Prestalk cells in monolayer cultures exhibit two distinct modes of cellulose synthesis during stalk cell differentiation in *Dictyostelium*

R. L. Blanton
Department of Biological Sciences, Box 43131, Texas Tech University, Lubbock, Texas 79409-3131, USA

SUMMARY

Stalk formation in *Dictyostelium discoideum* involves the synthesis of a stalk tube by the prestalk cell population and stalk cell walls by the individual prestalk cells. Cellulose is a major structural component of the stalk tube and stalk cell walls. The DIF-deficient strain HM44 was used to study the events of stalk formation in monolayer cultures. The induction of cellulose synthase activity was shown to require both DIF and cAMP. Microscopical observations of monolayer cultures using the cellulose-indicating fluorochrome Tinopal LPW demonstrated the presence in these cultures of two cellulose-containing materials: the stalk cell walls and an intercellular material found between cells and around cell clumps. The synthesis of intercellular material precedes that of stalk cell walls in induced cultures. Cells committed to stalk cell formation were delayed in doing so if they were switched to medium containing cAMP but no DIF. During this delay the cells synthesized large quantities of the intercellular material. The intercellular material was shown to be microfibrillar, was sensitive to cellulase, and labelled with a colloidal gold-conjugated cellulase. The intercellular material may have the same mode of cellulose synthesis as that involved in stalk tube formation. If so, that mode would be favored by DIF and cAMP in combination, whereas the cellulose synthesis involved in stalk cell wall formation would be DIF-dependent but delayed or repressed by cAMP.

Key words: *Dictyostelium*, cellulose synthesis, stalk cells, DIF, cAMP

INTRODUCTION

Cellulose synthesis is a significant activity of the differentiating cells of the cellular slime mold *Dictyostelium discoideum*. During development, cellulose is first detected biochemically just prior to the slug stage (Sussman, 1972) and cytochemically in late aggregates (Harrington and Raper, 1968). By the completion of the developmental program, all cells will have contributed cellulose to at least one of the following structures: (1) the slime sheath, which surrounds the slug (Hohl and Jehli, 1973); (2) the stalk tube, which encloses the stalk cell population (Raper and Fennell, 1952); (3) the stalk cell walls (Raper and Fennell, 1952); and (4) the inner layers of the spore walls (Erdos and West, 1989). The predominant role of cellulose is most likely structural, although there is evidence for its involvement in slug motility (Freeze and Loomis, 1977) and in cell type determination (Farnsworth, 1974). No mutant strains deficient in cellulose synthase activity have yet been isolated. However, mutants deficient in UDPG-pyrophosphorylase activity cannot synthesize UDPG, which is the substrate for the cellulose synthase, and hence do not synthesize cellulose. These strains form slugs that migrate inefficiently and are incapable of culminating (Dimond et al., 1976).

Raper and Fennell (1952) observed that the stalk tube (called by them the sorophore sheath) was the product of the prestalk cell population as a whole, whereas the stalk cell wall was the product of an individual prestalk cell. They drew an analogy between stalk tube formation and *Acetobacter xylinum* cellulose synthesis, and between stalk cell wall formation and plant cell wall synthesis (see also Rosness and Wright, 1974). This analogy was adopted by Brown (1978; Brown et al., 1983), and the two modes of cellulose synthesis were explained in terms of the plasma membrane-associated cellulose synthase complexes being either fixed or mobile with respect to the membrane surface. A fixed complex would tend to synthesize cellulose that moved away from the cell (as in *Acetobacter*); a mobile complex would tend to synthesize cellulose that formed a cell wall (Brown et al., 1983). Most cellulose-synthesizing organisms can be categorized in either the fixed or the mobile site group; *Dictyostelium* is the only organism that may use both modes of cellulose synthesis (Brown et al., 1983).

