CELL DIFFERENTIATION DURING FRUITING BODY FORMATION IN POLYSPHONDYLIUM PALLIDUM

DANTON H. O'DAY

Hubrecht Laboratory, Universiteitscentrum 'De Uithof', Uppsalalaan 8,
Utrecht, The Netherlands

SUMMARY

The spatial pattern of cellular differentiation was studied during fruiting body formation in Polysphondylium pallidum using 3 different staining methods: Calcofluor fluorescence (cellulose accumulation), neutral red (prestalk cells) and immunofluorescence (prespore cells). Neutral-red staining revealed the existence of a clear prestalk region which becomes evident during aggregation and continues throughout culmination. Immunofluorescent staining demonstrated that cells in the prestalk region gradually lose their presporeness (fluorescence) as they are transformed into differentiated stalk cells. Calcofluor staining revealed that stalk cell differentiation begins during the mid-aggregation phase and that the mode of formation of the main stalk and the side branches differs slightly in morphology. Calcofluor staining also demonstrated the development, during aggregation, of a thick cellulosic girdle with lateral tubular extensions which surround the aggregation streams. The above results are discussed in terms of our present knowledge about differentiation and morphogenesis in cellular slime moulds.

INTRODUCTION

Polysphondylium species belong to a group of eucaryotic soil microorganisms called the cellular slime moulds (Dictyosteliaceae). Individual amoebae feed on bacteria and after exhaustion of the local food supply may undergo one of three developmental events: fruiting body formation, macrocyst development or microcyst differentiation. During the fruiting body mode of development thousands of amoebae aggregate on a solid substratum to form multicellular pseudoplasmodia or slugs. During pseudoplasmodial migration stalk cells are continually produced at the slug apex so that the slug leaves behind a continuous stalk, composed of cellulose-stalk cells enclosed in a cellulose sheath, as it migrates. At culmination the slug lifts off the substratum leaving behind small clumps of cells at regular intervals as the stalk continues to grow in length. Each of these small cell masses reorganizes into several smaller sub-masses each of which produces stalk cells to form the lateral side branches. Spore masses differentiate from cells remaining at the ends of each branch and at the very top so that the completed fruiting body resembles a spindly Christmas tree.

Morphologically, the development of Dictyostelium discoideum, the most actively studied species of the Dictyosteliaceae, differs from that of Polysphondylium in 2 main aspects: the absence of stalk formation during pseudoplasmodial migration and the lack of side branch formation during fruiting. These morphogenetic processes are detailed by Bonner (1967).
Early work by Shaffer (1962) indicated that aggregation in *Polysphondylium* species was directed by a different acrasin (P-acrasin) than *Dictyostelium* species (D-acrasin) since aggregation centres of each of the genera only attract homologous amoebae. After aggregation, however, the *Polysphondylium* pseudoplasmodial tip gains the competence to attract amoebae of *D. discoideum* indicating a switch in tip secretory characteristics from P-acrasin to D-acrasin (Shaffer, 1962). Recently, Wurster, Pan, Tyan & Bonner (1976) have revealed that the acrasin of *P. violaceum* is likely a small peptide. The acrasin of *D. discoideum* is cyclic-3',5'-adenosine monophosphate (cyclic AMP) (Konijn, Barkley, Chang & Bonner, 1968; Konijn, Van Der Meene, Bonner & Barkley, 1967). Cyclic AMP induces amoebae of both genera to differentiate into stalk cells (Bonner, 1970; Francis, 1975; Hohl, Honegger, Trank & Markwalder, 1977) and the pseudoplasmodial tip is the site of differentiation of cells that form the stalk (Bonner, 1967).

In this regard, histochemical and immunofluorescent staining has clearly revealed a small prestalk cell region at the front and a larger prespore area at the rear of slugs of *D. discoideum* (Bonner, Chiquoine & Kolderie, 1955; Takeuchi, 1963). On the other hand, work on the spatial patterns of cell differentiation in pseudoplasmodia of *Polysphondylium* species has yielded contradictory results. Bonner et al. (1955), using the periodic acid/Schiff procedure and a histochemical stain for alkaline phosphatase, could not distinguish prestalk and prespore regions in *P. violaceum* pseudoplasmodia, while O'Day & Francis (1973) did obtain preferential staining of prestalk cells in *P. pallidum* using a different histochemical stain for alkaline phosphatase. Using pre-spore specific vacuoles (PV) as a criterion for prespore cells, Hohl et al. (1977) have shown that essentially all cells are prespore cells except for a small portion of the apical tip of pseudoplasmodia and culminating sorogens in *P. pallidum*. In this region cells lose their PVs as they are transformed into stalk cells. Hohl et al. (1977) also report that this pattern of differentiation is the same for side branch formation while O'Day & Francis (1973) could not histochemically define cell specific regions in side branches.

