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Cross-induction of cell types in *Dictyostelium*: evidence that DIF-1 is made by prespore cells

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SUMMARY

To investigate how cell type proportions are regulated during *Dictyostelium* development, we have attempted to find out which cell type produces DIF-1, a diffusible signal molecule inducing the differentiation of prestalk-O cells. DIF-1 is a chlorinated alkyl phenone that is synthesized from a C_{12} polyketide precursor by chlorination and methylation, with the final step catalysed by the *dmtA* methyltransferase. All our evidence points to the prespore cells as the major source of DIF-1. (1) *dmtA* mRNA and enzyme activity are greatly enriched in prespore compared with prestalk cells. The chlorinating activity is also somewhat prespore-enriched. (2) Expression of *dmtA* is induced by cyclic-AMP and this induction is inhibited by DIF-1. This regulatory behaviour is characteristic of prespore products. (3) Short-term labelling experiments, using the polyketide precursor, show that purified prespore cells produce DIF-1 at more than 20 times the rate of prestalk cells. (4) Although DIF-1 has little effect on its own synthesis in short-term labelling experiments, in long-term experiments, using $^{36}Cl^-$ as label, it is strongly inhibitory (IC_{50} about 5 nM), presumably because it represses expression of *dmtA*; this is again consistent with DIF-1 production by prespore cells. Inhibition takes about 1 hour to become effective.

We propose that prespore cells cross-induce the differentiation of prestalk-O cells by making DIF-1, and that this is one of the regulatory loops that sets the proportion of prespore-to-prestalk cells in the aggregate.

Key words: *Dictyostelium*, DIF-1, Proportioning mechanism, Cell differentiation

INTRODUCTION

In *Dictyostelium* development, cell-type proportioning is achieved by assigning uncommitted cells to the stalk or spore fates in the correct ratio. The proportioning mechanism can produce fruiting bodies composed of roughly 75% spores and 25% stalk cells, irrespective of the size of the aggregate, from 20 to 100,000 cells (Bonner and Slifkin, 1949; Williams et al., 1981). It can also partially or fully restore correct proportioning in isolated fragments of prestalk or prespore tissue (Raper, 1940) by inducing cell-type conversion (Sakai, 1973; Nadin et al., 2000; Rafols et al., 2000).

It is generally accepted that cell-type proportioning requires communication between prestalk and prespore cells (MacWilliams et al., 1985; Loomis, 1993; Gross, 1994; Schaap et al., 1996; Aubry and Firtel, 1999; Kessin, 2001), but understanding the nature of this communication is complicated because there are four subtypes of prestalk cell to consider, each of which presumably has its own induction conditions (Berks and Kay, 1990; Thompson and Kay, 2000). Prestalk-A, prestalk-O and prestalk-AB cells (pstA, pstO and pstAB cells) constitute the anterior prestalk zone (Jermyn et al., 1989; Jermyn and Williams, 1991; Jermyn et al., 1996; Early et al., 1993; Early, 1999), whereas anterior-like cells (ALCs) form a scattered population in the posterior prespore zone (Sternfeld and David, 1981; Devine and Loomis, 1985).

One signal molecule involved in stalk cell formation is a small chlorinated alkyl phenone called DIF-1 (Morris et al., 1987; Kay et al., 1999). It was discovered as an inducer of mature stalk cell differentiation in culture, but shown subsequently to induce differentiation of prestalk cells (Kopachik et al., 1983; Williams et al., 1987; Early et al., 1995) and repress differentiation of prespore cells (Kay and Jermyn, 1983; Early and Williams, 1988). Mutants that lack DIF-1 also lack pstO cells and have an increased proportion of prespore cells (Thompson and Kay, 2000); surprisingly, pstA cells seem unaffected, suggesting that they have a separate inducer from DIF-1. Our working hypothesis is therefore that DIF-1 induces the differentiation of pstO cells in normal development and that DIF-1 levels in the aggregate regulate the ratio of pstO to prespore cells.

In order to understand the logic of the DIF-1 regulatory system, it is essential to know which cell type produces DIF-1. Originally, DIF-1 was viewed as a candidate for the activator of prestalk cell differentiation in a Gierer-Meinhardt reaction-diffusion scheme (Geirer and Meinhardt, 1972), which would imply production by prestalk cells (Gross et al., 1981). Later schemes had DIF-1 as a consumed substrate, which was made by all cells, but converted into the true activator by prestalk cells (Meinhardt, 1983); as an inhibitor of anterior-like cell formation, possibly made by these cells (MacWilliams et al., 1985); or as being produced by prespore cells, resulting in the cross-induction of prestalk cells (Loomis, 1993; Kay et al.,

1999); or they did not to specify which cells made DIF-1, because the location of its breakdown was considered to be more important than its source (Schaap et al., 1996).

Experimental attempts to determine which cells make DIF-1 have produced contradictory results. One approach has been to measure DIF-1 production by separated prestalk and prespore cells, using a bioassay. This suggested that DIF-1 is either made by pstAB cells (Kwong et al., 1990) or (using a different bioassay) by prespore cells (Inouye, 1989). Alternatively, micro-dissection experiments, where DIF-1 is extracted and assayed from prestalk and prespore fragments of migrating slugs, show that the highest level of DIF-1 is in the prespore zone, suggesting that it is made by prespore cells (Brookman et al., 1987). These experiments have been limited by the sensitivity of the bioassays and by lack of any knowledge of how DIF-1 is biosynthesized. However, recent progress in this area has made new tools available.