The spatial and temporal control of cellulose synthesis in *D. discoideum* is poorly understood despite its developmental importance. For instance, it is not known which cells are responsible for the synthesis of the cellulose found in the slug slime trail. The precise timing of spore cellulose synthesis has been debated in the past, although there is now evidence that it occurs late in spore development (Erdos and West, 1989). The cellulose found in the stalk tube and in stalk cell walls is clearly the product of prestalk cell activity,
although the precise point(s) of addition of cellulose to the stalk tube have not been determined. Studies of stalk formation have shown a portion of the prestalk cell population to be tightly appressed to the stalk tube (George et al., 1972), presumably adding cellulose to its growing tip (Raper and Fennell, 1952; George et al., 1972). Furthermore, it was proposed that the prestalk cells also add cellulose to the inner layer of the stalk tube, once they have migrated into it and before they start to synthesize their own cell walls (Mühlenthaler, 1956; George et al., 1972).

Most of the published work on cellulose synthesis in D. discoideum predates the recent advances in the understanding of stalk cell differentiation (reviewed in Inouye, 1990; Williams and Jermy, 1991). For instance, given that the prestalk cell population is heterogeneous, an obvious question concerns which subpopulation(s) is(are) responsible for stalk tube and stalk cell wall cellulose synthesis. Two of the identified subpopulations, pstA and pstB cells, are distinguished by different patterns of expression of the ecmA and ecmB genes (Williams et al., 1989; Jermy and Williams, 1991) whose products are extracellular matrix proteins detectable in the slime sheath, stalk tube, and stalk cell walls (McRobbie et al., 1988a,b). The cellulose in these structures is being synthesized by the prestalk cells at the same time as they are secreting the ecmA and ecmB proteins. These same cells are also in the process of switching from stalk tube to stalk cell wall synthesis; analysis of the pstB-specific promoter demonstrated that a key event in the conversion of a pstA to pstB cell is entry of the cells into the stalk tube (Cecarelli et al., 1991).

It is clear that pstB cells or a successor subpopulation, rather than pstA cells, are involved in stalk cell wall cellulose synthesis. However, it is not clear which subpopulation is responsible for stalk tube cellulose synthesis. Ecma-β-galactosidase histochemistry studies of culminants showed (Jermyn and Williams, 1991) the pstA population to occupy the region that includes the cells that are thought to be associated with stalk tube synthesis (George et al., 1972), suggesting that cellulose synthesis in that region could be the responsibility of the pstA cells. However, not all of the cells in the stained region would be contributing to the stalk tube, just those tightly appressed (George et al., 1972). Cellulose could therefore be a very late marker of the progressive differentiation of pstA cells, which do demonstrate increasing expression of the ecmA gene over time (Jermyn and Williams, 1991). A strain transformed with a mutant regulatory subunit of protein kinase A under the control of the ecmA promoter (ecmA-Rm) is capable of forming pstA cells, but is blocked in pstB differentiation (Harwood et al., 1992). The ecmA-Rm cells are not able to form a stalk tube (or stalk cell walls), suggesting that pstB cells are responsible for the cellulose synthesis in both structures (Harwood et al., 1992). Perhaps a subpopulation intermediate between pstA and pstB cells is involved in stalk tube synthesis and its differentiation is also blocked in the ecmA-Rm cells.

Clearly, a full understanding of prestalk cell and stalk cell differentiation requires a more detailed understanding of cellulose synthesis in D. discoideum. This paper begins to place cellulose synthesis in the context of stalk cell differentiation by seeking to answer two questions: (1) can the different modes of cellulose synthesis (stalk tube versus stalk cell wall) be observed in the monolayer culture system (Kay, 1987)?; and (2) is cellulose synthesis during stalk formation induced by DIF, cAMP, a combination of these signals, or by some other mechanism? Relevant experiments have been aided by the HM44 mutant, which is deficient in DIF production but responds to it upon addition (Kopachik et al., 1983). Therefore, precise control of the initiation of stalk cell differentiation is possible.

MATERIALS AND METHODS

Strain and culture conditions

Strain HM44 was from Dr. R. R. Kay (Laboratory for Molecular Biology, MRC, Cambridge, UK). Amoebal stocks were stored in liquid nitrogen; working cultures were renewed every 5-8 weeks and maintained at 8°C on SM agar (Sussman, 1966) with Klebsiella aerogenes.

