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In vitro stalk cell differentiation in wild-type and 'slugger' mutants of *Dictyostelium discoideum*

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SUMMARY

In 'slugger' mutants of *Dictyostelium discoideum*, aggregates of cells remain for an abnormally long time in the migratory phase under conditions where wild-type aggregates form fruiting bodies. In the present work, we have examined the relationship between the defect in fruiting body formation in these mutants and their ability to form mature stalk cells. We dissociated anterior cells from slugs of the mutants and their parents and tested their ability to form stalk cells when incubated at low density in the presence of (1) the stalk cell morphogen Differentiation Inducing Factor-1 (DIF-1) together with cyclic AMP, or (2) 8-Br-cAMP, which is believed to penetrate cell membrane and activate cAMP-dependent protein kinase (PKA).

Most of the mutants were markedly defective in forming stalk cells in response to DIF-1 plus cAMP, confirming a close relationship between fruiting body formation and stalk cell maturation. On the other hand, many of these same mutants formed stalk cells efficiently in response to 8-Br-cAMP. This supports evidence for an essential role of PKA in stalk cell maturation and fruiting body formation. It also indicates that many of the mutants owe their slugger phenotype to defects in functions required for optimal adenylyl cyclase activity.

Key words: cell differentiation, *Dictyostelium*, slugger mutant, DIF, PKA

INTRODUCTION

During development in *Dictyostelium discoideum*, starved cells collect together by chemotaxis to cyclic AMP to form aggregates that rise up to form cylindrical structures which either transform directly into fruiting bodies or collapse onto the substratum for a time to form migratory 'slugs'. Slugs are differentiated along their length, with prestalk cells at the front, prespore cells at the back. Formation of prestalk cells is dependent on the prestalk inducer, Differentiation Inducing Factor (DIF), a hydrophobic hexaphenone derivative produced during development in *D. discoideum* (Town et al., 1976; Morris et al., 1987, 1988). During culmination, prestalk cells transform into dead, vacuolated stalk cells, while prespore cells mature into spores and are held aloft by a column of stalk cells. There is considerable evidence that the accumulation of ammonia, which is liberated in large amounts by the developing cells, is responsible for maintaining aggregates in the migratory state, and conversely that reduction in the ambient level of free ammonia leads to cessation of migration followed by fruiting body formation (Schindler and Sussman, 1977).

A number of observations point to a close relationship between the process of culmination and the maturation of prestalk cells. (1) Both processes, as well as DIF-dependent gene transcription, are inhibited by weak bases (Schindler and Sussman, 1977; Inouye, 1988; Wang and Schaap, 1989; Wang et al., 1990) and promoted by weak acids and other agents that tend to acidify the cell (Inouye, 1988). (2) A very early event at the onset of culmination is the vacuolation of the cells expressing both the ecmA and ecmB genes to become mature stalk cells (Jermyn and Williams, 1991). (3) The behaviours of transformants bearing a construct encoding a mutant form of the regulatory subunit of cAMP-dependent protein kinase (PKA) that acts as a dominant inhibitor of the catalytic subunit of PKA, under the control of a prestalk promoter, has shown that PKA activity is required in prestalk cells for culmination, as well as for formation of mature stalk cells in vitro (Harwood et al., 1992).

Mutants affecting pattern formation have been powerful tools in elucidating the mechanism of developmental processes in a varieties of organisms. 'Slugger' mutants of *D. discoideum* (Sussman et al., 1978; Newell and Ross, 1982) show a pronounced tendency to remain in the migratory phase rather than giving rise to mature fruiting bodies. Newell and Ross (1982) analysed 32 slugger mutants by parasexual genetic techniques and placed them

into ten complementation groups. In view of the possible relationship between culmination and stalk cell maturation, we have examined the formation of stalk cells from prestalk cells in representative slugger mutants and their parental strains. Such experiments should throw light on the interactions between the various components controlling culmination. We used two different experimental systems both starting with prestalk cells isolated microsurgically from the anterior zone of slugs and dissociated to single cells. In one system, stalk cell formation was measured in response to DIF-1 and cAMP and, in the other, in response to 8-Br-cAMP, a membrane permeable analogue of cAMP, which can be used for artificially activating PKA in living cells. 8-Br-cAMP has previously been shown to induce stalk cell formation from prestalk cells (Maeda, 1988).

MATERIALS AND METHODS

Strains

Dictyostelium discoideum strain NC4 (a wild-type strain), X22, XP55, NP187 (strains with genetic markers with the wild-type phenotype with respect to the 'slugger' characteristics), and the 'slugger' mutants derived therefrom were used. The details of the mutants are described in Newell and Ross (1982).