DIF-1 appears to be synthesized from a 12-carbon polyketide skeleton, which is decorated by chlorination and methylation (Fig. 1) (Kay, 1998). The polyketide synthase producing the DIF-1 precursor has not yet been identified, but the enzymes carrying out the last two steps of the biosynthetic pathway can be detected in cell lysates: the chlorinating enzyme is particulate and uses hydrogen peroxide as oxidant, whereas the methylating enzyme, des-methyl DIF-1 methyltransferase, is soluble and uses S-adenosyl methionine as methyl donor (Kay, 1998). The des-methyl DIF-1 methyltransferase gene, *dmtA*, has been identified and knocked out by homologous recombination (Thompson and Kay, 2000). Detectable des-methyl DIF-1 methyltransferase activity is abolished in the mutant and it has less than 1% of wild-type levels of DIF-1, proving that DmtA does catalyse the last step in DIF-1 biosynthesis. Using the tools made available by this work, all our evidence suggests that DIF-1 is made largely by prespore cells.

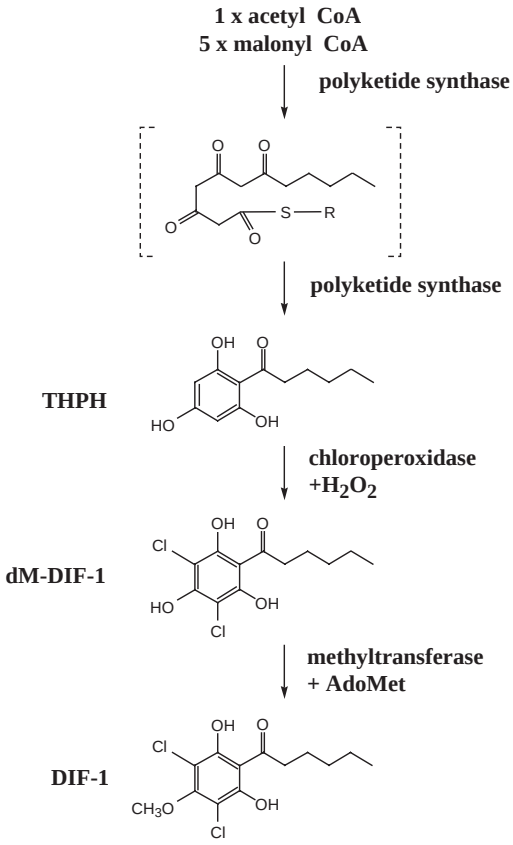

Fig. 1. Outline of the DIF-1 biosynthetic pathway. The C_{12} skeleton of DIF-1 is proposed to be assembled by a polyketide synthase and then modified, first by chlorination and then by methylation, to give DIF-1 (Kay, 1998). The methylation step is carried out by the methyltransferase encoded by the *dmtA* gene (Thompson and Kay, 2000).

MATERIALS AND METHODS

DIF-related chemicals

DIF-1 and related compounds were synthesized as described (Masento et al., 1988; Morandini et al., 1995). [^{3}H]THPH (the polyketide precursor of DIF-1, labelled in its alkyl side chain: 2,4,6-trihydroxy-1-hexan[3,4-^{3}H]-1-one) was prepared by reductive tritiation of 2,4,6-trihydroxyphenyl-1-hex-1-one-3-ene (Amersham custom synthesis) and the products further purified by HPLC using a C18 column.

Cell growth and labelling

Cells were grown and developed at 22°C. Strain Ax2 was grown in axenic medium with shaking (Watts and Ashworth, 1970), strain V12M2 was grown on nutrient plates in association with *Klebsiella aerogenes* and washed free of bacteria in KK2 (20 mM $K_1K_2PO_4$, 2 mM $MgSO_4$, pH 6.2) before use (Kay, 1998). Slugs were produced by streaking 10^7 cells on to 1.8% L28 agar (Oxoid) containing 10% NS (100% is 20 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$) and allowed to develop in unilateral light. They were dissected using a sharpened insect needle and fragments were accumulated in ice-cold KK2 before freezing.

Prestalk and prespore cells were purified from 40 hours V12M2 slugs, after pronase/2,3-di-mercapto-propanol disaggregation, by Percoll gradient centrifugation (Ratner and Borth, 1983), with prespore cells re-purified on a second Percoll gradient. The purity of the fractions was monitored by staining with an antibody against prespore cells (Hayashi and Takeuchi, 1976). All steps were at 4°C, except for the disaggregation, which was for 5 minutes at 22°C. Separated cells were washed twice by centrifugation and resuspended in 10 mM MES pH 6.2/NS. After 10 minutes equilibration at 22°C, cell suspensions at 10^7 per ml were incubated at 22°C with [^{3}H]THPH (1.9 nM and 110,000 cpm/ml) and 40 μM ancymidol (an inhibitor of DIF-3 breakdown) in a final volume of 0.75-1 ml. At the indicated times, the reaction was terminated by adding an equal volume of stop solution (90/2 ethyl acetate/acetic acid, containing 0.05 mg/ml butylated hydroxytoluene and 0.25 mg/ml tocopherol). The aqueous phase was extracted twice more with ethyl acetate and the combined organic phases dried down and analysed by HPLC using a 25 cm Sperisorb S5ODS 2 column (solvent A=2% acetic acid; B=2% acetic acid/methanol; gradient: 65-71% B in 36 minutes; 71-91% B in 30 minutes; 1 ml/minute). Internal standards were included to identify the radioactive peaks. The cpm in each fraction was determined by scintillation counting and that in the peaks corrected by subtraction of the background given by adjacent fractions of the HPLC. Fractional chlorination of THPH was calculated from the cpm in each peak fraction: (Cl-THPH + 2×(dM-DIF-1+DIF-1+DIF-3)) divided by the cpm in (THPH+Cl-THPH+dM-DIF-1+DIF-1+DIF-3). This allows for the double chlorination of dM-DIF-1 and DIF-1 and for the derivation of DIF-3 from DIF-1. Fractional methylation of THPH was calculated in a similar way.