Development

For monolayer cultures of HM44, amoebae were collected, washed, and diluted in stalk salts medium (Kay, 1987) containing 5 mM cAMP (adenosine 3’,5’-cyclic monophosphosphate, K+ salt; Sigma). Cultures (5×10² cells cm⁻²; 60 mm or 150 mm tissue culture dishes) were incubated in the dark at 22°C (additional details in the figure legends). Synthetic DIF-1 [1-{3,5-dichloro-2,6-dihydroxy-4-methoxy}phenyl]hexan-1-one] was initially from Dr. R. R. Kay and subsequently from Molecular Probes, Inc.

Cellulose synthase assays

Cellulose synthase activity was determined by a method modified from Blanton and Northcote (1990) to accommodate limited numbers of monolayer cells. Cells (150 mm dish) were collected by removing the culture medium, adding 2 ml disruption buffer, and scraping with a tissue culture cell scraper (Baxter Scientific), then lysed by sonication. The lysate was centrifuged (15 minutes, 18,000 rpm, SS-34 rotor) and the pellet was resuspended in wash buffer and re-centrifuged. The washed pellet was resuspended in H₂O. Protein concentrations of fresh membrane preparations were determined using the BioRad protein assay reagent with bovine serum albumin as a standard. Enzyme assay mixtures were as described in Blanton and Northcote (1990) except for the use of 0.1 mM UDP-[¹⁴C]G (uridine 5’-diphosphate-D-[¹⁴C]glucose; 2.64 GBq mmol⁻¹; ICN Radiochemicals). The reaction was stopped after 30 minutes incubation at 22°C by adding 75 μl H₂O followed by boiling. The reaction tube contents were spotted onto Whatman no. 1 filters, which were air-dried, washed 4 times with H₂O and twice with 100% methanol, and counted using Fisher Scintiverse BOA scintillation fluid.

Light microscopy of monolayer cultures

Cells in 60 mm dishes were fixed for light microscopy in culture medium containing 0.5% (v/v) glutaraldehyde and stored at 4°C until all dishes of each experiment had been fixed. After a minimum of 30 minutes fixation, dishes were washed twice with H₂O and incubated in the dark at room temperature for 5 minutes with 0.1% (w/v) aqueous Tinopal LPW (a gift from Ciba-Geigy; this compound is Fluorescent Brightening Agent 28; Colour Index No. 40622; equivalent to Calcofluor White ST). Dishes were washed twice with H₂O and 0.5 mM n-propyl gallate (Sigma) was added as an anti-quenching agent (modified from Giloh and Sedat, 1982). Stained dishes were stored for up to a week at 4°C.

Dishes were examined with an Olympus IMT-2 inverted microscope and a 40x long-working distance phase contrast objective with a coverslip correction collar, allowing high resolution obser-
vations through the dish bottom. Epifluorescence was performed with the violet filter cube pack.

**Photomicrography and quantitation of fluorescence intensity**
Photomicrographs were taken on Kodak TMax-400 film using a spot-metering automatic photomicrography system (Olympus PM10-ADS). In its averaging (integrating) mode, the exposure meter provided an accurate measure of the fluorescence intensity (as the inverse of the exposure time) in the central 30% of a 0.29 mm$^2$ area (when using a 20× objective, 1× intermediate lens, and 2.5× projection eyepiece). Three regions were chosen at random in each culture and the exposure time noted after a 5 second delay (to control for the slight fading that occurs upon initial illumination).

**Shadow-casting and electron microscopy**
The numerous floating cells in monolayer cultures differentiated identically to attached cells and could be easily collected onto Formvar-coated nickel grids for shadow-casting. The specimens were treated with 1 mg/ml proteinase K (Sigma) in 20 mM Na Heps, pH 7.0 for 1-2 hours at 37°C in a humid chamber to remove proteins that obscure the microfibrils, washed well with H$_2$O, and air-dried.

Shadow-casting was performed in a Cressington Model CFE-50 freeze-fracture apparatus with deposition of platinum/carbon (45° angle) followed by carbon replication (85° angle). Electron microscopic observations were made with either an Hitachi HU 11E or an Hitachi HS 9 electron microscope operating at 75 kV.

