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Ammonia depletion and DIF trigger stalk cell differentiation in intact *Dictyostelium discoideum* slugs

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Summary

The differentiation-inducing factor, DIF, was previously shown to induce stalk cell differentiation in *Dictyostelium discoideum* cells incubated as submerged monolayers. We investigated the mechanism that regulates the differentiation of stalk cells in the intact organism. It was found that in migrating or submerged slugs DIF cannot induce stalk cell differentiation, which is most likely due to the presence of a DIF antagonist. Cyclic AMP and ammonia were earlier reported to act as DIF antagonists *in vitro*. We show here that ammonia, but not cAMP, acts as an antagonist for DIF-induced stalk cell differentiation *in vivo*. DIF can induce stalk cell differentiation when ammonia levels in the slug are enzymically depleted. However, depletion of cAMP levels does not increase the efficacy of DIF. We propose that the induction of stalk cell differentiation during early culmination may be triggered by a drop in ammonia levels inside the organism.

Key words: pattern formation, stalk cell differentiation, differentiation inducing factor, ammonia, cAMP.

Introduction

The life cycle of the cellular slime mould *Dictyostelium discoideum* represents a comparatively simple model system to study the regulation of cell differentiation and pattern formation. The system becomes increasingly attractive, because several compounds that can induce differentiation *in vitro* have been identified (see Williams, 1988).

D. discoideum development can be subdivided into several major events of stage- or cell-type-specific gene expression. Starvation initiates the synthesis of a large number of gene products involved in the aggregation process (see Kessin, 1988). This type of gene expression is accelerated by the chemotactic signal itself, i.e. nanomolar cAMP pulses (Darmon *et al.* 1975; Gerisch *et al.* 1975).

Spore-specific gene expression is first evident after aggregation and remains restricted to the basal part of the aggregate and later to the posterior region of the slug (Hayashi & Takeuchi, 1976; Morrissey *et al.* 1984; Krefft *et al.* 1984). Spore-specific gene expression can be induced *in vitro* by cAMP concentrations in the micromolar range (Kay, 1982; Schaap & Van Driel, 1985; Oyama & Blumberg, 1986) and elevated extracellular cAMP levels are essential for prespore differentiation *in vivo* (Wang *et al.* 1988). Adenosine, a cAMP degradation product, inhibits prespore differentiation (Weijer & Durston, 1985; Schaap & Wang, 1986; Spek *et al.* 1988) and is considered to be essential for the establishment of the anterior prespore-free region in slugs (Schaap & Wang, 1986; Wang *et al.* 1988).

Stalk cell differentiation is specifically initiated during early fruiting body formation at the apex of the culminating structure. The process involves the synthesis of at least 10 new proteins, (Kopachik *et al.* 1985; Morrissey *et al.* 1984), extreme vacuolization of the cells and the synthesis of a fibrous cellulose cell wall (Raper & Fennell, 1952). Two stalk-specific genes are expressed several hours earlier and are specifically present at the anterior region of migrating slugs (Williams *et al.* 1987; Jermyn *et al.* 1987).

The synthesis of stalk-specific proteins as well as the expression of the two early stalk genes can be induced *in vitro* by the stalk-inducing factor DIF (Kay & Jermyn, 1983; Kopachik *et al.* 1985; Williams *et al.* 1987; Sobolewski & Weeks, 1988). However, the regulation of stalk cell differentiation *in vivo* is not immediately evident from the spatiotemporal distribution of this factor. DIF levels increase after aggregation and are maximal at the slug stage of development (Brookman *et al.* 1982), which is several hours before culmination takes place. Furthermore, no evidence for increased DIF levels at the apex of slugs and fruiting bodies has yet been found; it rather appears that DIF concentrations are somewhat higher at the posterior than at the anterior region of the slug (Brookman *et al.* 1987).

