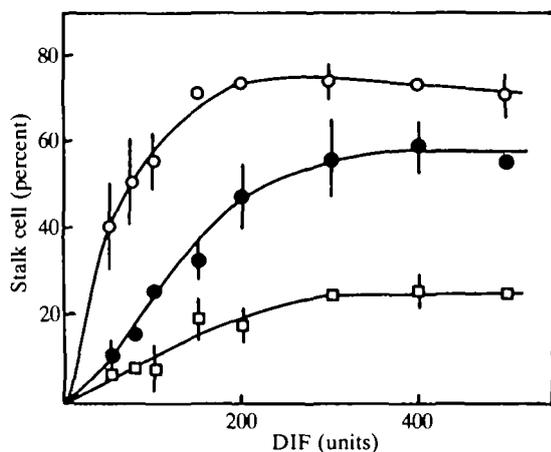### (A) The requirements of DIF and cyclic AMP for stalk cell formation

Prestalk and prespore cells were separated on Percoll gradients and then tested for their ability to form stalk cells in low-cell-density monolayers. Both cell types required DIF (Table 1), a result consistent with our finding that cells developing *in vivo* did not become independent of DIF until the culmination stage of development (Sobolewski & Weeks, 1988). Prespore cells also exhibited an absolute requirement

Table 1. *Stalk cell formation in low-cell-density monolayers of prestalk and prespore cells*

Cell type	Addition*	Percent stalk cell formation†
prestalk	none	0
	DIF	19·0
	DIF + cyclic AMP	90·0
prespore	none	0
	DIF	1·0
	DIF + cyclic AMP	81·0

* All incubations contained 2 ml BBS, and 150 units DIF and 1 mM-cyclic AMP were added where indicated.

† Stalk cell formation was assessed after 48 h incubation at 22 °C.

for cyclic AMP for stalk cell formation (Table 1). In contrast, prestalk cells were capable of forming stalk cells in the absence of cyclic AMP, although addition of cyclic AMP considerably increased the number of stalk cells formed (Table 1). This phenomenon was not due to insufficient levels of DIF since stalk cell formation in the absence of cyclic AMP reached a maximum value at relatively low concentrations of DIF (Fig. 1). This value was only a fraction of the maximum number of stalk cells that were formed in the presence of cyclic AMP (Fig. 1). These results suggest the possibility that there are two populations of prestalk cells that differ in their requirements for cyclic AMP.
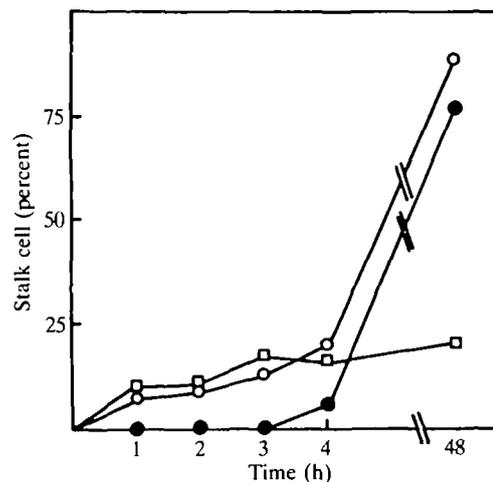
(B) *The effect of DIF and cyclic AMP removal on stalk cell formation*

Further evidence for the existence of two populations of prestalk cells was obtained by determining the time required for cells to be induced by DIF (Fig. 2). Regardless of the presence or absence of cyclic AMP, only part of the prestalk cell population was induced to form stalk cells by a short incubation in the presence of DIF. In contrast, the period of DIF induction required for stalk cell formation from the remaining prestalk cells was considerably longer. These data are consistent with the idea that there are distinct cyclic-AMP-independent and cyclic-AMP-dependent prestalk cell populations. Prespore cell conversion to stalk cells also required the longer incubation period in the presence of both cyclic AMP and DIF (Fig. 2).