Specimens mounted on grids and treated with protease were labelled with a colloidal gold/cellulase conjugate (15 nm particle size) and shadowed and observed in the electron microscope as described above. The preparation of the colloidal gold-cellulase conjugate and specimen labeling procedure were as described by Berg et al. (1988). Cellulase (65 units mg$^{-1}$) was from Worthington Biochemicals.

**RESULTS**

**Cellulose synthesis during monolayer development**
The induction of stalk cells in mutant HM44 by addition of exogenous DIF (Kopachik et al., 1983) was exploited to determine the time-course of induction of cellulose synthase activity. Cellulose synthase assays were performed at 2 hour intervals from HM44 cells developing in the presence of cAMP and either the presence or absence of DIF; initial enzyme activity was detected at 4 hours and peak activity at 8 hours postinduction (Fig. 1). The stalk cells were similar to those formed during normal development: the cells were highly vacuolated (Fig. 2A); the cell walls stained intensely with Tinopal LPW (Fig. 2B); and the cell wall microfibrils were randomly oriented (Fig. 2C).

In addition to stalk cell wall fluorescence (Fig. 2B), the monolayer cultures were also observed to have a Tinopal-

![Fig. 1. Kinetics of cellulose synthase activity during development in the monolayer culture system. Amoebae of the DIF-deficient strain HM44 were plated in monolayers in the presence of 5 mM cAMP as described in the Materials and methods. 9-10 hours after plating ($t_0$ in the graph) the original medium was removed, the plates washed twice with stalk salts, and new medium with 5 mM cAMP and either 0 (open squares) or 100 nM (open circles) DIF added to the plates. At the indicated times the cells were collected from the plates and enzyme assays performed on crude membranes prepared as described in Materials and methods. Data points represent the mean of three replicates; this is a representative example of an experiment performed four times.]

![Fig. 2. Stalk cells formed in monolayer cultures (strain HM44). Amoebae were plated in tissue culture dishes and induced with DIF and cAMP as described in the Materials and methods. (A,B) The amoebae can be seen to have formed stalk cells at high efficiency. The walls of the stalk cells stain brightly with Tinopal LPW (A, differential interference contrast photomicrograph; B, epifluorescence photomicrograph). (C) The walls of stalk cells formed in monolayer cultures have microfibrils that are oriented randomly (electron micrograph of replica of material shadowed at 45° with platinum-carbon). Bars, 25 µm for A, B and 100 nm for C. The small arrow next to the bar in C indicates the direction of shadowing.]
positive intercellular material surrounding and between the stalk cells (Fig. 3B,D,H). Formation of stalk cell walls could be first detected microscopically 8 hours postinduction; the intercellular material could be observed as early as 6 hours postinduction, becoming more abundant in older cultures (data not shown). Monolayer cultures of amoebae of strains V12M2, NC-4, and Ax-2 (Kay, 1987; Berks and Kay, 1988) also formed significant amounts of the intercellular material, particularly V12M2 (data not shown).

The effect of cell density on the synthesis of stalk cell walls and the intercellular material was tested by plating HM44 cells at densities ranging from $2 \times 10^4$ to $7 \times 10^5$ cells cm$^{-2}$. At the time of the medium change, the cultures were washed twice with stalk salts and incubated in new medium containing 100 nM DIF and either 5 mM cAMP or 10 µM cAMP-S (a non-hydrolysable analogue of cAMP). At lower cell densities, clumping of cells was less extensive, but stalk cell formation was enhanced by the clumping at higher densities. The intercellular material was also more abundant around cell clumps. However, stalk cell walls and intercellular material could be observed at all cell concentrations, and the amount of intercellular material appeared qualitatively proportional to cell number in the presence of either cAMP or cAMP-S (data not shown).