V12M2 cells were labelled with $^{36}Cl^-$ in submerged monolayers (Kay, 1998). At different times, the medium was taken off and non-polar compounds extracted using a C18 SepPak cartridge (Waters). Labelled DIF-1 was eluted with methanol, resolved by TLC and quantitated using a Phosphorimager. Plates were exposed for 2-6 days in a lead safe, to

reduce background, and signal converted to cpm (after subtracting background of the TLC plate) by comparison with standards spotted onto the plate. Recovery of DIF-1 during work-up of the samples was monitored using [^{3}H]-DIF-1 in some experiments and averaged 45%.

In situ hybridization and *dmtA* reporter construct

In situ hybridization was as described previously (Escalante and Loomis, 1995), except that a Riboprobe from *dmtA* cDNA was used. Specimens were bulk harvested at different stages of development and fixed with methanol. The green fluorescent protein reporter construct, plasmid pCT7, was constructed by inserting 2.5 kb of genomic sequence upstream of the *dmtA*-coding region (Thompson and Kay, 2000) into *Bgl*II/*Bam*HI digested plasmid 63-GFP. The first six amino acids from *dmtA* are fused in-frame with green fluorescent protein GFP.

Enzyme assays

Lysates were made by freeze/thawing. Desmethyl DIF-1 methyltransferase and DIF-1 dechlorinase were assayed in the high-speed supernatant as before (Kay, 1998; Nayler et al., 1992). A modified assay for the chlorinating enzyme (Kay, 1998) was used employing as substrate [^{3}H]THPH. Lysates were prepared in 50 mM $K_1K_2PO_4$ pH 7.5, 5 mM KCl, 2 mM $MgSO_4$, 10% glycerol, 1 mM dTT, 1× protease inhibitors (1000× is 5 mg/ml leupeptin, 2.5 mg/ml pepstatin, 150 mg/ml benzaminide). Each 25 μl assay contained 1 μM [^{3}H]THPH (0.1 μCi per assay) and 10 mM H_2O_2. After incubation at 25°C for 20 minutes, the reaction was terminated by adding 50 μl of stop (90/10/2 ethyl acetate/hexane/acetic acid, containing 5 mg/ml butylated hydroxytoluene and 1 mg/ml tocopherol), and the labelled compounds resolved by TLC using Whatman LK6D plates, developed with CH_2Cl_2, di-isopropyl ether, acetic acid (85/15/2). The solvent front was run for 15 cm, and the plate dried and developed a second time using the same solvent. Labelled bands were visualized by autoradiography after spraying with 3H-Enhance (NEN), scraped into scintillation vials and quantitated by scintillation counting.

General details

Protein was assayed using the BioRad dye-binding assay with bovine serum albumin as the standard. Extraction of RNA and northern blots were performed as previously described (Kay et al., 1993) using ^{32}P-labelled probes and a Phosphorimager for quantitation.

RESULTS

Cell-type location of the biosynthetic capacity for DIF-1

The final enzyme in DIF-1 biosynthesis is encoded by the *dmtA* gene, which is expressed at low levels during late aggregation and much more strongly in the slug (Kay, 1998; Thompson and Kay, 2000). In situ hybridization of *dmtA* mRNA shows that early mounds have a low, even level of staining, which is difficult to distinguish from background. By the tight mound stage, as the tip is forming, the prespore zone is strongly stained but there is little if any staining in the prestalk zone (Fig. 2). This prespore staining persists through the later developmental stages.

An interesting feature of these experiments is that the expression pattern is often graded within the prespore zone of the slug, with the highest level in the anterior. In this respect it resembles certain promoter constructs of the SP60 gene (Haberstroh and Firtel, 1990; Balint-Kurti et al., 1998) but differs from the uniform expression of the *cotB* and D19/psA prespore mRNAs (Escalante and Loomis, 1995; Maeda et al., 2000).

Although these experiments show that DmtA mRNA is preferentially expressed in prespore cells, they do not show whether it is also expressed in anterior-like cells, which are a minor population of prestalk cells scattered in the prespore zone. To investigate this possibility, we made a *dmtA* promoter fusion to green fluorescent protein (pCT7, carrying 2.5 kb of *dmtA* upstream sequence). As expected, transformants specifically express GFP in the prespore zone, from the tipped mound stage onwards (not shown). However, a non-staining population of cells could also be discerned within the prespore zone from migrating slugs. After manual dissection and disaggregation of the prespore zone, 87% of cells expressed GFP strongly and were clearly distinguishable from 13% that did not (using a strongly expressing clone, HM2121). This proportion of non-expressing cells in the prespore zone corresponds to the expected proportion of anterior-like cells (Sternfeld and David, 1982). As the GFP marker is relatively stable, it can be followed into the mature fruiting bodies. Squashes show expression in the spores, but not in the upper or lower cups [which derive from the ALCs (Sternfeld and David, 1982)] or in the mature stalk cells (Fig. 3). These results show that *dmtA* is much more strongly expressed in prespore cells than in anterior prestalk or ALC cells.

To quantify the degree of enrichment of *dmtA* mRNA in prespore cells, slugs of strain V12M2 were dissected into approximately one-third anterior and two-thirds posterior pieces, RNA extracted and analysed by northern blotting (Table 1). This gave a 5.7-fold enrichment in the prespore zone, a little less than that found for the PsA mRNA in exactly comparable experiments (Kay et al., 1993). This degree of enrichment is a minimum value, as it is impossible to avoid cross-contamination of the prestalk and prespore zones in these dissection experiments.