The induction of stalk cell differentiation by DIF is inhibited *in vitro* by cAMP (Berks & Kay, 1988) and by ammonia, which was proposed to act as a natural DIF

antagonist (Gross *et al.* 1983). It was shown much earlier by Schindler & Sussman (1977) that a drop in ammonia levels triggers the culmination process. These investigators proposed that stalk cell differentiation was induced by a combination of high cAMP and low ammonia levels (Sussman & Schindler, 1978). The role of cAMP in this model is contradicted by the observed inhibitory effects of cAMP on stalk cell differentiation (Berks & Kay, 1988), but if cAMP is replaced by DIF, the proposed role of ammonia may still be valid.

In order to gain insight in control mechanisms operative in the intact organism, we investigated whether DIF can induce stalk cell differentiation in migrating and submerged slugs and whether either cAMP or ammonia levels in the slug antagonize the effects of DIF. Our results show that ammonia, but not cAMP, acts as an natural DIF antagonist for stalk cell differentiation.

Materials and methods

Materials

DIF-1 was obtained as a kind gift from Dr Robert R. Kay, or isolated and HPLC purified as described by Kay *et al.* (1983). One unit of DIF is defined as the amount of DIF that induces one percent of *D. discoideum* V12M2 cells to differentiate as stalk cells in the DIF bioassay (Brookman *et al.* 1982). Calcofluor, 4',6'-diamidino 2-phenyl-indole (DAPI), L-glutamate dehydrogenase and α-ketoglutarate were from Sigma (St Louis, USA). Beef heart cAMP-phosphodiesterase and NADH were from Boehringer (FRG), cellulase Onozuka was from Serva (FRG) and FITC-conjugated swine anti-rabbit IgG was from Dakopatts (Denmark).

Culture and incubation conditions

D. discoideum NC4 cells were grown in association with *Escherichia coli* on glucose–peptone agar (Schaap & Spek, 1984). Vegetative cells were freed from bacteria by repeated washing with 10 mM-Na/K phosphate pH 6·5 (PB) and plated on non-nutrient agar (1·5 % agar in PB) or on dialysis membrane supported by PB agar. The cells were incubated at 22 °C until migrating slugs had formed. Membranes carrying slugs were then transferred to agar containing various DIF concentrations and allowed to migrate further for an additional 6 h period. Alternatively about 10 migrating slugs were carefully transferred from agar to 1 ml screw cap septum vials containing 400 μl PB pH 7·0 or 400 μl 10 mM-imidazole buffer pH 7·5 with various additives. The tubes were flushed for 30 s with O_2, closed and rotated at 10 rev min^{-1} at 21 °C during 6 to 12 h. Every hour the tubes were replenished with fresh O_2.

Histological procedures

In order to study effects of the different treatments on pattern formation, intact slugs were fixed in ice-cold methanol, embedded in paraplast and cut into serial sections of about 5 μm thickness. In the case of slugs migrating on dialysis membranes, slugs plus membranes were embedded to preserve the original orientation of the slugs. Slug sections were either stained with prespore-specific rabbit IgG (PSRI) and FITC-conjugated swine anti-rabbit IgG (SARFITC) or with 0·02 % Calcofluor in phosphate-buffered saline (freshly diluted before use from 1 % Calcofluor in ethanol).

To study effects on cell-type proportions, slugs were dissociated into single cells and small cell clumps by repeated aspiration through a 25-gauge needle. The cells were then allowed to adhere to glass slides, fixed in methanol and respectively stained with PSRI and SARFITC or with 0·02 % Calcofluor. Cells stained with prespore antiserum were counterstained with 0·2 μg ml^{-1} DAPI. Using a Leitz fluorescence microscope equipped with appropriate filters, the ratio of prespore cells (cell containing at least three FITC-stained vacuoles) versus total cells (cells stained with DAPI) was determined by counting. Calcofluor and DAPI fluorescence cannot be separated by filters; to determine the proportion of stalk cells, stalk cells were identified by Calcofluor fluorescence, while the total number of cells was measured by using the phase-contrast facility on the same microscope.