In an attempt to gain more insight into the pattern of differentiation during fruiting in *P. pallidum* we have studied: (1) the timing and pattern of stalk cell differentiation using Calcofluor (Bonner, 1970; Harrington & Raper, 1968); (2) the definition of a prestalk zone using neutral red (Bonner, 1952; Francis & O'Day, 1971); and (3) the prespore pattern using immunofluorescence (Takeuchi, 1963). In addition we also report on the discovery of a thick cellulosic girdle that develops around aggregates which may be related to the process of transition from aggregation to pseudoplasmodium formation in *Polysphondylium*.

**MATERIALS AND METHODS**

**Cultures**

*Polysphondylium pallidum* (strain WS 320) was cultured on 0·1 % L-agar (Raper, 1951) with *Escherichia coli* as a food source. Plates were maintained at 21 °C and specific developmental stages were selected from these growth plates. For neutral red staining, the contents of 3-day-old plates were scraped into one side of the plate, using a glass rod, and a drop of neutral red solution (6 mg/ml) was added so that a gradient of staining existed along the cell mound. These plates were then stored at 21 °C. Cell masses of different developmental stages, from stained
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and unstained cultures, were picked from the plates with a microscalpel, placed in a drop of distilled water and examined by light or phase-contrast microscopy. Photographs were taken with a Zeiss photomicroscope using Kodak Panatomic X film. The darkness of the neutral red staining was enhanced with a green filter.

**Calcofluor staining**

Cell masses of different developmental stages were placed in a 20-μl drop of 0.1% Calcofluor on a slide and a coverslip was added. In some cases the Calcofluor was added to aggregations in situ on culture plates. The fluorescence of stained cellulose was detected using a Zeiss standard microscope equipped with a Zeiss IV FI H650 epiluminator and appropriate filters (Zeiss combination 487703). Photographs were made using Kodak recording film.

**Immunofluorescence staining**

Cell masses were placed in a 30-μl drop of distilled water on a slide that had been previously coated with gelatin (Rogers, 1967). A coverslip was added and the preparation was left for 15-20 min to allow setting and adhesion of the cells. The preparations were placed in a freezer (-20 °C) until used. Before fixing, the coverslip was pried free from the frozen slide with a razor blade. The slide was then placed in cold methanol for 3-5 min and rinsed in phosphate-buffered saline (PBS). The cell masses were then stained with rabbit anti-spore serum, washed in PBS and conjugated with goat anti-rabbit FITC IgG (Nordic Pharmaceuticals). The spore antiserum, prepared against spores of *D. mucoroides*, was prepared and kindly supplied by Dr S. Brahma. The fluorescence of prespore cells was observed using a fluorescence microscope (see above) equipped with appropriate filters (Zeiss combination 487709). Photographs were made as described for Calcofluor fluorescence.

Slugs of *Dictyostelium discoideum*, selected from nutrient agar cultures (Durston, 1974), were used as controls and treated in an identical manner to *P. pallidum* cell masses.

**RESULTS**

**Calcofluor staining**

When aggregation stages were stained in situ fluorescence was observed during the early phases of the aggregation process as a detectable brightness in the centre of the aggregation mounds (Fig. 1A, B). As aggregation continued (mid-aggregation) the aggregation centre remained intact with increasing intensity while the aggregation streams, which were becoming more discrete, began to show a bright fluorescence (Fig. 1C). When mid-aggregation stages were picked from plates with a microscalpel it was observed that the aggregation streams close to the aggregation centre remained intact (Fig. 1D). After many manipulations it was clear that the aggregation streams were tightly bound to the main cell mass. Observation of these streams by phase microscopy revealed their smooth tubular shape close to the aggregation centre with a loss of this organization near the remaining ends of the streams (Fig. 2B, C). Observation of cell aggregation in situ showed that the amoebae at the extremities of the aggregation stream elongate so they appear to have a head-tail polarity and enter aggregation streams 'head-first' (Fig. 2A). The fluorescence of mid-aggregation stages placed on slides showed that the bright central fluorescence of aggregates is due to staining (Fig. 1D) around the base of the aggregates.

During mid to late aggregation, the first appearance of stalk cell production is evidenced by a bright fluorescence in the apical region of the aggregating cell mass (Fig. 3A). At this time about three quarters of the aggregate is covered in a fluorescent
sheath of material that is continuous with the aggregation streams. As aggregation continues and the cell mass increases in height, the stalk material continues to grow through the centre of the cell mass towards its base (Fig. 3B). At this time the entire cell mass is covered in a sheath of fluorescence. However, the apical fluorescence of the sheath never becomes as intense as the basal region and at times was difficult to observe. By the completion of aggregation the stalk has completely progressed through the cell mass so that in the slug stage the long stalk is clearly evident in both unstained and stained material (Fig. 3C). The fluorescent aggregation stream sheath material is still present. The production of stalk cells at all stages is evident at the apex of the cell masses, immediately behind the nipple-shaped tip that formed during aggregation, as a wide, tube-like assortment of stalk cells that condenses to around half its diameter a short distance behind its point of origin (Fig. 4D).

