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# Control of cell type proportions by a secreted factor in *Dictyostelium discoideum*

KEI INOUYE

*Department of Botany, Faculty of Science, Kyoto University, Kyoto 606, Japan*

## Summary

**It has been shown that, in *Dictyostelium discoideum*, conversion of prestalk cells to prespore cells in suspension cultures is inhibited by coexisting prespore cells. To examine whether the inhibition of conversion requires direct cell contact or is mediated by substances secreted by the cells, prestalk cells and prespore cells were incubated in shaken suspension, separated from each other by a dialysis membrane, and conversion of the prestalk cells to prespore cells scored after 24 h. Prestalk-to-prespore conversion was significantly inhibited if the density of the prespore cells was sufficiently high. In contrast, prestalk cells had little influence on prestalk-to-prespore conversion. Media conditioned by prespore cells, but not by prestalk cells, also inhibited the conversion of prestalk cells. Adenosine, propionate, diethylstilboestrol and differentiation inducing factor (DIF), all of which are known to influence the prestalk/prespore differentiation, were examined for their effects on prestalk-to-prespore conversion. Among these, all except adenosine significantly inhibited the conversion. Based on these results, possible mechanisms for maintenance of the constant cell-type ratio in *D. discoideum* slugs were discussed.**

Key words: cell-type ratio, proportion regulation, cellular slime mould, *Dictyostelium discoideum*.

## Introduction

The maintenance of constant proportions of differentiated cell types is a striking, but little understood, feature of multicellular development. In the pseudoplasmodium, or 'slug', of the cellular slime mould *Dictyostelium discoideum*, the relative numbers of prestalk cells and prespore cells are more-or-less invariant over a wide range of total size (Bonner, 1957). If undisturbed, the differentiated states of these cell types are stable (Akiyama and Inouye, 1987). However, removal of either cell type causes many of the remaining cells to redifferentiate so that the normal cell type ratio is restored (Raper, 1940; Bonner *et al.* 1955; Sakai, 1973; Oyama *et al.* 1983; Weijer *et al.* 1985).

We have attempted to elucidate the mechanism whereby cells recognize the presence or absence of the other cell type by examining the conversion of prestalk cells into prespore cells after removal of prespore cells. In a previous study, we showed that prestalk cells in suspension cultures as well as in migrating slugs were prevented from becoming prespore cells by the presence of prespore cells in the same culture medium or in the same slug (Akiyama and Inouye, 1987). To determine whether the inhibition of conversion requires direct cell contact, the present study investigates (1) whether prespore cells prevent the conversion of prestalk cells when the two cell types are separated by dialysis membrane, and (2) the effects of conditioned media of prestalk cells and prespore cells on the cell-type conversion. The results obtained indicate that prespore cells release a diffusible substance or substances that inhibit(s) the conversion of prestalk cells, thereby establishing a simple negative feedback system for maintenance of the constant cell-type ratio.

## Materials and methods

### *Growth and developmental conditions*

*Dictyostelium discoideum* (strain V12M2) was used in this study. Cells grown on *Klebsiella aerogenes* were starved and allowed to form slugs on buffered agar plates. Prestalk and prespore cells were obtained by dissociating migrating slugs using EDTA followed by Percoll-gradient centrifugation (Akiyama and Inouye, 1987). All the incubations were carried out at 21–22 °C in the dark.

### *Suspension cultures*

Fig. 1 shows the cylindrical vessel used in experiments in which prestalk cells and prespore cells were separated by a dialysis membrane. The vessel consists of inner and outer chambers separated by seamless cellulose dialysis tubing (pore size 24 Å, 18/32, Visking). Cells of the prestalk and prespore fractions were separately suspended in GAC medium (5 % glucose, 2 % albumin, 1 mM-cAMP, 2 mM-EDTA, 20 mM-potassium phosphate, pH 6.0; see Okamoto, 1981) at the densities indicated. Normally, 1 ml suspension of $5\times10^6$ prestalk cells was put in the inner chamber of the vessels as