Although *dmtA* mRNA is highly enriched in prespore cells, it might be argued that this is misleading because unknown post-transcriptional or post-translation controls result in much less enzyme activity being present in prespore than in prestalk cells. We therefore measured enzyme activity directly in lysates prepared from dissected prestalk and prespore zones of slugs. Table 1 shows that DmtA methyltransferase specific activity is 9.7-fold higher in lysates from the prespore zone compared with the prestalk zone and is as strongly prespore-enriched as a standard prespore marker, psA/D19 mRNA (Barklis and Lodish, 1983). The chlorinating enzyme was also assayed in the lysates, by measuring the conversion of [^{3}H]THPH into mono- and di-chlorinated THPH (see Materials and Methods). It too was enriched in the prespore zone, though to a lesser extent than

Table 1. Distribution of DIF-1 biosynthetic activity in the slug

Marker	*n*	Anterior:posterior ratio	Cells labelled	Reference
dmtA mRNA	3	1:5.7		This work
DmtA methylase enzyme	4	1:9.7		This work
Chlorinating enzyme	4	1:4.2		This work
DIF-1 dechlorinase	3	11.5:1	Prestalk cells	This work
DIF-1 dechlorinase		12.9:1	Prestalk cells	Kay et al., 1993
ecmA mRNA		4.4:1	Prestalk cells	Kay et al., 1993
PsA/D19 mRNA		1:8.6	Prespore cells	Kay et al., 1993

Migrating slugs of strain V12M2 were cut into approximately one-third anterior and two-thirds posterior pieces, and the specific activity of each marker determined in these pieces, relative to protein levels.

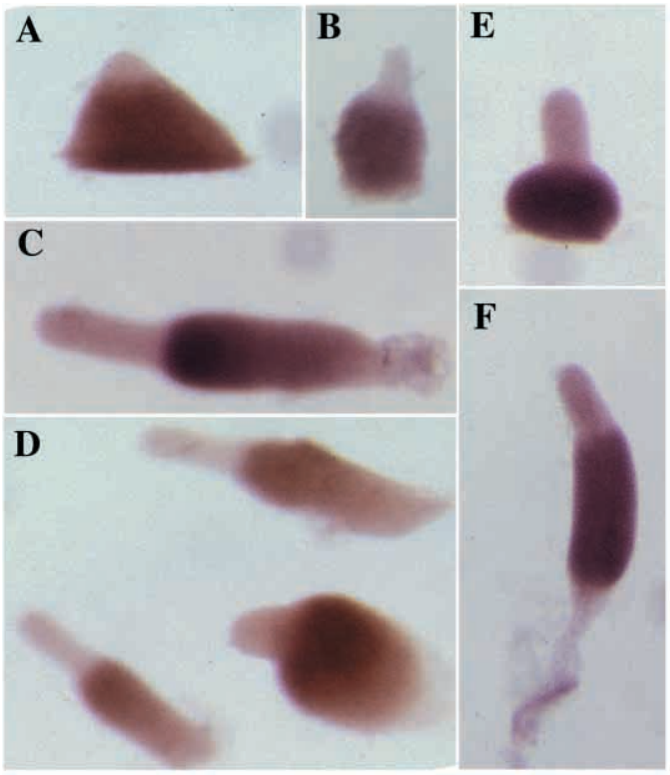

Fig. 2. Distribution of *dmtA* mRNA in developing structures of strain Ax2. This mRNA encodes the methyltransferase catalysing the last step in DIF-1 biosynthesis and is clearly prespore specific from the time when a specific signal can be first detected. (A) Mound just before tip formation; (B) tipped mound; (C,D) slugs (a graded distribution of *dmtA* mRNA is apparent in the prespore zone); (E) Mexican hat; (F) early culminant.

the methylating enzyme. DIF-1 dechlorinase was measured as a prestalk marker and for comparison with earlier work (Kay et al., 1993); its anterior enrichment was confirmed.

Regulation of the expression of the DIF-1 biosynthetic enzymes

Expression of all tested prespore products can be induced by cAMP and this induction is invariably inhibited by DIF-1. The behaviour of prestalk products is more varied: many are induced by DIF-1, but there are some exceptions, and cAMP can be either stimulatory or inhibitory, depending on the gene in question (Kay et al., 1999). To test the response of the DIF-1 biosynthetic enzymes, cells were first pulsed with low levels of cAMP to bring them to a responsive state and then the effects of high, constant cAMP levels and of DIF-1 were determined. Fig. 4 shows that *dmtA* mRNA, methyltransferase enzyme activity and the chlorinating activity are all stimulated by cAMP addition but are little affected by DIF-1 alone. When both compounds are added together, DIF-1 strongly represses the induction of *dmtA* expression by cAMP and to a lesser extent, the induction of chlorinating activity.

DIF-1 synthesis by separated prestalk and prespore cells

Although the DmtA methyltransferase activity is enriched in prespore cells, it could still be argued that this activity is not

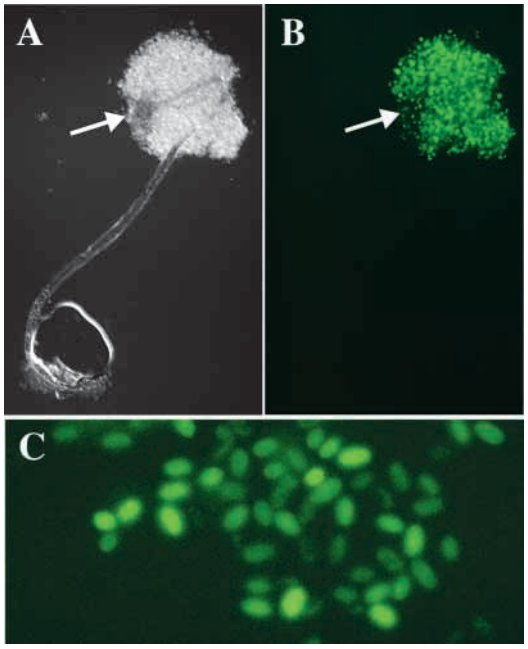

Fig. 3. Expression pattern of a green fluorescent protein reporter driven by the *dmtA* promoter. (A,B) Fruiting body squash, in phase contrast (A) and fluorescence (B), the upper cup is arrowed (it derives from AL cells); (C) mature spores.

used to make DIF-1 in vivo, owing to a level of control only effective in living cells. An example might be an allosteric inhibitor, which is diluted out or lost when cell lysates are assayed. To address this possibility, we examined DIF-1 synthesis in living cells.

