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Formation and anatomy of the prestalk zone of *Dictyostelium*

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Summary

The pDd63 and pDd56 genes encode extracellular matrix proteins which, respectively, surround the migratory slug and mature stalk cells. Both genes are dependent for their expression upon, and rapidly induced by, DIF, the stalk cell inducer. Using these genes as cell-autonomous markers, we have defined three distinct kinds of 'prestalk' cells localized to different parts of the anterior region of the slug. At least one, and probably both, prestalk cell types initially differentiates at the base of the aggregate. The most abundant of the two prestalk cell types then migrates into the tip, the precursor of the prestalk zone which arises at the apex of the aggregate. Thus we believe that morphogenesis of the prestalk zone, the primary pattern-forming event in *Dictyostelium* development, involves a combination of positionally localized differentiation and directed cell migration. To account for the positionally localized differentiation of prestalk cells, we invoke the existence of gradients of the known antagonists of DIF – cAMP and NH3. We further suggest that differences in the motility of pstA and pstB cells might result from differences in their chemotactic responsiveness to cAMP signals propagated from the tip.

Key words: *Dictyostelium*, prestalk zone, extracellular matrix, DIF, inducer.

Introduction

At the end of aggregation, which in *D. discoideum* occurs in response to pulsatile emissions of cAMP from a signalling centre, a hemispherical cell mass, known as the tight aggregate, is formed. A nipple-shaped tip then forms atop the tight aggregate. This elongates to form a structure known as the standing slug, or first finger. Under conditions inappropriate for culmination, a migratory slug is formed. This is exquisitely phototactic and thermotactic and these sensitivities direct it to the surface where culmination occurs. The tip of the aggregate is retained at the anterior of the slug where it displays many of the properties of an embryonic organizer (Raper, 1940; Rubin and Robertson, 1975). The front 20 % of the slug is composed of prestalk cells. The rear 80 % is predominantly composed of prespore cells but there are also scattered cells, throughout the prespore zone, with the characteristics of prestalk cells. These are termed anterior-like cells. (Sternfeld and David, 1982).

In one sense *D. discoideum* is an exception amongst slime moulds, in that the migratory slug contains a population of prestalk cells which act as the direct precursors of stalk cells. In other species the majority of cells in the slug express prespore markers and the stalk is formed by 'transdifferentiation' of cells as they reach the extreme tip of the slug (Gregg and Davis, 1982; Schaap *et al.* 1985). In these situations it is clear that differentiation occurs in response to positional signals at the tip which direct cells to differentiate into stalk cells. However, in *D. discoideum* it has been a matter of great debate whether initial differentiation, to form prestalk cells, occurs randomly throughout the aggregate, with subsequent cell sorting to form a distinct prestalk zone.

Cells sort when morphogenesis is subverted (Sternfeld and David, 1981; MacWilliams, 1982; Oyama *et al.* 1983) and it is possible to generate populations of cells biased to one or other pathway of differentiation, by manipulation of the growth conditions (Leach *et al.* 1973; Tasaka and Takeuchi, 1981) or by selecting cells at defined phases of the cell cycle. (MacDonald and Durston, 1984; Van Lookeren Campagne *et al.* 1984). In an analysis using an antibody directed against a prespore marker, prespore cells were first detected, at the time of tip formation, in the lower part of the aggregate (Krefft *et al.* 1984). This was taken to indicate that prespore cell differentiation occurs in response to positionally localized, morphogenetic signals. However, spore cells are essentially passive participants in *Dictyostelium* pattern formation and it is the differentiation and directed movement of prestalk cells that primarily shapes the fruit. In order to understand *Dictyostelium* morphogenesis, it is therefore necessary to determine how the expression of stalk-specific markers is induced and to use these markers to study the differentiation of individual prestalk cells. We have isolated definitive prestalk cell markers by identifying genes responsive to an inducer specific for stalk cell differentiation.