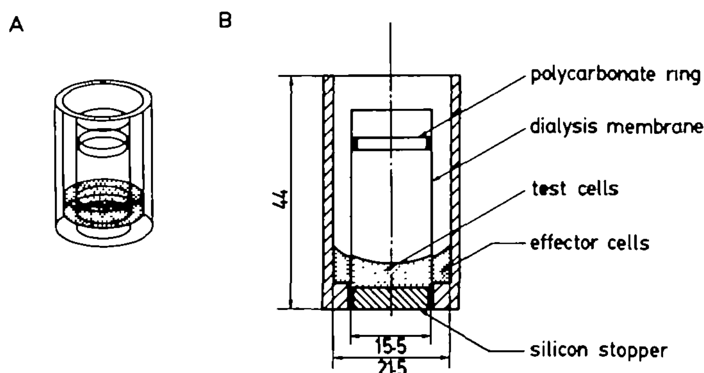


**Fig. 1.** The cylindrical vessel used in the experiments the results of which are shown in Figs 1B and 3A. (A) A line sketch, (B) a vertical section. Units are in mm. In each experiment, pieces of dialysis membrane were cut out from seamless cellulose dialysis tubing (pore size 24 A, 18/32, Visking), which were then boiled and washed extensively in $H_2O$ to remove preservatives. Each dialysis membrane was tightly fitted in the hole at the bottom of the vessel with a slice of silicon stopper. Bottoms of plastic Petri dishes (3 cm diameter) were used as lids of the vessels.

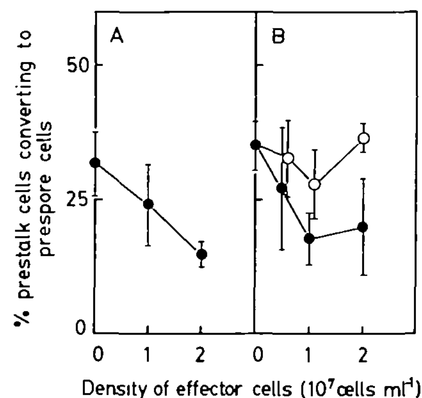


**Fig. 2.** Fraction of prestalk cells converting to prespore cells in 24 h. (A) Mixed suspension cultures of prestalk cells and prespore cells (data replotted from Akiyama and Inouye, 1987 for comparison). (B) Suspension cultures in which test (prestalk) cells and effector (prestalk ○ or prespore ●) cells were separated by a dialysis membrane. Data points represent means of 3–7 determinations with standard deviations. The difference in the extent of conversion between (○) and (●) is significant at the cell densities over $10^7$ cells ml$^{-1}$ ($P<0.10$ for $10^7$ cells ml$^{-1}$, $P<0.01$ for $2\times10^7$ cells ml$^{-1}$). In A, one or other initial cell type was labelled with tetramethylrhodamine isothiocyanate in order to detect conversion between cell types. B shows the results of experiments in which the test cells ($5\times10^6$ ml$^{-1}$ prestalk cells) were in the inner chamber and the effector cells in the outer chamber (see Fig. 1). The result was the same with the reverse combinations (data not shown).

test cells and 1 ml suspension of prestalk or prespore cells at various densities in the outer chamber as effector cells. The vessels were placed on a rotary shaker (New Brunswick) and shaken at 120 revs min$^{-1}$. For incubation in conditioned media, prestalk cells were suspended in the conditioned media supplemented with 1 mM-cAMP and rotary shaken in small glass vials at 120 revs min$^{-1}$.

After 24 h of rotary shaking, cells were collected, cell clumps formed dissociated in 10 mM-EDTA solution (pH 6.5) containing 5 % glucose, and fixed in cold methanol. Prespore differentiation was detected using a specific antibody as described in the previous paper (Akiyama and Inouye, 1987).

Hydrolysis of the added cAMP was monitored in parallel experiments using [$^3$H]cAMP. The fraction of cAMP hydrolysed during the incubation period was estimated to be less than 10 % at the highest cell density used and no detectable amount of adenosine was formed.