Growth and development

Cells were grown on *Klebsiella aerogenes* on SM medium (Sussman, 1987). Cells were collected when the bacterial lawn started to be cleared, and washed free of bacteria by repeated centrifugation. Washed cells were deposited as streaks or spots of thick cell suspension on buffered agar plates (20 mM NaCl, 2 mM $MgSO_4$, 20 mM potassium phosphate buffer, pH 7.0, 1.8% Difco agar) and incubated in a moist chamber to allow slug formation.

In vitro stalk cell differentiation

Prestalk region of slugs were cut off with a microknife and collected in a drop (50 ml) of ice-cold 20 mM potassium phosphate buffer, pH 7.0 containing 1 mM EDTA and 0.1 mM cAMP, transferred to an Eppendorf tube containing 100 ml of the same solution, and dissociated into single cells by several passages through a 25G needle using a 1 ml plastic syringe. The relative size of the prestalk zone had been examined beforehand for each strain by neutral red staining. After cell counts, the cell suspension was microcentrifuged and resuspended at a cell density of 10^4 cells/ml in the stalk salt solution (Kay, 1987) containing 1 µM cAMP (10 µM was also used in some experiments with similar results) and the indicated concentrations of DIF-1 or 8-Br-cAMP. 0.1 ml aliquots of the cell suspensions were then plated in tissue culture dishes (NUNC, 5 cm diameter) or on coverslips precleaned with ethanol. The resulting cell density was approximately 2×10^3 cells/cm^2. Cells were then incubated at 21-22°C in the dark. After 24 hours of incubation, the cells were examined for their differentiation into mature stalk cells using a phase-contrast microscope. Cell wall formation was examined in representative plates using Calcofluor. The purity of the prestalk cell preparations was checked in most of the experiments by staining a portion of the cells with a fluorescein-conjugated prespore-specific antibody.

RESULTS

(1) In vitro cell differentiation in wild-type strains

Without added DIF or 8-Br-cAMP, prestalk cells of parental, non-slugger strains remained amoeboid even in the presence of cAMP. If DIF-1 was added (with 1-10 µM cAMP), up to 40% of the cells became mature stalk cells with a large vacuole and cell wall (Fig. 1A). 8-Br-cAMP induced stalk cell maturation very efficiently and the maximal yield of stalk cells was invariably very high, reaching, in some cases, almost 100% (Fig. 1B). It should be noted that prestalk cells have been exposed to DIF, and this probably explains why DIF does not need to be added to the medium if 8-Br-cAMP is present. Cells before the tip stage (i.e. non-prestralk cells) are not induced by 8-Br-cAMP to form stalk cells (see Maeda, 1988).

The majority of prespore cells treated in the same way with 8-Br-cAMP were induced to form spores (not shown). It can be concluded therefore that 8-Br-cAMP induces both maturation of prestalk cells to stalk cells and of prespore cells to spores, confirming the observation of Maeda (1988).

(2) In vitro stalk cell formation in mutants

Representatives of all the complementation groups have been examined for the formation of stalk cells in response to DIF-1 and cAMP, and in response to 8-Br-cAMP. The relative sizes of the anterior prestalk zone differed from one strain to another as judged from neutral red staining patterns. Accordingly approximately 1/5 to 1/15 fragments of the length of slugs of each strain were collected, dissociated and plated in the same way as the wild-type strains.

Many of the mutants were deficient in responding to DIF-1. Fig. 2A shows the dose-response curves for several mutants together with their parent. Only two strains, NP435 (*slgI*) and NP445 (*slgB*), out of 22 examined formed stalk cells as efficiently as their parent (see Fig. 3).

On the other hand, many, but not all, of the mutants formed stalk cells very efficiently in response to 8-Br-