The stalk cell morphogen, DIF-1 (Morris *et al.* 1987)

acts rapidly to induce the expression of two closely related proteins, ST430 and ST310 (Jermyn *et al.* 1987; McRobbie *et al.* 1988*a*,*b*; Williams *et al.* 1987). They were first identified by two-dimensional gel electrophoresis of total cellular proteins (Morrissey *et al.* 1984) and are composed of tandem repeats of a twenty four amino acid, cysteine-rich sequence (Ceccarelli *et al.* 1987; Williams *et al.* 1987). They are extracellular proteins of the slime sheath and stalk tube (McRobbie *et al.* 1988), the protein–cellulose matrices which respectively surround the migrating slug and stalk cells of the mature fruit. We have shown, using the 'DIF-less' mutant HM44 (Kopachik *et al.* 1983), that the pDd63 gene and the pDd 56 genes, which encode these two proteins, are dependent upon DIF for their expression (Jermyn *et al.* 1987). Both genes are induced at the transcriptional level and expression of the pDd63 gene is detectable within fifteen minutes of the addition of DIF (Williams *et al.* 1987; Ceccarelli *et al.* 1987). The speed of this induction suggests it is mediated by a pre-existing receptor, or second-messenger pathway, directly responsive to DIF. Consistent with their induction by DIF, both mRNA sequences first appear just before the time a tip forms on the aggregate and both mRNA sequences persist into mature stalk cells (Jermyn *et al.* 1987; Williams *et al.* 1989).

Within the limits of purity achieved when prestalk and prespore cells are separated by gradient centrifugation, the pD56 and pD63 mRNA species appear to be completely prestalk-specific (Jermyn *et al.* 1987). As such they differ from most previously described 'prestalk' markers, which are also expressed in prespore cells (Jermyn *et al.* 1987). With the exception of the recently described cDt100 cDNA clone of Ozaki *et al.* 1988, which is as highly enriched in prestalk cells as pDd63 and pDd56, these other markers seem likely to be expressed in all cells and selectively stabilized in prestalk cells. They cannot, therefore, be used as cell-autonomous markers. The pDd56 and pDd63 provide such markers but, because they encode extracellular proteins, we are unable directly to identify cells expressing them. We have created cell-autonomous markers from them, by fusing their promoters to a 'reporter' gene containing a nuclear translocation signal, and transforming the constructs into *Dictyostelium* cells (Jermyn *et al.* 1989). We have shown there to be multiple classes of prestalk cells located in different parts of the anterior zone whose ontogeny and fate we have investigated (Williams *et al.* 1989). Here we outline these results and discuss their significance.

Discussion

(1) Multiple prestalk cell types in the migrating slug

The pDd63-Tag-CAt and pDd56-Tag-CAT constructs contain the pDd63 and pDd56 promoters (Ceccarelli *et al.* 1987; Early *et al.* 1988) coupled to reporter genes (Fig. 1). They display correct temporal, cell-type-specific and DIF-inducible expression after transformation into *Dictyostelium*. We have therefore been able

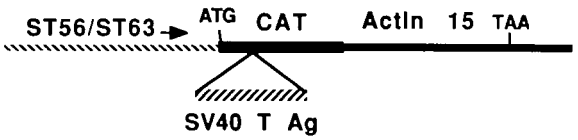


Fig. 1. Structure of the reporter gene constructs used to establish cell autonomous markers. The pDd56 and pDd63 genes were fused at a point just downstream of their ATG initiation codons to the chloramphenicol acetyl transferase (CAT) gene. Termination and polyadenylation signals are provided by the *Dictyostelium* actin15 gene. After transformation into *Dictyostelium* the fusion genes were correctly regulated (Ceccarelli *et al.* 1987; Early *et al.* 1988). They were further modified by the insertion of a fragment of the SV40 T antigen gene containing the nuclear localization signal (Jermyn *et al.* 1989). The fusion proteins are efficiently transported to the nucleus of *Dictyostelium* cells expressing the two genes.

to use them to monitor expression of the endogenous pDd56 and pDd63 genes. Cells expressing the pDd63 gene are restricted to the front 10 % of the length of the slug (Fig. 3A and Jermyn *et al.* 1989). The prestalk zone, defined using vital dyes that selectively stain prestalk cells (Bonner, 1951), occupies the front 20 % of the length of the slug. Hence there is a region, between the anterior 10 % and the prespore zone which does not contain cells expressing the pDd63 gene. In slugs derived from pDd56 transformants there is an anterior, central 'funnel' of staining cells, which occupies about one fifth to one tenth of the length of the slug (Fig. 3B and Jermyn *et al.* 1989).