Transmission electron microscopy

Slugs were fixed in a mixture of 83 mM-glutaraldehyde, 26 mM-OsO_4 and 60 mM-sucrose in 100 mM sodium-cacodylate buffer pH 7·4. After postfixation in 1 % OsO_4, the slugs were dehydrated, embedded in Agar 100 epoxy resin, sectioned and observed with a Jeol CX 100 transmission electron microscope as described before (Schaap *et al.* 1982).

Results

Effects of DIF on cell differentiation in migrating and submerged slugs

We first studied the effects of DIF on migrating *D. discoideum* NC4 slugs. Early migrating slugs were transferred to agar which contained 3000 units ml^{-1} of DIF. After 6 h of migration, the slugs were fixed in methanol, embedded in paraffin and sectioned. Fig. 1A show a section of a DIF-treated slug, which was stained with prespore-specific antiserum. DIF treatment has induced the disappearance of prespore antigen in the region proximal to the DIF agar, but no formation of mature stalk cells were observed. Similar effects of DIF have also been found by Kay and co-workers (personal communication).

To obtain a more efficient penetration of DIF, intact slugs were submerged in oxygenated buffer (Sternfeld & Bonner, 1977) containing DIF (Fig. 1C). Submersion in buffer did not affect the anteroposterior pattern significantly in the absence of DIF (Fig. 1B). After 6 h of incubation with DIF, a general reduction of prespore antigen was observed (Fig. 1C). It must, however, be noted that the effects of DIF on prespore pattern as represented in Fig. 1A and C represent the more extreme examples of loss of prespore antigen.

To quantify the effects of DIF on prespore proportions, slugs were dissociated into single cells after DIF treatment. After 6 h of submersion in 10 000 units ml^{-1} DIF the proportion of prespore cells was reduced from 66 to 56 %, while 30 000 units ml^{-1} DIF caused a reduction to 38 %. At higher DIF concentrations, cell viability was affected. Prolonged incubation with DIF induced a somewhat further decrease in the proportion of prespore cells, but even after 10 h of DIF treatment, virtually no mature stalk cells could be detected (Table 1).

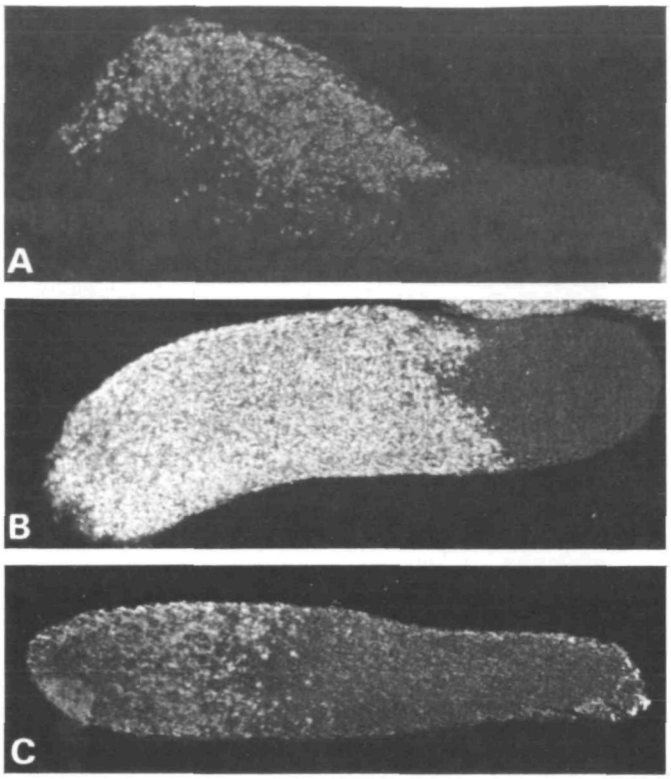

Fig. 1. Effects of DIF on slug pattern. (A) *D. discoideum* NC4 slugs, developed on dialysis membrane, were transferred with the membrane to agar that contained 3000 units ml^{-1} of DIF. After 6 h of migration, the slugs were sectioned and stained with prespore antiserum. DIF induced a loss of prespore antigen at the side of the slug, proximal to the DIF agar. Alternatively, slugs were submerged in phosphate buffer (B) or buffer containing 5000 units ml^{-1} DIF (C) and incubated in roller tubes for 6 h at 10 rev min^{-1} in an oxygen atmosphere. In control slugs, the prespore-staining pattern remained preserved under these conditions (B). In slugs submerged in DIF, a general decrease in prespore staining occurred. However, a complete loss of prespore antigen was never observed and neither could any differentiation of vacuolated stalk cells be detected (A: ×190; B: ×160; C: ×180).