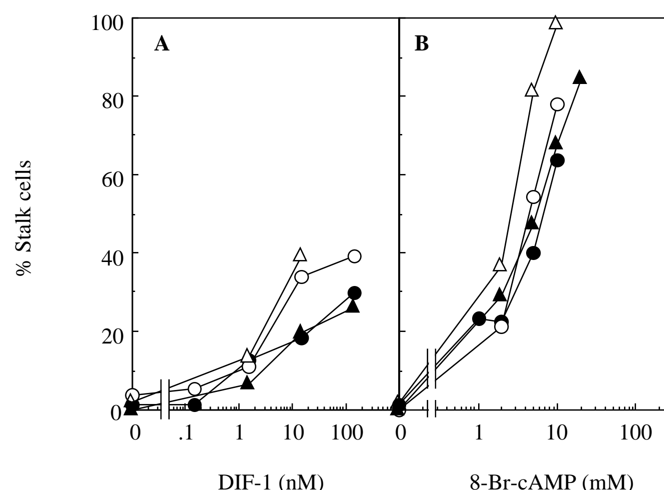

Fig. 1. Stalk cell maturation in response to DIF-1 and cAMP (A) and to 8-Br-cAMP (B) in the parental strains of the slugger mutants. As the response to DIF-1 was variable the experiments giving the best responses are shown in A. Means of 2 to 3 determinations are shown in B. Standard deviations are omitted for clarity. ● NC4, ○ X22, ▲ XP55, △ NP187.

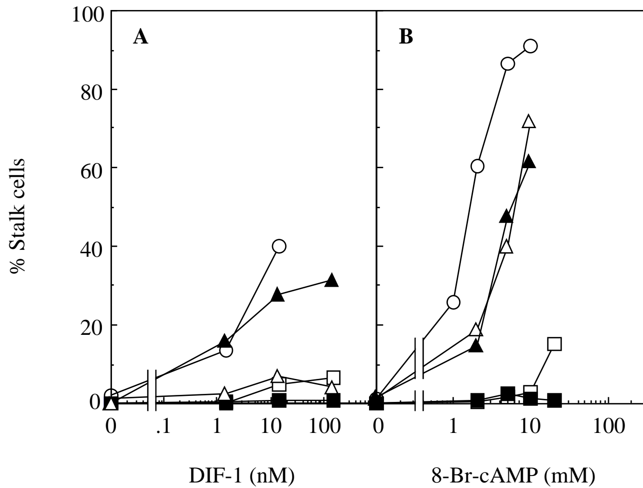

Fig. 2. Stalk cell maturation in response to DIF-1 and cAMP (A) and to 8-Br-cAMP (B) in slugger mutants. The best response for each strain is shown in A. Means of 2 to 4 determinations are shown in B. Standard deviations are omitted for clarity. ○ NP187 (parent), △ NP449 (*slgH*), ▲ NP445 (*slgB*), □ NP439 (*slgJ*), ■ NP441 (*slgF*).

cAMP. This is illustrated in Fig. 2B, which shows the various degrees of responses by some mutants and their parent.

Fig. 3 summarizes responses to the two conditions for all the mutants tested, relative to the responses of their parental strains. It is interesting to note that all the mutants capable of fruiting, albeit very much delayed, responded well to 8-Br-cAMP, whereas all the mutants that could not be rescued by 8-Br-cAMP never formed normal fruiting bodies (with a stalk penetrating a sorus) under conditions that immediately induce fruiting in the wild type (see also Newell and Ross, 1982).

DISCUSSION

We have found that anterior regions of slugs of wild-type (non-slugger) strains of *Dictyostelium discoideum* NC4, when dissociated and incubated in buffer in the presence of cAMP and DIF-1, give rise to substantial numbers of stalk cells (Fig. 1). When exposed to cAMP alone the cells remain amoeboid. Similar observations have been reported for another wild-type strain V12M2 (Kwong et al., 1988) which has a considerably higher tendency to form stalk cells in vitro (Berks and Kay, 1988). In contrast, anterior cells from most of the slugger mutants are markedly defective in forming stalk cells under this condition. This finding supports the evidence for a close relationship between fruiting body formation and stalk cell maturation. Among the few exceptions, NP435 (*slgI*) formed fruiting bodies relatively well, contrary to the original description, and its high efficiency of DIF-induced stalk cell formation may simply be a manifestation of this weak slugger phenotype. Probably the same argument is applicable to the E mutants because these strains also form fruiting bodies after a somewhat prolonged migration (Newell and Ross, 1982). However, NP445 (*slgB*) is totally defective in the initiation of fruiting, like the other strains in the same complementation group, whereas it is normal in terms of in vitro stalk cell formation, quite the opposite of the other strains in the group. We have presently no explanation to this anomaly.