Prestalk and prespore cells of strain V12M2 were separated by density-gradient centrifugation, with all steps at 4°C (except disaggregation for 5 minutes at 22°C), to minimize re-differentiation of the cells. For maximum sensitivity, DIF-1 synthesis was measured by the use of the labelled polyketide precursor [^{3}H]THPH, with the labelled products resolved by HPLC (Fig. 5). THPH was successively monochlorinated, then dichlorinated and methylated by the cells to make DIF-1. DIF-1 was then metabolized, to give DIF-3, though further metabolism was inhibited by using the cytochrome P450 inhibitor, ancymidol (Morandini et al., 1995).

Fig. 5 shows qualitatively that prespore cells make DIF-1 from THPH much more efficiently than do prestalk cells. Though prestalk cells have some capacity to perform the chlorination reactions, they are largely deficient in methylation capacity. This is quantitated in Fig. 6A,B: the prespore fraction performs chlorination of THPH at four times the rate of the prestalk fraction and methylation (that is DIF-1 production) at nearly 20 times the rate. Similar results were obtained in a second experiment, analysed in the same way (prespores performed chlorination at 2.8 times and methylation at 35 times the rate of prestalk cells) and in three others analysed by TLC (not shown). When allowance is made for cross-contamination of the prestalk and prespore fractions, methylation (and DIF-1 production) by prestalk cells would be undetectable.

This assay system was also used to check for other regulatory effects on DIF-1 synthesis. In short-term incubations (5-20 minutes), 1 mM cAMP did not influence the synthesis of DIF-

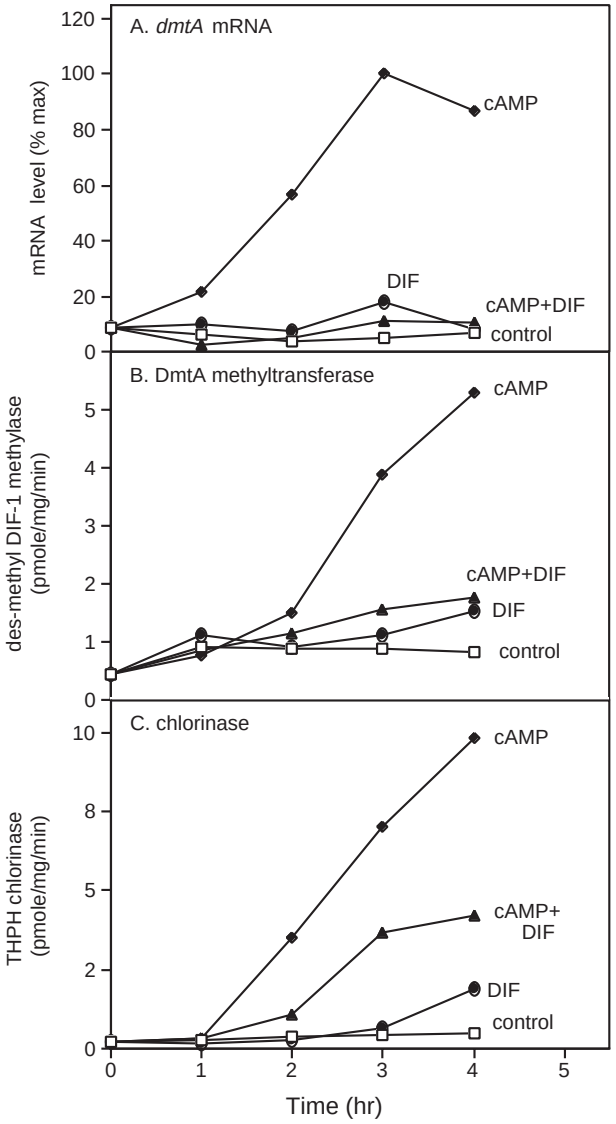

Fig. 4. Regulation of DIF-1 synthetic capacity by cAMP and DIF-1. Cells of strain Ax2 were starved in shaken suspension at 2×10^7/ml in KK2 buffer and after the first hour, pulsed with 50 nM cAMP every 6 minutes for a further 6 hours. These cells were subdivided, 4 mM cAMP and 100 nM DIF-1 added as indicated, and samples taken for northern analysis and enzyme assays. A second experiment gave a similar result.