Effect of elimination of possible DIF antagonists on pattern formation in slugs

It was previously reported that both cAMP and ammonia antagonize DIF-induced stalk cell differentiation *in vitro* (Gross *et al.* 1983; Berks & Kay, 1988). To investigate whether secretion of cAMP or ammonia by slug cells inhibits the induction of stalk cell differentiation by DIF *in vivo*, we depleted extracellular cAMP levels by treatment with cAMP-phosphodiesterase, while ammonia levels were depleted by means of a glutamate dehydrogenase mixture which use ammonia as a substrate (Schindler & Sussman, 1977). It was necessary to perform these experiments on slugs submerged in buffer, since application of the glutamate dehydrogenase mixture to migrating slugs was reported to rapidly induce the transition of migrating slugs into early fruiting structures (Schindler & Sussman, 1977).

Treatment of slugs with increasing amounts of cAMP-phosphodiesterase induced a progressive reduction of the proportion of prespore cells (Table 1) as was reported before (Wang *et al.* 1988), but no significant differentiation of stalk cells could be observed. The small amount of stalk cells, which appears after 10 h treatment with 15 000 units ml^{-1} DIF is not further increased by addition of cAMP-phosphodiesterase.

More striking results were obtained when slugs were incubated with DIF in combination with the glutamate dehydrogenase mix. Irregularly shaped clumps of stalk cells appeared within 6 h of incubation. After 10 h of incubation, DIF treatment (in the presence or absence or glutamate dehydrogenase mix) generally caused a severe disruption of slug morphology. In the presence of glutamate dehydrogenase large amounts of fully differentiated stalk cells had been formed (Fig. 2A–D), as was evident by Calcofluor staining (Harrington & Raper, 1968) and by ultrastructural characteristics as large vacuoles and a fibrous cell wall (Fig. 2E,F). As far as could be judged from the disrupted cell masses, the stalk cells tended to be formed preferentially at the periphery of the cell mass (Fig. 2A,B). Isolated small groups of mature stalk cells were also often observed (Fig. 2B,C).

Control experiments, in which cells were incubated

Table 1. *Cell-type proportions in submerged slugs after various treatments*

Addition	Stalk cells (%)	Prespore cells (%)
None	0·2 ± 0·7	61·8 ± 3·1
DIF (15 000 units ml^{-1})	2·8 ± 1·1	36·9 ± 3·0
Ammonia depletion	3·2 ± 0·2	60·2 ± 4·0
DIF+ammonia depletion	21·9 ± 7·3	36·7 ± 6·1
cAMP-PDE (0·05 units ml^{-1})	0·5 ± 0·7	40·6 ± 4·9
cAMP-PDE (0·25 units ml^{-1})	0·5	14·6
DIF+cAMP-PDE (0·05 units ml^{-1})	1·3 ± 1·8	39·7 ± 1·8
DIF+cAMP-PDE (0·25 units ml^{-1})	2·0	10·1

10–15 slugs were incubated in 400 μl PB containing the indicated amounts of DIF and beef-heart cAMP phosphodiesterase, or in 400 μl of 70 mM-α-ketoglutarate and 0·1 mM-NADH, in 10 mM-imidazole buffer pH 7·5 containing 0·03 units ml^{-1} L-glutamate dehydrogenase (ammonia depletion), 15 000 units ml^{-1} DIF, or a combination of both (DIF+ammonia depletion).