Thus the slug has a more complex organization than hitherto suspected, with two kinds of prestalk cells, located in different parts of the anterior 10 % of the slug (Fig. 3C). We term these prestalk A (pstA) and prestalk B (pstB) cells. The pstA cells express the pDd63 gene but there is no detectable level of the pDd56 gene product. The pstB cells express the pDd56 gene and possibly also the pDd63 gene. We call the cells in the region immediately posterior to the front 10 %, which express neither marker, prestalk 0 (pst0) cells.

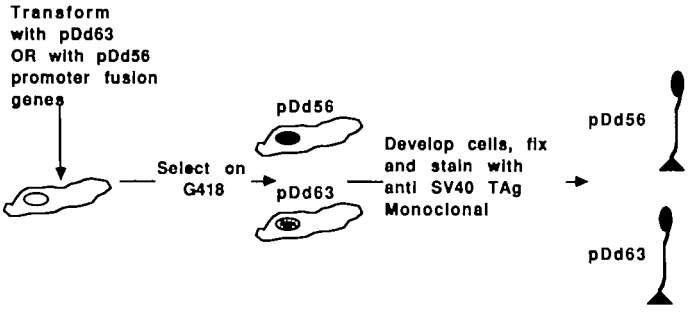
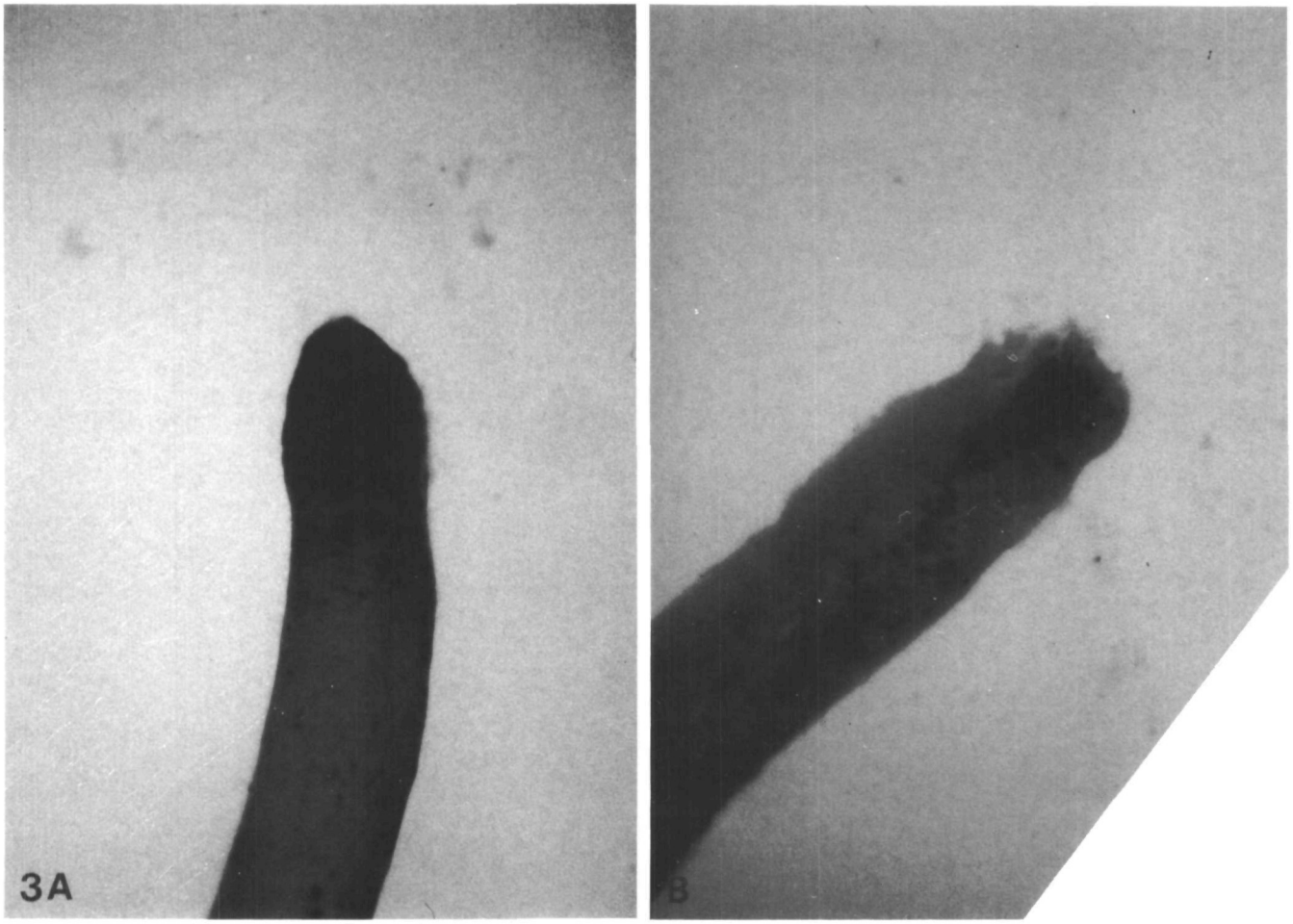