Testing for the potential effects of cAMP and DIF on the formation of the intercellular material and stalk cell walls was complicated by the effects of these molecules upon both the early events of stalk cell differentiation (such as commitment to the prestalk cell pathway) and late events such as stalk cell maturation (Berks and Kay, 1988, 1990; Sobolewski and Weeks, 1988). Double medium shift experiments were performed to determine the effects of cAMP and DIF upon the synthesis of the intercellular material and stalk cell walls by cells committed to stalk cell differentiation (Fig. 4). Sample cultures fixed at the time of the second medium change (Fig. 3A,B) and at 16 (Fig. 3C-H) and 36 (data not shown) hours after the first medium change were analyzed for Tinopal LPW fluorescence. Little difference was observed in the extent of stalk cell formation in the 36 hour cultures of conditions A, B, and C (see Fig. 4), although intercellular material formation was higher in treatments A and C than in B (data not shown). However, in 16 hour cultures, conditions A (Fig. 3C,D) and C (Fig. 3G,H) had only a few stalk cells and extensive intercellular material, while condition B (Fig. 3E,F) had high percentages of stalk cells and little additional intercellular material.

Determining percentages of stalk cells formed was hindered by the large clumps in high density cultures and would not have indicated the amounts of intercellular material formed in a culture. Instead, an automatic photomicrographic exposure device was used to determine relative fluorescence intensity (Fig. 5). Although the data were variable because of the heterogeneity introduced by cell clumping, they reflected the results obtained by parallel microscopic observations. The fluorescence intensity in the condition B cultures increased rapidly, reflecting the rapid

Fig. 3. HM44 monolayer cultures prepared by the double medium shift method. Cultures were prepared as described in Fig. 4. (A,B) Cells at the time of the second medium change. (C-H) Cultures 8 hours after the second medium change incubated in condition A (C,D), B (E,F), or C (G,H). Matched phase contrast/epifluorescence photomicrographs of the same field for each treatment are shown. Bars, 25 µm for A-H.
synthesis of stalk cell walls in the absence of cAMP. Condition A cultures eventually reached nearly the same intensity as the condition B cultures, but were delayed in reaching the maximum rate of intensity increase by about 2 hours; condition C cultures had not reached the same rate by 20 hours.

Cellulose synthase specific activity was determined at the time of the second medium shift (t₈) and in condition A, B, and C cultures two hours following the shift (t₁₀; Table 1). Only the condition A cultures had a significant increase in specific activity between the t₈ and t₁₀ time points.

**Characterization of the intercellular material**

Electron microscopic observation of stalk cells prepared by high-resolution shadowing revealed the presence of sheets of fibrillar material surrounding the stalk cells (Fig. 6A). The material was often oriented (Fig. 6B), giving the impression of having been synthesized while a cell was moving. Stalk cells often had narrow bands of material that extended multiple cell lengths away (data not shown). Grids stained with Tinopal LPW and examined with the fluorescence microscope were then shadowed and examined with the electron microscope. The extended material revealed by Tinopal LPW fluorescence corresponded to the fibrillar sheets and bands seen in the electron microscope. The fibrils of intercellular material labeled with a cellulase conjugated to colloidal gold (Fig. 6C,D) and were sensitive to cellulase treatment (Fig. 7A-D).

Immunocytochemistry of non-protease treated cells using an antibody that recognizes the ecmA and ecmB proteins did not indicate their colocalization with the microfibrils (data not shown); however, since the proteins are secreted into the culture medium in monolayer cultures (Berks and Kay, 1990), they may not be able to associate with cellulose in these conditions.

**DISCUSSION**

**Cellulose synthesis during monolayer development**

Both DIF and cAMP are required for the induction of cellulose synthase activity. The induction is not as rapid as that of known DIF-induced proteins such as the ecmB protein (Berks and Kay, 1988) or DIF-1 dechlorinase (Insall et al., 1992), suggesting that the induction of the cellulose synthase is not directly by DIF, but rather through another process that is itself DIF-induced. However, this conclusion is premature since it is based upon the timing of enzyme activity and not gene expression.