After 10 h of incubation, the slugs were dissociated into single cells and stained with prespore antiserum and Calcofluor. The proportion of prespore and stalk cells versus the total number of cells were determined by counting. With two exceptions (cAMP-PDE 0·25 units ml^{-1} ± DIF), the data represent the means and S.D. of two experiments. In each experiment, 600 cells were counted for each variable.

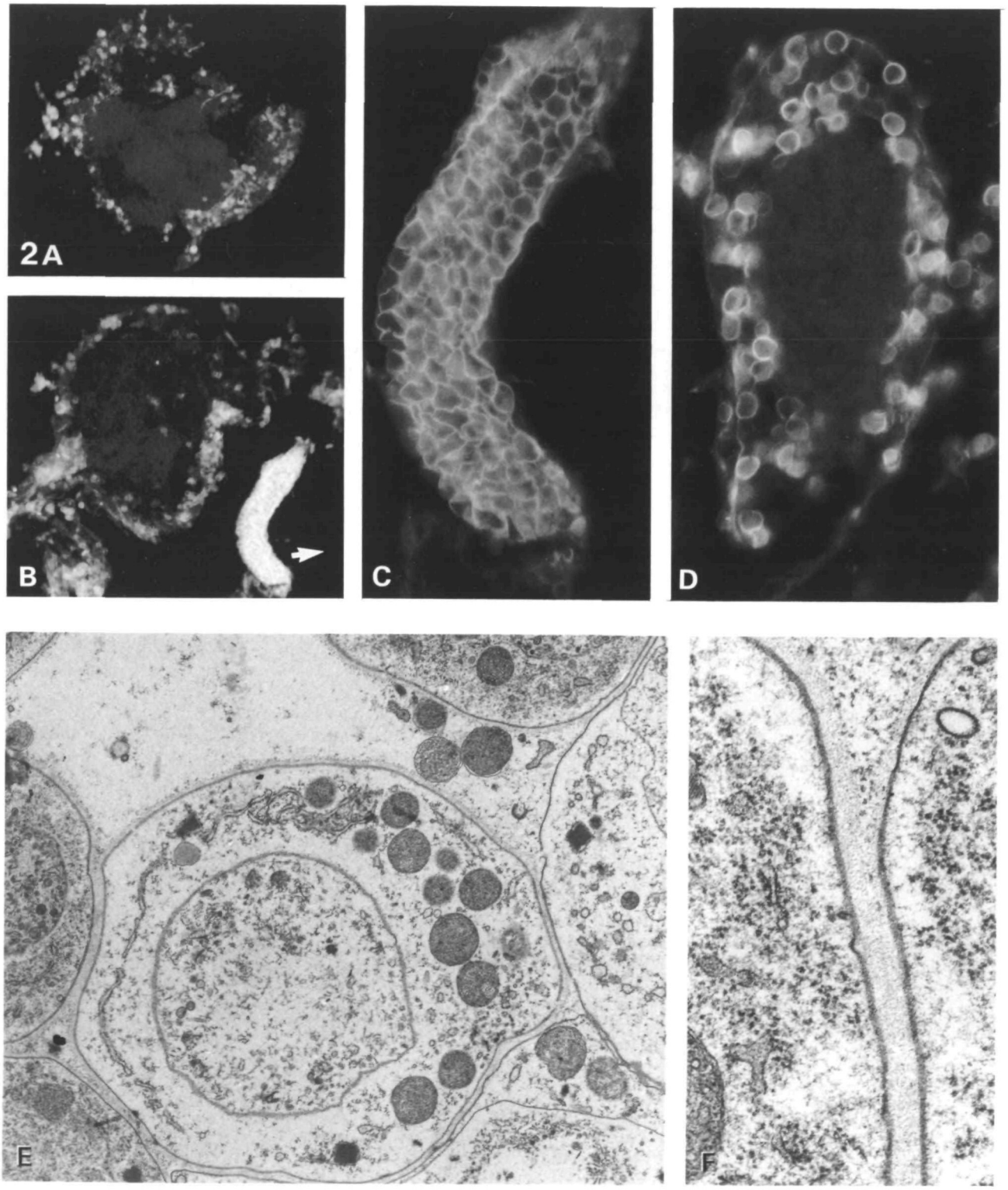

Fig. 2. Effects of DIF and ammonia depletion on slug pattern. Migrating slugs were submerged during 10 h in 400 μl of 70 mM-α-ketoglutarate, 0·1 mM-NADH, 0·03 units ml^{-1} L-glutamate dehydrogenase in 10 mM-imidazole buffer pH 7·5 to which 6000 units DIF were added. (A–D) Sections of methanol-fixed slugs stained with 0·02 % calcofluor (A,B: ×50; C: ×240; D: ×280). (E,F) TEM preparations of slugs fixed in glutaraldehyde and OsO_4, showing the extreme vacuolization and fibrous cell wall that is characteristic for mature stalk cells (E: ×13 000; F: ×52 000).

with DIF in combination with boiled glutamate dehydrogenase, or in which the substrate α-ketoglutarate was replaced by the product L-glutamate did not result in any significant differentiation of stalk cells.

Quantification of the effect of the different treatments on the proportion of stalk cells and prespore cells showed that ammonia depletion combined with DIF addition causes 22% of the cells to differentiate into stalk cells (Table 1). Both treatments alone yielded about 3% stalk cells. Ammonia depletion does not affect the proportion of prespore cells, while 15000 units ml^{-1} DIF reduces the proportion of prespore cells from 62 to 40% after 10 h of incubation.

Discussion

We investigated whether DIF can induce stalk cell differentiation *in vivo* and whether cAMP and ammonia function as DIF antagonists. It was found that DIF causes a moderate reduction in the proportion of prespore cells in migrating and submerged slugs, but causes only 2% of the cells to differentiate into stalk cells. In combination with enzymic depletion of ammonia, DIF induces stalk cell differentiation in 22% of the cells within 10 h of incubation. Enzymic depletion of ammonia in the absence of added DIF leads to stalk cell differentiation in about 3% of the cells, without affecting the proportion of prespore cells. Depletion of cAMP, another putative DIF antagonist, does not promote stalk cell differentiation, but results in the almost complete disappearance of prespore cells as was previously reported (Wang *et al.