Fig. 2. Use of the reporter genes as cell autonomous markers. The pDd56 and pDd63 gene fusions contain a region of the SV40 genome recognized by four potent monoclonal antibodies and staining nuclei are detected by secondary incubation with an antibody coupled to horse radish peroxidase (Jermyn *et al.* 1989).



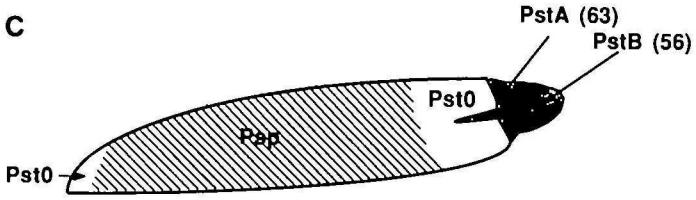


Fig. 3. Expression of the pDd63 and pDd56 fusion genes in different parts of the slug. (A) Expression of the pDd63 fusion gene. Migrating slugs, transformed with the pDd63–Tag–CAT construct were fixed as whole mounts and stained as described in the legend to Fig. 2. (B) Expression of the pDd56 fusion gene. This figure shows the front portion of a whole mount of pDd56 transformant slug. (C) Schematic representation of the distribution of prestalk and prespore cells in the slug. The pDd56 gene is expressed only in the central core (pstB cells) while the pD63 gene is expressed in cells surrounding them (pstA cells) and possibly also in the pstB cells. (Jermyn *et al.* 1989). The pstO cells in the zone behind the front 10% of the length of the slug express neither marker. The prespore (psp) cells constitute the major fraction of cells. The rearguard cells, which express neither marker are classed here as pstO cells.

(2) Ontogeny and fate of prestalk cells during slug formation

We have recently shown that initial pstA and pstB cell differentiation occurs prior to appearance of the tip and that pstA cells move to it (Williams *et al.* 1989 and Fig. 4). Furthermore, quite contrary to expectation, pstB cells are predominantly localized to, and pstA cells become enriched at, the base of the tipped aggregate. Expression of the pDd56 gene, which defines a cell as pstB, is totally dependent upon DIF (Jermyn *et al.* 1987). Since pstB cells appear initially to differentiate at the base of the tight aggregate, this strongly implies the base to be a region of high effective DIF concentration from the very earliest stages of morphogenesis. Expression of the pDd63 gene is also dependent upon DIF (Jermyn *et al.* 1987) and we would therefore expect expression of the pDd63 gene to be initiated in the base of the aggregate. Because pstA cells rapidly migrate to the tip, we are unable to determine whether they also initially differentiate there but their enrichment in the basal zone of the tipped aggregate is certainly consistent with this idea.