Protein kinase A (PKA) is known to be required for spore maturation (Kay, 1989; Maeda, 1988; Simon et al., 1992) and recent evidence indicates that it also plays an essential role in stalk cell formation (Maeda, 1988; Harwood et al., 1992; Mann et al., 1992). Our results with anterior cells from wild-type slugs confirm that 8-Br-cAMP is an efficient inducer of stalk cell formation and show that it is indeed substantially more efficient than DIF-1. (Essentially the same result was recently obtained

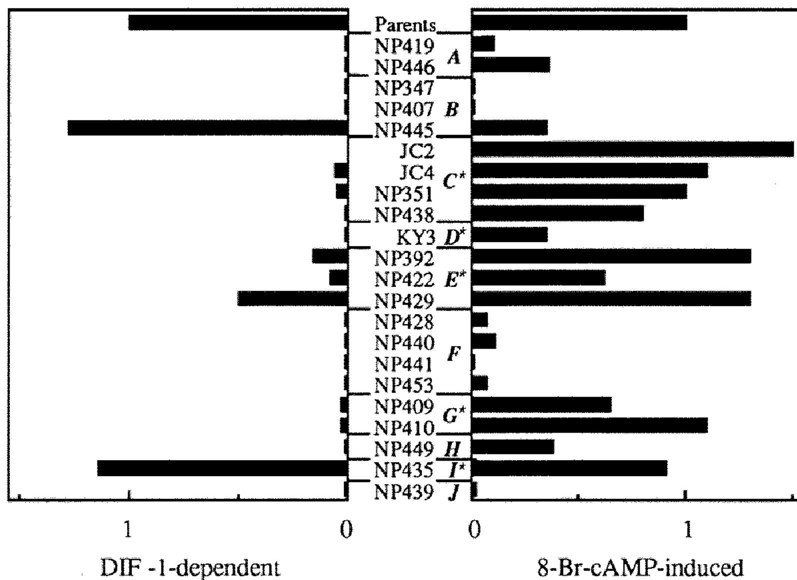

Fig. 3. Responsiveness of the slugger mutants to DIF-1 (left panel) and to 8-Br-cAMP (right panel) relative to their parents. Complementation groups are indicated on the right-hand side of the mutant names. As a measure of responsiveness to the two conditions, reciprocals of the concentration of DIF-1 or 8-Br-cAMP giving rise to 20% stalk cell differentiation (x) were calculated for each mutant and divided by the reciprocal of the corresponding x for their parent. x values for NC4, X22, XP55 and NP187, respectively, are 2.5, 4.0, 15.8, 3.2 for DIF-1 (in nM) and 1.9, 1.8, 2.0, 0.8 for 8-Br-cAMP (in mM). For those mutants never attaining 20% stalk cell differentiation, x was taken to be zero. Maximum stalk cell differentiation in each mutant in each condition closely parallel the values indicated. * on the names of some complementation groups indicate that the mutants in these groups are capable of fruiting on LPS (Newell and Ross, 1982). NP435 (*slgI*) eventually formed fruiting bodies in our hands.

using gradient-separated prestalk cells of NC4 (Kubohara, Maeda and Okamoto, personal communication)). This, together with the evidence for a close relationship between culmination and stalk cell formation, suggests that fruiting body formation is prevented during slug migration because of partial inhibition of cAMP production and hence partial inhibition of PKA activity. Harwood et al. (1992) have shown that transformants bearing a construct encoding a dominant inhibitor of the catalytic subunits of PKA are defective in the stalk cell-specific gene expression and proposed a model in which during migration a protein repressor that is subject to inactivation by PKA inhibits DIF-dependent transcriptional processes required for stalk cell formation.

We have reported that many of the slugger mutants that are defective in stalk cell formation in monolayer culture in the presence of DIF-1 do respond to 8-Br-cAMP. This behaviour implies that the functions affected in these mutants are required for optimal PKA activation, and underscores the contribution of this pathway to stalk cell maturation and culmination. Sussman and his collaborators have shown that it is the accumulation of the weak base ammonia that is responsible for the maintenance of the slug state (Schindler and Sussman, 1977) and that ammonia rapidly inhibits adenylyl cyclase activation in response to extracellular cAMP (Williams et al., 1984). They have further shown that, in one of the slugger mutants, KY3, which we have found to respond reasonably well to 8-Br-cAMP, adenylyl cyclase activation is abnormally sensitive to ammonia inhibition (Schindler and Sussman, 1979). These findings supports the notion that some at least of these mutants have defects in functions required for optimal adenylyl cyclase activation. Further analysis of these mutants should therefore prove useful in identifying these functions.