### *Preparation of conditioned media*

Conditioned media were prepared in two different ways. The first method used the vessels described above (Fig. 1). Fresh GAC medium was put in the inner chamber of the vessels and GAC medium containing prespore cells in the outer chamber. After 24 h of rotary shaking at 120 revs min$^{-1}$, the media in the inner chambers were collected. For the second method, prestalk cells or prespore cells were suspended in GAC medium at the densities indicated and rotary shaken in glass vials at 120 revs min$^{-1}$. After 24 h of incubation, the cell suspensions were centrifuged for 2 min using a microcentrifuge (Eppendorf 5417) to remove cells and debris.

### *Chemicals*

DIF-1 was kindly provided by Dr R. Kay (MRC, Cambridge, England). Diethylstilboestrol was purchased from Sigma (St Louis, USA).

## Results and Discussion

### *Effects of coexisting cells on cell-type conversion*

In a previous study, we showed that conversion of prestalk cells to prespore cells in suspension cultures was inhibited by the presence of prespore cells in the same culture medium (Akiyama and Inouye, 1987). Fig. 2A shows an experiment in which prestalk cells were incubated alone or in the presence of increasing number of prespore cells in the medium containing cyclic AMP and scored after 24 h incubation for conversion to prespore cells. When incubated alone, a considerable fraction of prestalk cells turned into prespore cells, whereas if prespore cells coexisted in the same medium, prestalk cells were prevented to some extent from becoming prespore cells. In such mixed cultures, cells formed clumps in which prestalk cells and prespore cells were intermingled. To examine whether or not the inhibition of conversion requires direct cell contact between the different cell types, prestalk cells (as test cells) and prestalk or prespore cells (as effector cells) were incubated in such a way that they were separated by a cylinder of dialysis membrane (Fig. 1). It can be seen from Fig. 2B that conversion of prestalk cells to prespore cells was significantly inhibited if the density of the prespore cells was sufficiently high. In contrast, when prestalk cells were the effector cells, the extent of

conversion was comparable to that in the absence of effector cells.

*Effects of conditioned media on cell-type conversion*

The above results indicate that the inhibition of prestalk-to-prespore conversion is mediated by dialysable molecules released from the cells. There are at least two possibilities which could account for the results: (1) prestalk cells release dialysable molecules that enhance prestalk-to-prespore conversion, while prespore cells degrade or adsorb these molecules, or (2) prespore cells secrete dialysable inhibitors of the conversion. The former possibility is unlikely because in the absence of prespore cells, increasing the density of prestalk cells did not affect the conversion of prestalk cells. To examine the second possibility, fresh media were conditioned with prespore cells using the same sort of vessels used in the above experiments. Newly prepared prestalk cells were then incubated in these media. As shown in Fig. 3A, the conditioned media prepared in this way efficiently inhibited the conversion of prestalk cells. The effect of conditioned media prepared in the conventional way was also examined. Conversion of prestalk cells was significantly inhibited in the conditioned media of prespore cells, with stronger inhibition in the media of higher prespore densities. When the conditioned media were diluted, their inhibitory effect was also reduced. Media conditioned by prestalk cells, on the other hand, did not significantly affect the conversion of prestalk cells (Fig. 3B).

The activities of the conditioned media, prepared by either method, were comparable on a per-cell basis to the ability of prespore cells to inhibit conversion in mixed culture (compare Fig. 2A with Figs 2B, 3A and 3B). From this, it was concluded that the regulation of prestalk-to-prespore ratio in suspension cultures is mediated by dialysable molecules secreted predominantly by prespore cells. The following mechanism can then be proposed for the regulation of cell-type ratio in suspension cultures: if the cell-type ratio is normal, prestalk cells are prevented from becoming prespore cells by inhibitor molecules released from the prespore cells. If the prespore proportion is lower than normal, a

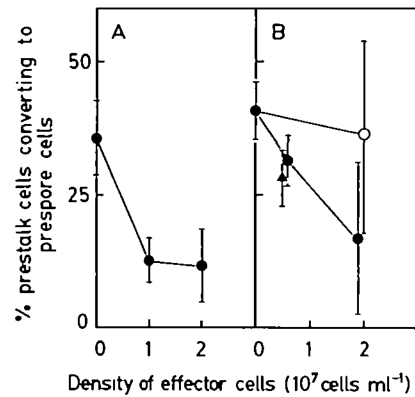


**Fig. 3.** Effects of conditioned media on prestalk-to-prespore conversion. (A) Effects of the conditioned media of prespore cells collected through dialysis membrane. (B) Effects of conditioned media from prestalk (○) or prespore cells (●) prepared in the ordinary way. ▲ represents conditioned medium from $2\times10^7$ ml$^{-1}$ prespore cells subsequently diluted fourfold with fresh GAC medium. In (B), the difference between prestalk and prespore cells at the cell density of $2\times10^7$ cells ml$^{-1}$ is significant at $P<0.10$.