* 1988). Apparently, despite its antagonistic effects on stalk cell differentiation *in vitro* (Berks & Kay, 1988), extracellular cAMP is not critically involved in the regulation of this type of differentiation *in vivo*. Possibly, the cAMP levels in the prestalk region of the slug are not sufficiently high to inhibit stalk cell differentiation.

During normal development, DIF levels are maximal at the early slug stage (Brookman *et al.* 1982), but the synthesis of the majority of stalk-specific proteins does not start earlier than the culmination stage (Kopachik *et al.* 1985). Our data suggest that ammonia depletion during early culmination may trigger the differentiation of stalk cells during normal development.

A sudden drop in ammonia levels could be a simple consequence of the culmination process and the position of the tip region during culmination, as was earlier suggested by Sussman & Schindler (1978). As soon as the slug stops migrating and erects itself, the exchange of volatile compounds between the cell mass and the atmosphere becomes more efficient. The tip region, which is obviously at the best position to exchange volatile compounds is furthermore relatively narrow compared to the rest of the cell mass, which results in a relatively large surface-to-volume ratio. Loss of ammonia due to evaporation may therefore occur most efficiently at this region. The expression of two stalk-specific genes, *pdD*63 and *pDd*56, coincides with the increase in DIF levels during normal development (Williams, 1988). It is possible that the expression of these genes is less sensitive to inhibition by ammonia than the expression of the majority of stalk genes.

The process of stalk formation does not only involve the differentiation of stalk cells, but also the construction of a cellulose stalk tube at the centre of the prestalk region. (Raper & Fennell, 1952). It is as yet difficult to imagine that such a topologically complicated process could simply result from local ammonia depletion. It is likely that a drop in ammonia levels permits the transition of prestalk into stalk cells but that the actual formation of the stalk, is controlled by a more intricate regulatory mechanism.

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