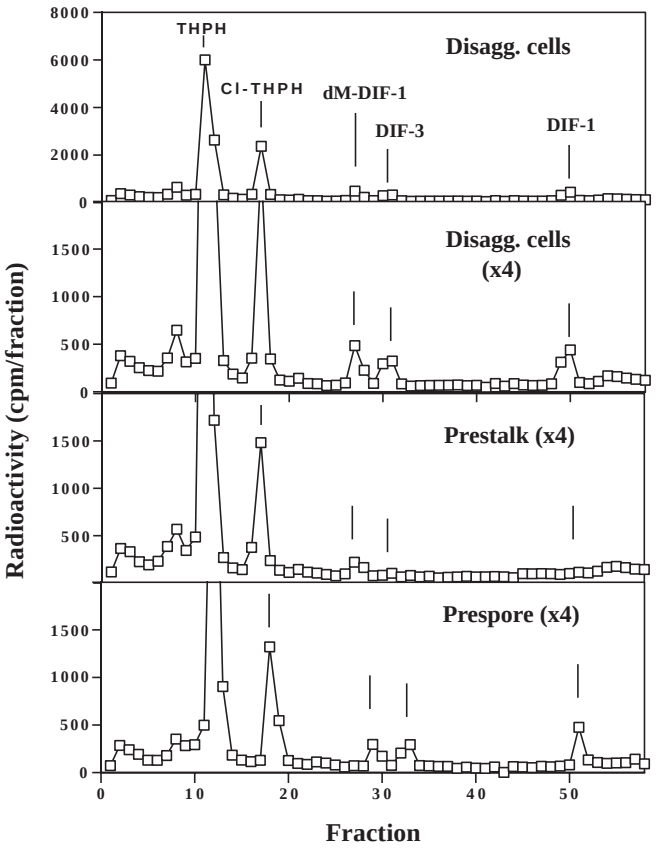

Fig. 5. Synthesis of DIF-1 made from the labelled polyketide, [^{3}H]THPH, by prestalk and prespore cells. The products made from [^{3}H]THPH by disaggregated slug cells (top two panels) or the prestalk and prespore fractions purified from them, were analysed by HPLC. THPH is successively converted to Cl-THPH, dM-DIF-1, DIF-1 and then to DIF-3, all of which are resolved (the unlabelled peaks preceding THPH are either impurities or produced in the work up). It can be seen that the prestalk fraction is capable of chlorinating THPH to Cl-THPH and dM-DIF-1, but methylation to give DIF-1/3 is barely detectable. By contrast, prespore cells and the starting mixture of cells (Disagg. cells) can perform all of these reactions. Cells were incubated at 10^7/ml in 1 ml NS/MES containing 1.9 nM [^{3}H]THPH and 40 μM ancymidol (to inhibit breakdown of DIF-3). After 5 or 10 minutes at 22°C, labelled compounds were extracted, concentrated and resolved by HPLC, together with unlabelled standards. Each sample was analysed in duplicate.

1 from [^{3}H]THPH (not shown). DIF-1 caused more labelled DIF-1 to accumulate, but this was at the expense of DIF-3, and there was no major change in the chlorination or methylation rates (Fig. 6C); it seems that the effect of DIF-1 was simply to protect the labelled DIF-1 from breakdown to DIF-3. By contrast, DIF-3 strongly inhibited DIF-1 formation by blocking the chlorination reaction (IC_{50} about 20 nM DIF-3, not shown) in both prestalk and prespore cells. In cell lysates, DIF-3 also inhibited the chlorination of THPH, suggesting a direct effect on the chlorinating enzyme (not shown).

A final test

It is predicted that, if DIF-1 is a prespore product, then it should inhibit its own synthesis in longer-term experiments, where there is time for it to repress expression of its biosynthetic machinery. For these experiments cells were developed as monolayers with cAMP and labelled with $^{36}Cl^-$, so that DIF-1 production would depend on endogenous production of the polyketide precursor, as it does in normal development. Labelled DIF-1 becomes detectable in the medium at 8 hours and reaches a peak at around 12 hours of 16.8±13.6 pmole/10^8 cells, n=5, which corresponds to a concentration in the medium of about 0.7 nM (corrected for losses during work-up, determined using [^{3}H]DIF-1). Fig. 7 shows that DIF-1, added at the start of development, strongly inhibits the production of labelled DIF-1 (and its metabolites DIF-3 and DM3) at 12 hours. Half-maximal inhibition requires 4.7 nM DIF-1 (mean of three experiments; range 1-10 nM), which may be an underestimate, because as much as 50% of 10 nM DIF-1 is