(C) *Comparison of the rates of stalk cell formation from prestalk and prespore cells*

The time required for the first appearance of stalk cells in the monolayers was also determined. Stalk cell formation from prestalk cells was more rapid than that from prespore cells (Fig. 3), suggesting the possibility that prespore cells were converted to prestalk cells prior to their terminal differentiation into stalk cells. However, all attempts at detecting the formation of the prestalk-cell-specific acid phosphatase II during the induction of stalk cell formation

Fig. 1. The effect of DIF concentration on stalk cell formation from isolated prestalk and prespore cells. Prestalk (□, ○) and prespore cells (●) were separated on Percoll gradients, washed by centrifugation and plated in 2·0 ml BBS at the indicated concentrations of DIF, either in the presence (○, ●) or absence (□) of 1 mM-cyclic AMP. Stalk cell formation was determined after 24 h of incubation at 22 °C. The values are the means ± the standard deviations for two separate experiments.

Fig. 2. Effect of factor removal on stalk cell formation in monolayers of prestalk and prespore cells. Prestalk cells were plated in 2 ml BBS containing either 1 mM-cyclic AMP and 150 units DIF (○) or 150 units DIF (□). Prespore cells were plated in the presence of 150 units of DIF and 1 mM-cyclic AMP (●). At the indicated times supernatants were removed, the monolayers were carefully rinsed three times and replenished with 2·0 ml of BBS. Stalk cell formation was assessed 48 h after the beginning of the experiment.
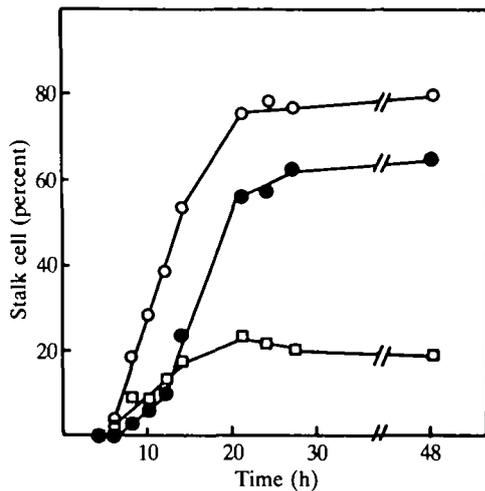
Fig. 3. Rate of formation of stalk cells from isolated prestalk and prespore cells. Prestalk cells (O, □) and prespore cells (●) were separated on Percoll gradients, washed by centrifugation and plated in either 2 ml of BBS containing 150 units of DIF and 1 mM-cyclic AMP (O, ●) or 2 ml of BBS containing 150 units DIF (□). The proportion of stalk cells was determined at the indicated times.

from prespore cells in these low-cell-density mono-layers were unsuccessful (data not shown).
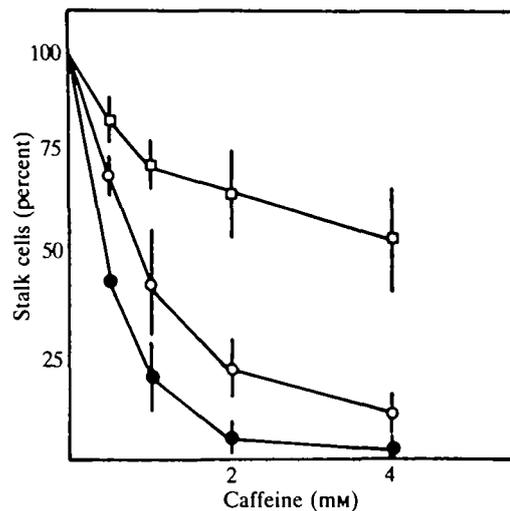
(D) *Effect of caffeine on stalk cell formation*

Previous studies have shown that stalk cell formation from vegetative cells in low-density monolayers was sensitive to inhibition by caffeine (Kwong *et al*. 1988). The formation of stalk cells from prestalk cells was more sensitive to caffeine in the presence of cyclic AMP than in its absence (Fig. 4), again supporting the idea that prestalk cells comprise cyclic-AMP-dependent and -independent cells. The formation of stalk cells from prespore cells was even more sensitive to caffeine inhibition (Fig. 4).