certain fraction of the prestalk cells will turn into prespore cells until the number of prespore cells becomes high enough to produce a sufficient amount of the inhibitor molecules to prevent further conversion of prestalk cells. In a previous study, we have shown that, in migrating slugs too, the conversion of prestalk cells into prespore cells is prevented by the presence of prespore cells (Akiyama and Inouye, 1987). It is therefore suggested that the same inhibitor molecules that act in suspension cultures are also responsible for the maintenance of the prestalk cell population in normal development.

*Possible candidates for the inhibitor of cell-type conversion*

The nature of the inhibitor molecules is not yet known. A number of substances influence the cell-type ratio in favour of prestalk cells, such as $Li^+$ (Maeda, 1970),

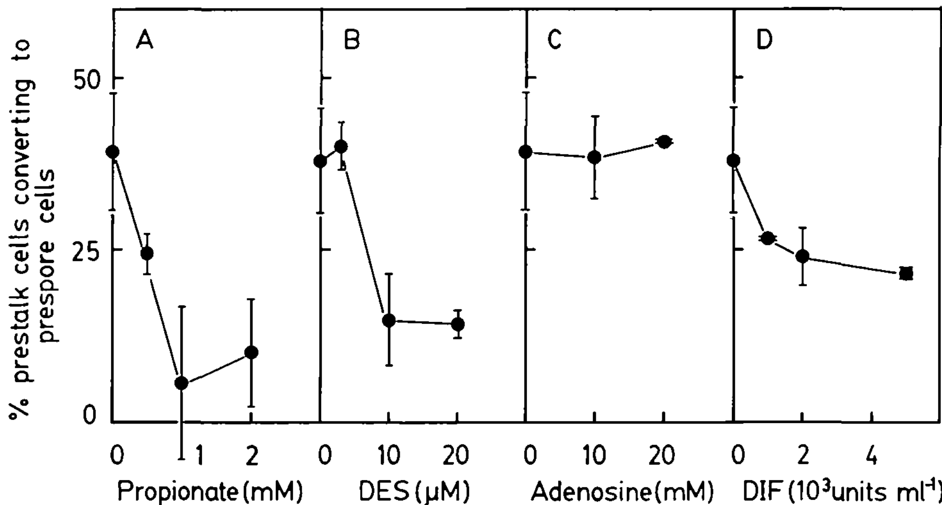


**Fig. 4.** Effects of potassium proprionate (A), diethylstilboestrol (B), adenosine (C), and DIF (D) on prestalk-to-prespore conversion. In C, prestalk cells were suspended in GAC medium containing indicated amounts of adenosine. In A, B and D, appropriate amounts of concentrated stock solutions were added to the cell suspensions at the beginning of incubation. In B and D, ethanol, a solvent of the stock solutions of DES and DIF, was also added so that the ethanol concentration becomes 0.2 % in all concentrations of DES and DIF.

weak acids (Gross *et al.* 1983), proton pump inhibitors (Gross *et al.* 1983), adenosine (Weijer and Durston, 1985), differentiation inducing factor (DIF) (Town and Stanford, 1977; Kay and Jermyn, 1983; Morris *et al.* 1987) etc., and any of these that are released by the cells could be candidates for the endogenous inhibitor of prestalk-to-prespore conversion. Weak acids, such as propionate, and proton pump inhibitors, such as diethylstilboestrol (Pogge-von Strandmann *et al.* 1984; Gross *et al.* 1988), significantly inhibited the prestalk-to-prespore conversion (Fig. 4A and B). Since these substances have been shown to lower the cytoplasmic pH of slug cells (Inouye, 1988), it can be argued that prestalk-to-prespore conversion can be prevented by lowering cytoplasmic pH. However, it is not known whether *D. discoideum* cells release a sufficient amount of weak acids or whether they produce an endogenous inhibitor of the plasma membrane proton pump, and there is no evidence suggesting that any of these substances might be an active component of the conditioned medium.