**Cells in monolayer cultures synthesize two types of fibrillar structures**

Cells in monolayer cultures not only synthesize cell walls, but can also synthesize substantial amounts of an intercellular material. The microfibrillar nature of the material, its sensitivity to cellulase treatment, its labelling with a colloidal gold-cellulase conjugate, and its staining with Tinopal LPW are all suggestive of cellulose. However, further characterization (for instance, by selected area electron diffraction; see Blanton and Chanzy, 1985) is required before the intercellular material may be conclu-

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<th>Condition*</th>
<th>Specific activity ± s.d. (nmoles min⁻¹ mg protein⁻¹)</th>
<th>Relative activity†</th>
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<tr>
<td>A; original medium returned</td>
<td>1.487±0.123</td>
<td>1.66</td>
</tr>
<tr>
<td>B; new medium with DIF only</td>
<td>0.903±0.055</td>
<td>1.00</td>
</tr>
<tr>
<td>C; new medium with cAMP only</td>
<td>0.977±0.110</td>
<td>1.09</td>
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*Refer to Fig. 4 for culture conditions. Enzyme assays were performed on cells 2 hours following the second medium change.
†The specific activities of the cellulose synthase at 12 hours was normalized to that at 8 hours (0.897±0.064 nmoles min⁻¹ mg protein⁻¹) when the second medium shift occurred. The values represent the means of triplicate reactions of a single experiment and the standard deviations of those means.
sively stated to be cellulose. It is intriguing to speculate that the intercellular material represents the stalk tube mode of cellulose synthesis. The synthesis prior to stalk cell wall formation of the intercellular material and its orientation as if left behind by a moving cell are both consistent with this hypothesis. In normal development, the microfibrils in the stalk tube are oriented parallel to the long axis of the stalk; those in the stalk cell walls are oriented randomly (Mühlethaler, 1956).

**The effects of DIF and cAMP on stalk cell wall and intercellular material synthesis**

Both DIF and cAMP were required for efficient stalk cell conversion and for synthesis of the intercellular material. However, cells committed to the stalk cell pathway incubated in medium containing DIF alone formed stalk cells rapidly and synthesised minimal intercellular material, whereas those incubated in medium containing only cAMP were delayed in stalk cell wall formation and synthesized extensive intercellular material in the interim. Therefore, the synthesis of intercellular material directly precedes that of the stalk cell wall; by delaying stalk cell wall synthesis the amount of intercellular material synthesized is greater. Whether the cells are transferred to medium containing only DIF or only cAMP does not affect the cellulose synthase specific activity; therefore, differences in synthesis of stalk cell walls and intercellular material synthesis are not likely attributable to changes in relative activity levels.

The results presented here correlate well with observations by others. Sobolewski and Weeks (1988) found the timing of cAMP-independence was similar in vivo (development on filters) and in vitro (monolayer cultures), but the timing of DIF-independence was much different. Stalk cell formation in vitro occurred immediately after prestalk cell differentiation; the appearance of a normally late stalk antigen occurred much earlier in monolayer cultures than in vivo (Sobolewski and Weeks, 1988). They proposed that the delay in stalk cell formation until culmination was constrained by an inhibitor; Ceccarelli et al. (1991) also postulated that an inhibitor was responsible for preventing stalk cell differentiation outside of the stalk tube. The identity of
the inhibitor is unknown, although cAMP was suggested as a potential candidate (Sobolewski and Weeks, 1988; Ceccarelli et al., 1991). The presence of cAMP during the later stages of stalk cell differentiation delays the formation of stalk cells with some differences in extent and timing between strains (Berks and Kay, 1988). Strain NC-4, for instance, was exceptionally sensitive to the inhibitory effects of cAMP; V12M2 and its derivatives (such as HM44) much less so (Berks and Kay, 1988). The precise in vivo role of this inhibition is not known, although it is possible that cAMP may serve to inhibit the formation of stalk cells in slugs (Berks and Kay, 1988) and in culminants until the cells are contained within the stalk tube (Ceccarelli et al., 1991). If the intercellular material represents the mode of cellulose synthesis that in vivo is involved in stalk tube formation, then cAMP could not only serve to prevent premature stalk cell maturation, but also to promote the synthesis of the stalk tube cellulose.

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