In a minority of the mutants, 8-Br-cAMP fails to induce stalk cell formation with the same high efficiency as it does in the parental cells. These mutants may have defects in PKA itself, or in components acting downstream from PKA. A defect in PKA itself, however, is unlikely, at least in *slgF* and *slgJ*, since spore maturation, which also requires PKA activation (Simon et al., 1992), was efficiently induced by 8-Br-cAMP in representative strains of these complementation groups (unpublished). A defect in the DIF-receptor/signal transduction system might also result in such a phenotype because stalk cell formation is induced by 8-Br-cAMP only after prestalk cells have differentiated (Maeda, 1988). It is not known, however, if a tip can form without prestalk cell differentiation.

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REFERENCES

Berks, M. and Kay, R. R. (1988). Cyclic AMP is an inhibitor of stalk cell differentiation in *Dictyostelium discoideum*. *Dev. Biol.* **126**, 108-114.

Harwood, A. J., Hopper, N. A., Simon, M.-N., Driscoll, D. M., Veron, M. and Williams, J. G. (1992). Culmination in Dictyostelium is regulated by the cAMP-dependent protein kinase. *Cell* **69**, 615-624.

Inouye, K. (1988). Induction by acid load of the maturation of prestalk cells in *Dictyostelium discoideum. Development* **104**, 669-681.

Jermyn, K. A. and Williams, J. G. (1991). An analysis of culmination in *Dictyostelium* using prestalk and stalk-specific cell autonomous markers. *Development* **111**, 779-787.

Kay, R. R. (1987). Cell differentiation in monolayers and the investigation of slime mold morphogenesis. *Methods in Cell Biology* **28**, 433-448.

Kay, R. R (1989). Evidence that elevated intracellular cyclic AMP triggers spore maturation in *Dictyostelium. Development* **105**, 753-759.

Kwong, L., Sobolewski, A., Atkinson, L. and Weeks, G. (1988). Stalk cell formation in monolayers from isolated prestalk and prespore cells of *Dictyostelium discoideum*: evidence for two populations of prestalk cells. *Development* **104**, 121-127.

Maeda, M. (1988). Dual effects of cAMP on the stability of prespore vesicles and 8-bromo cAMP-enhanced maturation of spore and stalk cells of *Dictyostelium discoideum. Develop. Growth and Differ.* **30**, 573-587.

Mann, S. K. O., Yonemoto, W. M., Taylor, S. S. and Firtel, R. A. (1992). *DdPK3*, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. *Proc Natl. Acad. Sci. USA* **89**, 10701-10705.

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* **328**, 811-814.

Morris, H. R., Masento, M. S., Taylor, G. W., Jermyn, K. A. and Kay, R. R. (1988). Structure elucidation of two differentiation inducing factors (DIF-2 and DIF-3) from the cellular slime mould *Dictyostelium discoideum. Biochem. J.* **249**, 903-906.

Newell, P. C. and Ross, F. M. (1982). Genetic analysis of the slug stage of *Dictyostelium discoideum. J. Gen. Microbiol.* **128**, 1639-1652.

Schindler, J. and Sussman, M. (1977). Ammonia determines the choice of morphogenetic pathways in *Dictyostelium discoideum. J. Mol. Biol.* **116**, 161-169.

Schindler, J. and Sussman, M. (1979). Inhibition by ammonia of intracellular cAMP accumulation in *Dictyostelium discoideum*: Its significance for the regulation of morphogenesis. *Dev. Genet.* **1**, 13-20.

Simon, M.-N., Pelegrini, O., Veron, M. and Kay, R. R. (1992). Mutation of protein kinase A causes heterochronic development of *Dictyostelium. Nature* **356**, 171-172.

Sussman, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods in Cell Biology* **28**, 9-29.

Sussman, M., Schindler, J. and Kim, H. (1978). 'Sluggers', a new class of morphogenetic mutants of *D. discoideum. Exp. Cell Res.* **116**, 217-227.

Town, C. D., Gross, J. D. and Kay, R. R. (1976). Cell differentiation without morphogenesis in *Dictyostelium discoideum. Nature* **262**, 717-719.

Wang, M. and Schaap, P. (1989). Ammonia depletion and DIF trigger stalk cell differentiation in intact *Dictyostelium discoideum* slugs. *Development* **105**, 57-62.

Wang, M., Roelfsema, J. H., Williams, J. G. and Schaap, P. (1990). Cytoplasmic acidification facilitates but does not mediate DIF-induced prestalk gene expression in *Dictyostelium discoideum*. *Dev. Biol.* **140**, 182-188.

Williams, G. B., Elder, E. M. and Sussman, M. (1984). Modulation of the cAMP relay in *Dictyostelium* by ammonia and other metabolites: possible morphogenetic consequences. *Dev. Biol.* **105**, 377-388.

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