PstA cells are the majority population and their behaviour presumably reflects the fact that prestalk cells are more chemotactially responsive and have a higher intrinsic motility than prespore cells (Bonner, 1951; Mee *et al.* 1986; Durston and Vork, 1979). We think that pstB cells may remain at the base because

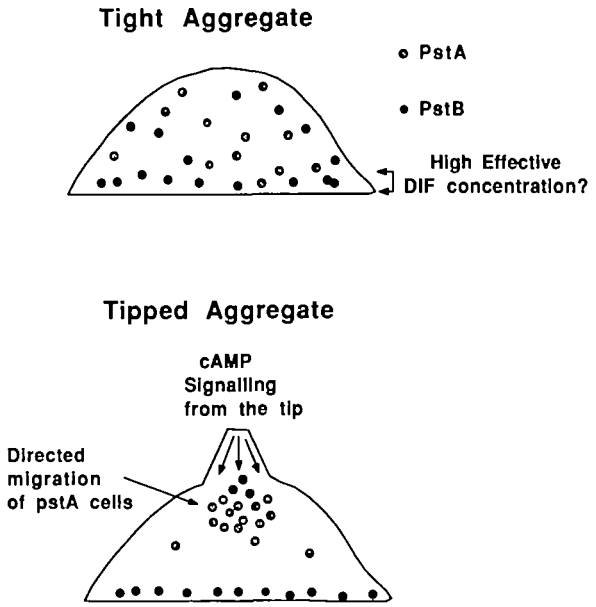


Fig. 4. A model for pstA and pstB cell differentiation. The pstA and pstB cells are proposed to differentiate in the base of the aggregate. PstA cells then migrate to the tip while pstB cells are left at the base to be lost in the slime trail as the slug migrates away from its site of formation (Williams *et al.* 1989). PstA cells within the tip then differentiate further to give the central core of pstB cells found in the slug tip.

they are chemotactically inactive (Fig. 5). We further believe that pstB cells are cells more advanced in their differentiation than pstA cells and that these are not two mutually exclusive states of differentiation – that there is potential progression, from pstA to pstB when the effective DIF concentration becomes high enough (Fig. 6).

Both cell sorting and positionally localized differentiation contribute to tip formation

Our observations suggest both models for morphogenesis to be partially correct. There *is* positionally localized differentiation but it initially occurs in the base of the aggregate and not, as logic would have suggested, in the tip. There is directed migration, of pstA cells, but pstB cells differentiate entirely *in situ* – an initial population in the base of the aggregate and a second population in the tip of the standing slug.

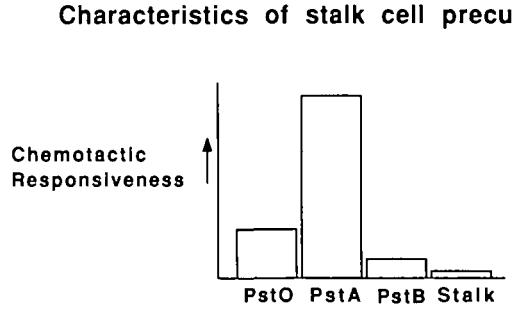


Fig. 5. A possible explanation for the different behaviour of pstA and pstB cells during slug formation.

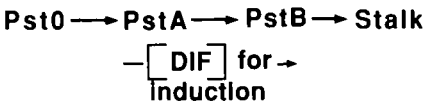


Fig. 6. Proposed stages in stalk cell differentiation. Terminal stalk cell differentiation is known to require a higher level of DIF than pstA cell differentiation, i.e. expression of the pDd63 gene (Jermyn *et al.* 1987). The evidence that a higher level of DIF is required for pstB cell differentiation derives from unpublished studies by Mary Berks and Rob Kay.