Adenosine is known to inhibit prestalk-to-prespore conversion in roller-tube cultures (Weijer and Durston, 1985), and a decrease in its level in the slug results in the differentiation of prespore cells in the prestalk zone (Schaap and Wang, 1986; Wang *et al.* 1988). However, adenosine is very unlikely to be involved in the inhibition by prespore cells of cell-type conversion in suspension cultures because it is most likely produced by prestalk cells (Otte *et al.* 1986; Wang *et al.* 1988) and had no effect on the conversion of prestalk cells in our *in vitro* system (Fig. 4C).

On the other hand, DIF meets the conditions for being a natural inhibitor of prestalk-to-prespore conversion. As shown in Fig. 4D, externally added DIF inhibited the conversion of prestalk cells. In the slug, the specific activity of DIF is higher in the prespore zone than in the prestalk zone (Brookman *et al.* 1987), suggesting that it might be produced by prespore cells. Preliminary characterization of our conditioned media shows that its conversion-inhibiting activity is adsorbed by XAD-2 resin which has been shown to adsorb DIF (Kay *et al.* 1983). However, the amount of DIF needed to suppress the conversion of prestalk cells appears much larger than the amount produced by the cells, estimated to be about 300 units per $10^7$ cells (Brookman *et al.* 1982). This may indicate that prespore cells release, in addition to DIF, substances other than DIF that help to prevent the conversion of prestalk cells. Alternatively, a high extracellular concentration of cAMP used in this study (1 mM) may enhance the production of DIF by prespore cells, as is the case for DIF production by aggregation-phase cells (Brookman *et al.* 1982).

### *A possible mechanism for the control of cell-type ratio*

Although the identification of the inhibitor molecules must await further study, the results presented here demonstrate that diffusible substances secreted predominantly by prespore cells actually regulate proportions by controlling cell-type conversion. On the other hand, in migrating slugs, unlike in the *in vitro* condition used in this study, adenosine stabilizes prestalk differentiation (Schaap and Wang, 1986; Wang *et al.* 1988). Possibly, neither adenosine nor the inhibitor of this study is sufficient to prevent prestalk-to-prespore conversion in the slug, but together they may suppress the appearance of excessive prespore cells. This would explain why removal of prespore zone of a slug (Bonner *et al.* 1955; Sakai, 1973) or removal of endogenous adenosine by applying adenosine deaminase (Schaap and Wang, 1986) both induce the conversion of prestalk cells into prespore cells; in the former case, removal of the prespore zone would lower the inhibitor level in the prestalk zone of the same slug, which would give rise to prespore differentiation in that region; whereas, in the latter case, the inhibitor alone without adenosine would be insufficient to prevent the prestalk cells from becoming prespore cells. From a theoretical point of view, such a combination of two factors, both inhibiting the differentiation of one cell type (prespore) but produced by both cell types, provides a novel kind of model in which spatial segregation of different cell types is reconciled with the maintenance of constant proportion (details of the model will be published elsewhere; for reviews of other models, see MacWilliams and Bonner, 1979; Williams, 1988).

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## References


Akiyama, Y. & Inouye, K. (1987). Cell-type conversion in normally proportioned and prestalk-enriched populations of slug cells in *Dictyostelium discoideum*. *Differentiation* **35**, 83–87.

Bonner, J. T. (1957). A theory of the control of differentiation in the cellular slime moulds. *Q. Rev. Biol.* **32**, 232–246.

Bonner, J. T., Chiquoine, A. D. and Kolderie, M. Q. (1955). A histochemical study of differentiation in the cellular slime molds. *J. exp. Zool.* **130**, 133–157.

Brookman, J. J., Jermyn, K. A. and Kay, R. R. (1987). Nature and distribution of the morphogen DIF in the *Dictyostelium* slug. *Development* **100**, 119–124.