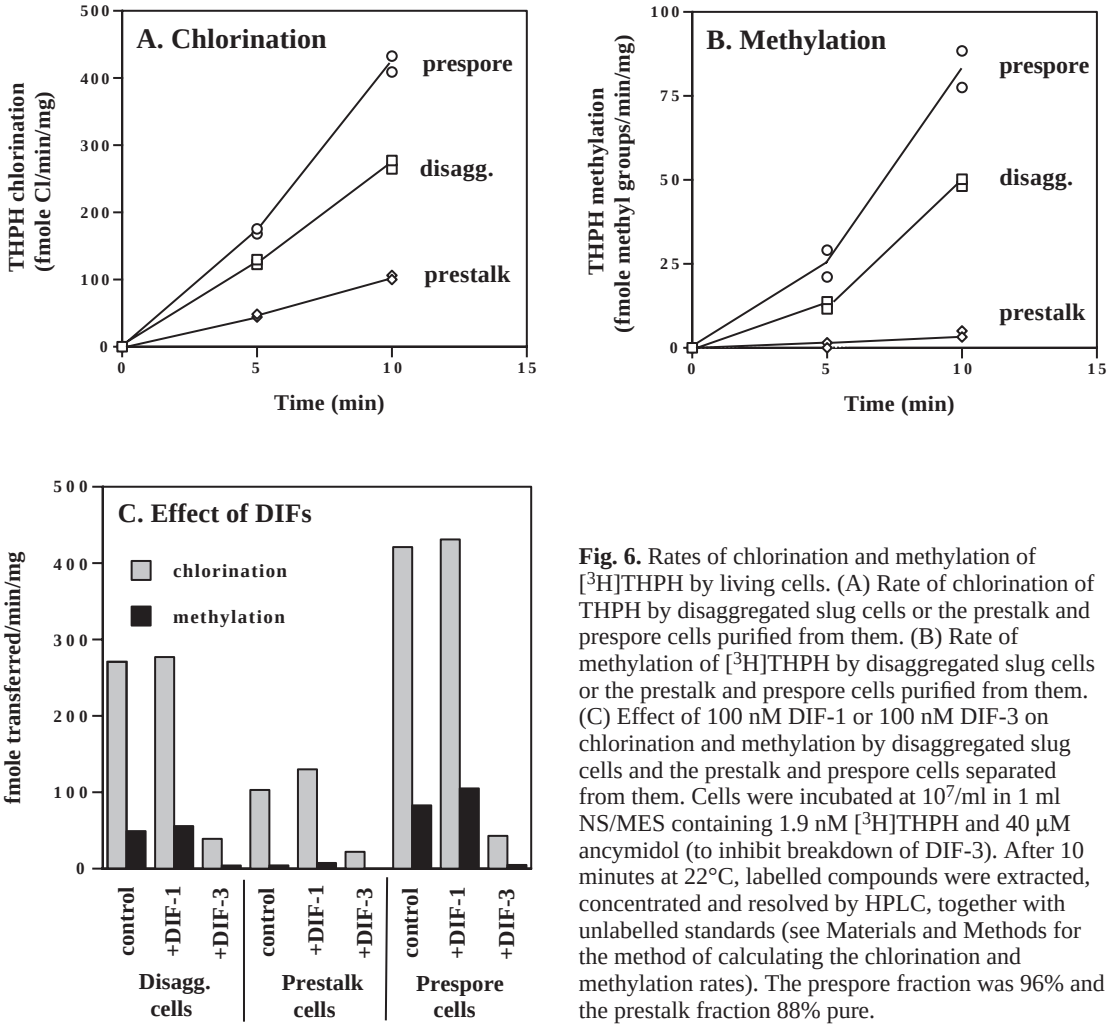

Fig. 6. Rates of chlorination and methylation of [^{3}H]THPH by living cells. (A) Rate of chlorination of THPH by disaggregated slug cells or the prestalk and prespore cells purified from them. (B) Rate of methylation of [^{3}H]THPH by disaggregated slug cells or the prestalk and prespore cells purified from them. (C) Effect of 100 nM DIF-1 or 100 nM DIF-3 on chlorination and methylation by disaggregated slug cells and the prestalk and prespore cells separated from them. Cells were incubated at 10^7/ml in 1 ml NS/MES containing 1.9 nM [^{3}H]THPH and 40 μM ancymidol (to inhibit breakdown of DIF-3). After 10 minutes at 22°C, labelled compounds were extracted, concentrated and resolved by HPLC, together with unlabelled standards (see Materials and Methods for the method of calculating the chlorination and methylation rates). The prespore fraction was 96% and the prestalk fraction 88% pure.

broken down during the incubation (monitored with [^{3}H]DIF-1). DIF-2, a homologue of DIF-1 with one fewer carbon atom in the alkyl side chain, and DIF-3 both inhibited DIF-1 synthesis in these experiments, whereas DM3, the third metabolite produced from DIF-1 (Traynor and Kay, 1991) is without effect, or slightly stimulatory.

To investigate how quickly DIF-1 inhibits its own synthesis, cells were developed for 12 hours with medium containing $^{36}Cl^-$ (to equilibrate the pools) and then fresh medium added, with or without DIF-1 (and with fresh $^{36}Cl^-$). In these circumstances, DIF-1 continues to accumulate at the control rate for the first hour after addition of 40 nM DIF-1, but then accumulation essentially ceases (Fig. 8).

DISCUSSION

The proportion of stalk to spore cells formed during *Dictyostelium* development is set by a strong homeostatic mechanism, which operates over a wide range of cell numbers and can restore proportions in isolated pieces of prestalk or prespore tissue. DIF-1 levels rise strongly in development at the time of prestalk and prespore cell differentiation (Brookman et al., 1982; Sobolewski et al., 1983) and it is our working assumption that DIF-1 levels within the aggregate control the proportion of pstO cells that differentiate. As a step towards understanding the cell-proportioning mechanism, it is important to identify the cell type making DIF-1.

We showed first that the final enzyme of DIF-1 biosynthesis, encoded by the *dmtA* gene, is as strongly enriched in prespore cells as a standard prespore marker and that its expression is induced by cAMP and repressed by DIF-1, as expected of a prespore product. The chlorinating enzyme is less highly enriched in prespore cells than DmtA, suggesting that it must also be expressed in prestalk cells, though at a lower level than in prespores.

Using a sensitive labelling technique, we found that separated prespore cells make DIF-1 at more than 20 times the rate of prestalk cells, over incubation times of 5-20 minutes. Prestalk cells chlorinated the polyketide precursor with reasonable efficiency, but are almost completely deficient in the final methylation step that produces DIF-1, as expected from the distribution of chlorinating and methylating enzyme activity between the two cell types.

Finally, we used metabolic labelling with $^{36}Cl^-$ (where DIF-1 production depends on endogenous polyketide synthesis) to show that DIF-1 inhibits its own synthesis, after a delay of about 1 hour. This is as expected if DIF-1 is a prespore product