By showing that prestalk differentiation occurs prior to tip formation and that one of the two classes of prestalk cell, pstA cells, migrate into it, we have provided direct proof that directed prestalk cell migration plays a role in normal development. While we have confirmed its involvement, we do not believe that our observations show cell-sorting to play the primary role in patterning. We certainly do not subscribe to the view that cells are determined to one or other pathway of differentiation at the time they enter development by virtue of their position in the cell cycle (Gomer and Firtel, 1987). A critical property of the pDd56 and pDd63 genes is their dependence upon, and rapid induction by, DIF. Cells are inevitably heterogeneous and may, for example, differ in their sensitivity to DIF. This could affect the probability that they will form prestalk cells. However, the fact that cells transcribing these two genes constitute the two definitive prestalk cell populations, provides compelling evidence that exposure to DIF, or some active metabolite of it (Meinhardt, 1983), is the over-riding signal directing cells into the stalk cell pathway.

How is the initial positionally localized differentiation achieved and how are the cell types regulated during slug migration? There are a host of potential explanations and the eventual distinction between them will require a knowledge of the effective concentrations of all of the potential morphogens and their antagonists. However, it is worthwhile to attempt to formulate a model if only to serve as the basis for further experiment.

Molecules regulating the differentiation of pstA and B cells

Both the DIF inducible genes require DIF for their expression but the pDd56 gene may require a higher level of DIF for activation than the pDd63 gene (Mary Berks and Rob Kay personal communication). Presumably, therefore, the effective DIF concentration is highest at the base. We do not believe this is likely to be due to a higher level of DIF itself. Rather, we favour an alternative explanation; that an antagonist of DIF is present at a lower concentration and we suggest that this might be ammonia (Fig. 4). This is produced in large amounts because of the very active catabolism accompanying development. Ammonia acts as a DIF antagonist *in vitro*, acting to prevent stalk cell differentiation (Gross *et al.* 1983). There is also good evidence for it playing a similar role *in vivo*. A reduction in

ammonia levels appears to be the normal trigger for culmination (Schindler and Sussman, 1977) and depletion of ammonia, induced artificially within the slug (Inouye, 1988; Wang and Schaap, 1989), leads to stalk cell differentiation *in situ*. We suggest that loss of ammonia might occur selectively at the base of the aggregate, perhaps because it has a higher rate of loss to, or inactivation in, the substratum than DIF. The formation of a stable, prestalk zone in the slug is more difficult to explain. First we must account for the existence of the pst0 cells.

There is abundant evidence to show that prespore gene expression is induced and maintained by cAMP signalling (reviewed by Schaap, 1986; Gerisch, 1987; Williams, 1988). Cyclic AMP signalling is amplified in a relay reaction and, in principle, all cells in the slug should respond. How then are cells at the front of the slug prevented from expressing prespore genes? DIF may play a part in this, since it represses the expression of prespore markers. (Kay and Jermyn, 1983; Early and Williams, 1988; Wang *et al.* 1986). However, adenosine, a competitive inhibitor of cAMP signalling and prespore cell differentiation (Newell, 1982; Newell and Ross, 1982; Schaap and Wang, 1986; Theibert and Devreotes, 1984; Schaap and Wang, 1986), appears very likely to be the primary repressor *in vivo*. The enzymes generating adenosine from cAMP are present at a higher concentration in the prestalk zone (Brown and Rutherford, 1980; Armant and Stetler, 1980; Armant and Rutherford, 1979) and enzymatic depletion of adenosine from the intact slug leads to prespore gene expression in the anterior, prestalk region (Schaap and Wang, 1986). We suggest that the pst0 zone is composed of cells blocked in prespore gene expression because of the presence of a high adenosine concentration (Fig. 7A,B). They are prevented from progressing into pstA cells because they are not exposed to a sufficiently high DIF concentration. They would therefore correspond in their properties to cells late in aggregation, i.e. they would be uncommitted to either pathway of differentiation. On this model adenosine would act only as an inhibitor of prespore differentiation. One prediction is that cells in the anterior zone should simultaneously express prespore and prestalk markers, when adenosine levels in the slug are depleted, and we are testing this.