(E) *Stalk cell formation from dissected segments of migrating pseudoplasmodia*

In order to determine if the cyclic-AMP-dependent and -independent prestalk cells were localized in different regions of the migrating pseudoplas-modium, the anterior prestalk cell region was dissected into two equal portions each constituting about 10 % of the total length of the pseudoplasmodium.

The cells from each segment were dissociated and their requirements for stalk cell formation in low-density monolayers were assessed. Cells from the anteriormost 10 % of the migrating pseudoplas-modium formed substantially more stalk cells in monolayers in the absence of cyclic AMP than cells from either the next 10 % or the posterior 80 % (Table 2). These data indicate that cyclic-AMP-

Fig. 4. The effect of caffeine on stalk cell formation from isolated prestalk and prespore cells. Prestalk cells (O, □) and prespore cells (●) were separated on Percoll gradients, washed by centrifugation and plated in 2·0 ml of BBS containing the indicated concentrations of caffeine, and either 150 units of DIF and 1 mM-cyclic AMP (O, ●) or 150 units DIF (□). Stalk cell formation was determined after 24 h incubation at 22°C. The values are the means ± standard deviations of three independent experiments.

Table 2. *Effect of cyclic AMP on stalk cell formation in monolayers of cells from dissected pseudoplasmodia*

	Percent stalk cell formation†		
Fraction of pseudoplasmodium*	1 mM cyclic AMP	no cyclic AMP	Ratio‡
anterior segment (prestalk)	42·1 ± 6·8	19·2 ± 3·7	0·46
intermediate segment (prestalk)	35·6 ± 4·6	8·2 ± 3·0	0·23
posterior segment (prespore)	36·3 ± 3·2	7·5 ± 3·2	0·21

* The dissection of the pseudoplasmodia was as described under Methods.

† All incubations contained 80 units DIF in 2 ml BBS and the indicated amount of cyclic AMP. The values are the means ± standard deviations of four independent experiments.

‡ The ratio between stalk cell formation in the absence of cyclic AMP to stalk cell formation in the presence of 1 mM-cyclic AMP.

independent cells are preferentially located in the anteriormost region of the migrating pseudoplasmo-dium.

Discussion

Studies on the requirements for stalk cell formation in

monolayers by prestalk and prespore cells separated by Percoll gradients revealed discrete differences between the two populations. Prestalk cells required only DIF for stalk cell formation in low-density monolayers, whereas prespore cells required both cyclic AMP and DIF (Table 1). However, stalk cell formation from prestalk cells was markedly enhanced by cyclic AMP at any given DIF concentration (Fig. 1), suggesting that only part of the prestalk cell population was independent of cyclic AMP for stalk cell formation. Furthermore, induction of cyclic-AMP-independent cells required only a short incubation in the presence of DIF, whereas the remaining prestalk cells that were dependent upon DIF and cyclic AMP required a longer incubation in the presence of both factors (Fig. 2). Finally, stalk cell formation from cyclic-AMP-independent prestalk cells was less sensitive to caffeine inhibition than that from cyclic-AMP-dependent cells (Fig. 4), suggesting that the cyclic AMP induction step is more sensitive to caffeine compared to the DIF induction step. Taken together, the simplest interpretation of these results is that there are two distinct prestalk cell populations within the low-density fraction isolated from Percoll gradients. At present there are no molecular markers that might distinguish between two populations of prestalk cells and so we cannot totally preclude the possibility that there is a single population that is extremely heterogeneous in its response to cyclic AMP and DIF.

Evidence for heterogeneity in the developing cell population was previously obtained from experiments designed to determine the stage at which cells became independent of cyclic AMP for stalk cell formation during development *in vivo* (Sobolewski & Weeks, 1988). Although cyclic AMP independence was first observed at the transition between the tipped aggregate and first finger stages of development, the number of cyclic-AMP-independent cells continued to rise as differentiation progressed (Sobolewski & Weeks, 1988).