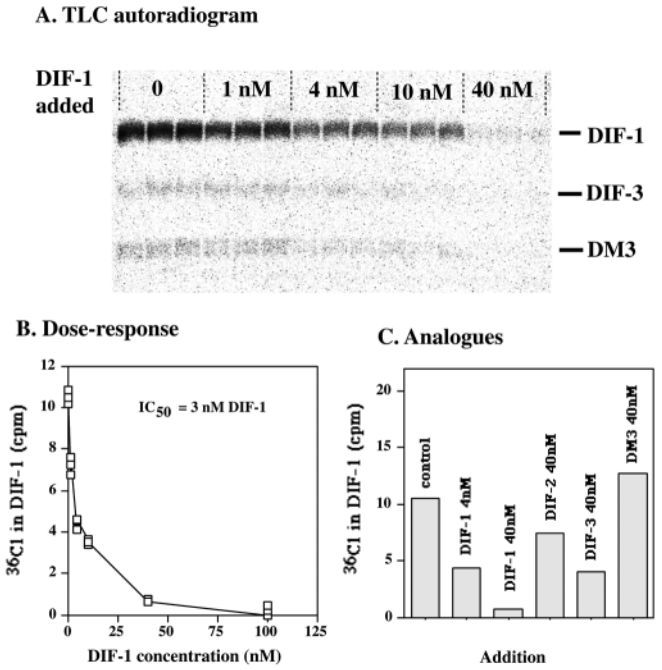

Fig. 7. The regulation of DIF-1 production by DIF in living cells. (A) TLC showing the inhibition of DIF-1 synthesis by DIF-1. Accumulation of the DIF-1 metabolites, DIF-3 and DM3, is inhibited in parallel with DIF-1. (B) Quantitation of the results shown in A; (C) effect of compounds related to DIF-1 on DIF-1 synthesis. All these compounds are produced during development and therefore are potential regulators of DIF-1 synthesis: DIF-2 is a homologue of DIF-1 with one fewer carbon atom in the alkyl side chain, DIF-3 is produced from DIF-1 by a single dechlorination; DM3 is produced from DIF-3 by an oxidation of the side chain. Cells of strain V12M2 were incubated for 12 hour in submerged culture with cAMP and $^{36}Cl^-$. DIF-1 or related compounds were added at the start of the experiment, as indicated. Labelled compounds were extracted from cells and media, resolved by TLC and quantitated using a Phosphorimager. Data are typical of three experiments.

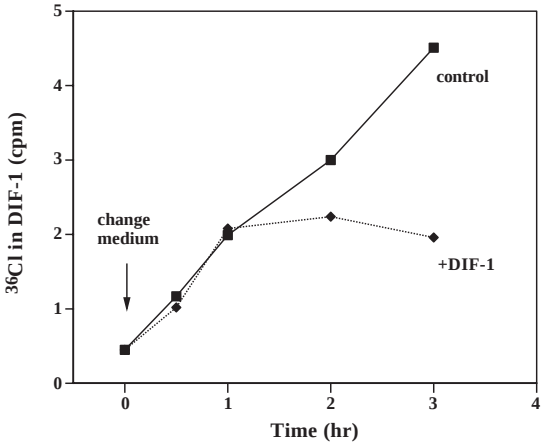

Fig. 8. Speed of inhibition of DIF-1 synthesis by DIF-1 in living cells. Cells of strain V12M2 were incubated in submerged culture with cAMP and $^{36}Cl^-$. At 12 hours, fresh medium (still containing $^{36}Cl^-$) was substituted, with 100 nM DIF-1 added as indicated. Labelled compounds were extracted from cells and media, resolved by TLC and quantitated using a Phosphorimager. Data are typical of three experiments.

and could largely be explained by repression of *dmtA* gene expression by DIF-1.

In previous work, gradient-separated prestalk cells were found to make two to three times more DIF-1 than prespores (Kwong et al., 1990). Only the relatively insensitive bioassay was available to measure DIF-1 production, and so incubations of 4-24 hours were required to produce detectable DIF-1. However, by 2 hours of incubation, the prestalk cell population had re-differentiated to express prespore markers at levels comparable with the original prespore population; thus the cell-type actually making remains DIF uncertain in these experiments. By contrast, cell re-differentiation has been avoided in our experiments by using incubations of as little as 5 minutes, which is too short a time to allow any major changes in gene expression.

The different approaches that we have used all concur and we therefore conclude that DIF-1 is predominantly made by prespore cells, in accordance with its enrichment in the prespore zone of the slug (Brookman et al., 1987).

A minor production of DIF-1 by non-prespore cells cannot be precluded from our results. Indeed this is likely in early development, when low levels of DIF-1 and *dmtA* mRNA are made before prespore cells are thought to have differentiated (Brookman et al., 1982; Sobolewski et al., 1983; Kay, 1998; Thompson and Kay, 2000). Thus *dmtA* may be expressed at a low level in all cells early in development, before later switching to the observed prespore specificity.

These results suggest a simple and robust mechanism for cell-type proportioning: prespore cells make DIF-1, and so cross-induce the differentiation of pstO cells. If there are too many prespore cells, DIF-1 levels will rise, favouring recruitment of pstO cells; if there are too few, DIF-1 levels will fall, favouring recruitment of prespore cells. An additional feedback mechanism, working in the same direction, is the inactivation of DIF-1 by prestalk cells, owing to their possession of the DIF-1 dechlorinase enzyme (Insall et al., 1992; Kay et al., 1993).

Finally, two unexpected observations deserve comment. First, we noticed that expression of *dmtA* is often graded in the prespore zone of the slug, with the highest level at the front. Such a graded expression pattern has only been described previously for certain prespore promoter constructs, and not for a natural mRNA (Haberstroh and Firtel, 1990; Balint-Kurti et al., 1998). As there is some evidence that DmtA is rate-limiting in converting the polyketide THPH to DIF-1 (Kay, 1998), this observation hints that DIF-1 production itself may be graded in the prespore zone. If such a gradient exists in synthetic capacity, it might help to explain why the front of the prespore zone has the greatest propensity to regenerate a prestalk zone in regulation experiments (Durston, 1976; MacWilliams, 1982; Lokeshwar and Nanjundiah, 1983). Second, we found that DIF-3 inhibits DIF-1 synthesis in living cells, apparently by inhibiting the chlorination of THPH. The enzyme making DIF-3 is found at high levels at the front of the slug (Kay et al., 1993) and it is therefore possible that the DIF-3 produced here helps to repress any residual DIF-1 synthesis in the prestalk zone.

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