The occurrence of pstA and pstB cells in the tip indicates that the effective DIF concentration must be relatively high there (Fig. 7A). The concentration of DIF has been determined in sub-sections of migrating slugs, with the apparently paradoxical observation of a higher concentration in the rear of migrating slugs than in the front. This has been suggested to indicate that the active species is not DIF itself but a metabolite produced from it (Meinhardt, 1983). However, the known breakdown products of DIF are much less active in stalk cell induction than DIF (Kay *et al.* this volume) and this model, therefore, remains unproven. Our results might appear to have some bearing on this paradox. The rear of the slug derives from the basal zone and, given the observation that prestalk cell differentiation appears to initiate there, DIF might also be expected to be present at a higher concentration in the base. However, metabolic studies show DIF to have a half-life *in vitro* of only minutes (Kay *et al.* this volume) and it therefore seems very unlikely that slugs, which had migrated for several days, would retain any vestiges of the distribution present in the aggregate.

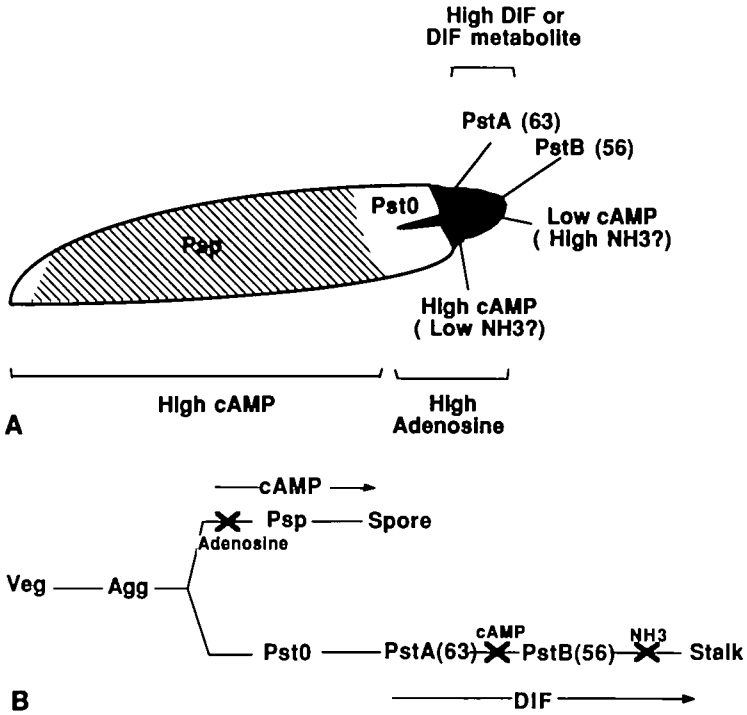


Fig. 7A,B. A scheme for the interactions between inducers of cellular differentiation and of their antagonists in the slug. The evidence supporting parts of this scheme has been presented elsewhere (Williams, 1988). The major new feature is the inclusion of pstA cells as an intermediate stage in prestalk differentiation and the suggestion that the progression from pstA to pstB is repressed by cAMP.

What of the relative distribution of pstA and pstB cells? We believe that there is a progression from pstA to pstB and that this occurs when the DIF concentration reaches a critical threshold (Fig. 6). It is therefore tempting to believe that the effective DIF concentration is higher in the core at the centre of the migrating slug. The distribution of pstB cells suggests the existence of a radially distributed antagonist. Loss from the outer layers of the tip, of a highly diffusible substance such as ammonia, would establish a radial gradient but, on the model presented above where DIF and ammonia are held to be directly antagonistic, the effective concentration of DIF would be higher in the outer part of the tip. Hence differentiation of pstB cells would occur to give the reverse distribution to that actually observed. There is a potential explanation but it requires invoking a more complex set of interactions:

Cyclic AMP is required in the early stages of an *in vitro* induction in order that cells become competent to respond to DIF (Sobolewski *et al.* 1983) but it then acts to repress accumulation of the pDd56 protein (Berks and Kay, 1988). Ammonia is known to repress the

intracellular accumulation of cAMP in response to an extracellular cAMP pulse (Schindler and Sussman, 1977). Hence it may be that, in the slug tip, ammonia is acting as a cAMP antagonist leading to a low effective cAMP concentration (Fig. 7A). It may also play a role as a DIF antagonist, preventing the progression of pstB cells into stalk cells (Fig. 7B). This would be consistent with the fact that depression of ammonia levels is the signal for terminal differentiation (Schindler and Sussman, 1977). Again, however, the speculative nature of these proposed interactions emphasizes the importance of establishing the effective concentrations of all of the signalling molecules involved in both chemotaxis and cellular differentiation.

Evolutionary considerations

The Dictyostelids present a fascinating opportunity to study the evolution of multicellularity, cellular differentiation and morphological diversification (Bonner, 1982; Schaap *et al.* 1985). The most remarkable feature of our results is the demonstration that prestalk cell differentiation appears to be initiated at the base of the aggregate and not at the tip, as logic would have suggested. How might this have arisen?

Primitive relatives of *Dictyostelium*, such as *Protostelium mycophaga*, form a single spore, supported by an acellular stalk (Fig. 8). In a more advanced species, such as *Actyostelium leptosomum*, a mass of spores is formed but again it is supported by an acellular stalk (Fig. 8). Thus *D. discoideum* presumably evolved from organisms in which stalk material, possibly containing a protein similar to ST310 the product of the pDd56 gene, was secreted down onto the substratum. We suggest that the initial burst of pDd56 gene expression in pstB cells at the base of the aggregate may reflect an intermediate stage in the evolution of the Dictyostelids, where cells were diverted into the stalk cell pathway of differentiation only at the base of the aggregate. Consistent with this hypothesis is our finding of a second wave of pDd56 gene expression during culmination at the base of the mexican hat (K. A. Jermyn and J. G. Williams, unpublished results). This is the stage at which the migrating slug re-enters the culmination pathway. Thus it is logical that the pstB cells, which are lost from the base when a migratory slug is formed, should be replaced as the slug sits down at culmination.

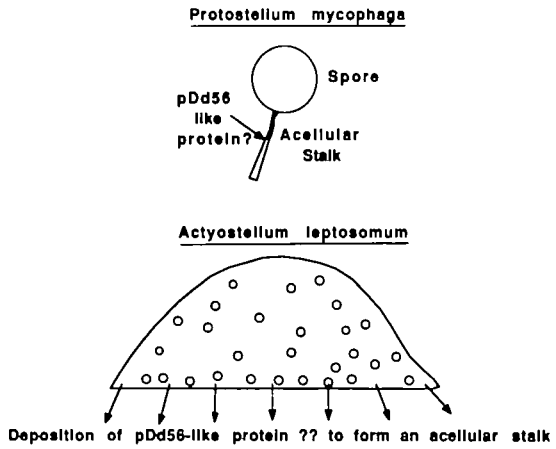


Fig. 8. Possible early stages in the evolution of the Dictyostelids.

At the point in time when a migratory slug stage evolved, it may have been necessary to provide a continuously generated source of slime sheath material at the front of the slug. Therefore, cells were arrested in prestalk cell differentiation at some equivalent of the present day pstA stage and endowed with enhanced chemotactic responsiveness to cAMP and the capacity to produce a pDd63-like protein. This gave the further possibility of producing a stalk tube internal to the spore cell mass, by the reverse fountain movement characteristic of culmination in present day Dictyostelids. The further differentiation of these pstA cells into pstB cells in the centre of the slug tip may then have evolved in order to facilitate the initial production of the stalk tube, which occurs at this point. Consistent with this suggestion is the observation that this group of pstB cells is the first to enter the stalk tube and that they form part of the basal disc, the expanded base of the stalk tube, which gives *D. discoideum* its name (K. A. Jermyn and J. G. Williams, unpublished results). Again, this model is speculative but it will be informative to seek the equivalents of the pDd63 and pDd56 proteins in other Dictyostelids and to compare their patterns of expression.

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