Our studies show an absolute requirement of cyclic AMP for the prespore-to-stalk-cell conversion. In contrast, Weijer & Durston (1985) studied the conversion of prespore cells to prestalk cells in shaken cell suspensions and found that cyclic AMP inhibited this transition. The reason for this difference is not known, but it may reflect the fact that Weijer & Durston studied the conversion process in multicellular clumps in the absence of added DIF, whereas our studies involve cells in low-cell-density monolayers in the presence of DIF.

We have shown previously that the conversion of vegetative cells to stalk cells in monolayers is markedly inhibited by caffeine and that this inhibition can be reversed by high concentrations of the cell-per-

meable analogue, 8-Br-cyclic AMP (Kwong *et al.* 1988). These results suggest that an elevated intracellular cyclic AMP concentration is necessary for stalk cell formation, since caffeine inhibits the activation of adenylate cyclase (Brenner & Thoms, 1984; Theibert & Devreotes, 1983). In the present study, the conversion of prespore cells to stalk cells was more sensitive to caffeine than the conversion of cyclic-AMP-dependent prestalk cells. Thus, although prespore cells and cyclic-AMP-dependent prestalk cells share similar requirements for stalk cell formation, namely the presence of both cyclic AMP and DIF, their conversion to stalk cells is slower (Fig. 3) and appears to be mechanistically distinct. The difference in sensitivity to caffeine exhibited by the prespore cells and the two types of prestalk cells may reflect differences in the requirements for elevated intracellular cyclic AMP concentrations for their conversion to stalk cells, although other mechanisms are not precluded.

The anterior prestalk segment of the migrating pseudoplasmodia was found to contain both cyclic-AMP-dependent and -independent cells (Table 2). Difference in morphology (Kopachik, 1982) and the distribution of antigen (Gomer *et al.* 1986) have indicated the existence of more than one cell population within the prestalk region of the migrating pseudoplasmodia, but it is not known if these differences correspond to the cyclic-AMP-dependent and -independent cells described in this report.

The pseudoplasmodial dissection experiments extend an earlier study by Town & Stanford (1977). They had shown that cells dissected from both front and rear segments of migrating pseudoplasmodia were capable of forming stalk cells when plated at high cell density in the presence of cyclic AMP, but only cells from the prestalk region of the pseudoplasmodia were able to form stalk cells in the absence of cyclic AMP. Our data show an enrichment of cyclic-AMP-independent cells in the anteriormost segment of the pseudoplasmodia, but a significant proportion of cyclic-AMP-independent cells was also detected in the posterior segment. Stalk cell formation from these cyclic-AMP-independent cells may have been missed under the high-cell-density conditions used by Town & Stanford (1977).

The presence of a significant number of cyclic-AMP-independent cells in the rearmost prespore region of pseudoplasmodia (Table 2) was unexpected. Given that the prespore cells isolated from Percoll gradients were totally dependent on cyclic AMP for stalk cell formation in monolayer, this result implies that the cyclic-AMP-independent cells in the posterior region of pseudoplasmodia correspond to the previously described anterior-like cells (Sternfeld & David, 1981, 1982; Devine & Loomis, 1985; Voet

et al. 1985; Kakutani & Takeuchi, 1986). Since only approximately 15 % of the posterior cells are anterior-like cells (Sternfeld & David, 1982; Voet *et al.* 1985), our data would indicate that a high proportion of these cells must be independent of cyclic AMP for stalk cell formation (Table 2). This raises the interesting possibility that the cyclic-AMP-independent cells in the anterior region might also be anterior-like cells. Anterior-like and anterior cells freely interchange positions during pseudoplasmodial migration (Kakatuni & Takeuchi, 1986; Odell & Bonner, 1986), but it is not known whether the anterior-like cells remain unchanged or convert to a distinct prestalk cell population when they pass into the anterior region.

Since there is only a slight distinction at the molecular level between anterior-like cells and the cells from the anterior prestalk region of the pseudoplasmodia and since the two cell types are not separated by Percoll gradients (Devine & Loomis, 1985), it is not possible at present to determine if the cyclic-AMP-independent cells in the anterior region are anterior-like cells.

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