Brookman, J. J., Town, C. D., Jermyn, K. A. and Kay, R. R. (1982). Developmental regulation of a stalk-cell differentiation-inducing factor in *Dictyostelium discoideum*. *Devl Biol.* **91**, 191–196.

Gross, J. D., Bradbury, J., Kay, R. R. and Peacey, M. J. (1983). Intracellular pH and the control of cell differentiation in *Dictyostelium discoideum*. *Nature, Lond.* **303**, 244–245.

Gross, J. D., Peacey, M. J. and Pogge-von Strandmann, R. (1988). Plasma membrane proton pump inhibition and stalk cell differentiation in *Dictyostelium discoideum*. *Differentiation* **38**, 91–98.

Inouye, K. (1988). Difference in cytoplasmic pH and the sensitivity to acid load between prespore cells and prestalk cells of *Dictyostelium*. *J. Cell Sci.* **91**, 109–115.

Kay, R. R., Dhokia, B. and Jermyn, K. A. (1983). Purification of stalk-cell-inducing morphogens from *Dictyostelium discoideum*. *Eur. J. Biochem.* **136**, 51–56.

Kay, R. R. and Jermyn, K. A. (1983). A possible morphogen controlling differentiation in *Dictyostelium*. *Nature, Lond.* **303**, 242–244.

MacWilliams, H. K. and Bonner, J. T. (1979). The prestalk-prespore pattern in cellular slime moulds. *Differentiation* **14**, 1–22.

Maeda, Y. (1970). Influence of ionic conditions on cell differentiation and morphogenesis of the cellular slime molds. *Dev. Growth Differ.* **12**, 217–227.

Morris, H. R., Taylor, G. W., Masento, M. S., Jermyn, K. A. and Kay, R. R. (1987). Chemical structure of the morphogen differentiation inducing factor from *Dictyostelium discoideum*. *Nature, Lond.* **328**, 811–814.

Okamoto, K. (1981). Differentiation of *Dictyostelium discoideum* cells in suspension culture. *J. gen. Microbiol.* **127**, 301–308.

Otte, A. P., Plomp, M. J. E., Arents, J. C., Janssens, P. M. W. and van Driel, R. (1986). Production and turnover of cAMP signals by prestalk and prespore cells in *Dictyostelium discoideum* cell aggregates. *Differentiation* **32**, 185–191.

Oyama, M., Okamoto, K. and Takeuchi, I. (1983). Proportion regulation without pattern formation in *Dictyostelium discoideum*. *J. Embryol. exp. Morph.* **75**, 293–301.

Pogge-von Strandmann, R., Kay, R. R. and Dufour, J.-P. (1984). An electrogenic proton pump in plasma membranes from the cellular slime mould *Dictyostelium discoideum*. *FEBS Letters* **174**, 422–428.

Raper, K. B. (1940). Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. *J. Elisha Mitchell Sci. Soc.* **56**, 241–282.

Sakai, Y. (1973). Cell type conversion in isolated prestalk and prespore fragments of the cellular slime mold *Dictyostelium discoideum*. *Dev. Growth Differ.* **15**, 11–19.

Schaap, P. and Wang, M. (1986). Interactions between adenosine and oscillatory cAMP signaling regulation size and pattern in *Dictyostelium*. *Cell* **45**, 137–144.

Town, C. D. and Stanford, E. (1979). An oligosaccharide-containing factor that induces cell differentiation in *Dictyostelium discoideum*. *Proc. natn. Acad. Sci. U.S.A.* **76**, 308–312.

Wang, M., van Driel, R. and Schaap, P. (1988). Cyclic AMP-phosphodiesterase induces dedifferentiation of prespore cells in *Dictyostelium discoideum* slugs: evidence that cyclic AMP is the morphogenetic signal for prespore differentiation. *Development* **103**, 611–618.

Weijer, C. J. and Durston, A. J. (1985). Influence of cyclic AMP and hydrolysis products on cell type regulation in *Dictyostelium discoideum*. *J. Embryol. exp. Morph.* **86**, 19–37.

Williams, J. G. (1988). The role of diffusible molecules in regulating the cellular differentiation of *Dictyostelium discoideum*. *Development* **103**, 1–16.

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