During culmination, the apex continues to produce stalk material and as the stalk
Fig. 2. Structure aggregation streams in *P. pallidum*. Light microscopy of aggregation stream formation (in situ) reveals elongate amoeba entering unsheathed streams (A). When a squash preparation of a mid-aggregate is observed by phase-contrast microscopy the aggregation streams close to the main aggregate appear very rigid (B) and adjacent to the aggregate (C) they have a smooth, tubular structure. A, x 200; B, C, x 360.
Fig. 3. Calcofluor staining during aggregation, young pseudoplasmodium stages. Squash preparations of mid-late aggregates (A) reveal the appearance of stalk cells in the apex of the cell mass. This stalk continues to grow in length as aggregation continues (B) reaching the substratum as the pseudoplasmodium stage is attained (C). Differentiation of the stalk cells shows that the stalk begins as a wide tube that condenses a short distance later (D). A–C, ×24; D, ×110.
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Fig. 4. Calcofluor staining of culmination stages. Squash preparations of culminating sorogens reveal the appearance of bright fluorescent spots in side-branch cell masses (A) which become clearly defined as stalk cells as the side branches (B, C) grow towards the main stalk, and finally adhere (D). A, B, × 24; C, D, × 110.
lengthens small parcels of tissue pinch off from the main cell mass. Stalk cell production for side branches is first apparent as small bright dots of fluorescence around the edge of the cell masses (Fig. 4A). These bright areas of fluorescence increase in size and number (Fig. 4B), growing towards the main stalk (Fig. 4C) and finally making contact as completed side branches (Fig. 4D). As side branches are being produced in the first separated cell masses, the new cell masses that are pinched off have not yet begun stalk cell differentiation (Fig. 4B).

During the whole process of fruiting the brightly staining basal material remains intact and tightly associated with the culminating sorogen. The exact relationship of the stalk with this material was difficult to assess due to the intensity of fluorescence and the complexity of its shape.

Stalk cell differentiation for side branch formation occurs in slightly different fashion from that of the main cell mass. Stalk cell differentiation at the apical tip begins as a wide, tube-like structure that condenses a few microns farther down. This pattern continues throughout morphogenesis. Branch formation begins at the most distal portions of pinched-off cell mass with each tip forming just a bulbous clump of stalk cells followed by the addition of fewer and fewer cells, giving a clear taper to the structure. When the side branches contact the main stalk they become attached in some yet-undefined manner.

Neutral-red staining

Cells developing in the presence of neutral red showed differences in staining during early aggregation, some mounds possessed one central bright red area while others possessed 2, 3, or more red spots. However, by mid-aggregation a clear pattern of differentiation was evident with the tip of the aggregate appearing dark red while the rear remained unstained (Fig. 5A). Although the intensity of staining varied, the darkly stained region always resided at the apical tip of cell masses, from the beginning of stalk differentiation (Fig. 5B) through sorogen culmination (Fig. 5C). The middle region possessed a small number of dispersed stained cells (Fig. 5D). Examination of squashed cell masses at high magnification revealed that the staining in different regions was due to variations in a population of cells that contained unstained cells, cells with small red granules and cells with many large red granules (Fig. 5E, F). The staining of side branches was diffuse and uniform.

Immunofluorescence

Slugs of *D. discoideum* stained with fluorescein-conjugated antispore serum displayed a typical pattern of fluorescence with the anterior region devoid of stain and the posterior regions showing significant fluorescence (Fig. 6A). Slugs and sorogens of
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*P. pallidum* showed only a barely discernible area of reduced fluorescence at their most apical part, while the stalk was totally devoid of stain (Fig. 6B). Under high magnification, the cells of *P. pallidum* revealed bright areas of fluorescence in their cytoplasm (Fig. 6C). Side branches stained uniformly. The cells of *P. pallidum* were not as brightly stained as those of *D. discoideum* control slugs. The uniform fluorescence was observed first at mid-aggregation. It was not possible to define a region of reduced intensity before late aggregation. As well, side branches stained uniformly with no prestalk differentiation evident.

**DISCUSSION**

The results of the present study have revealed some interesting facets of the fruiting body mode of development in *Polysphondylium pallidum*. The first of these aspects has to do with aggregation and pseudoplasmodial construction, while the second relates to the pattern of cellular differentiation.

In this study we have shown that amoebae commence aggregation by forming loose streams in which the cells demonstrate a polar behaviour both on entrance and within the aggregation streams. This agrees with the early work of Shaffer (1962). As the amoebae approach the main cell mass by mid-aggregation they enter rigid tubes which lead to a larger tube or girdle surrounding the main aggregate. The main girdle and its lateral tubular extrusions fluoresce brightly with Calcofluor, a specific fluorescent stain for cellulose (Harrington & Raper, 1968). Whether other materials, in addition to cellulose, are contained in this structure is not known. This cellulosic encasement gives an unusual rigidity to the main cell mass and to the aggregation streams. If this structure is developmentally important its function is transitory since it is left behind by the pseudoplasmodium. Thus its potential importance is restricted to the late phases of aggregation and early phases of pseudoplasmodial morphogenesis. It is possible that the function of this thick girdle is related to the change in acrasin-secretion characteristics of the pseudoplasmodial tip that occurs at this time (Shaffer, 1962). Thus the girdle and its tubular extensions may act structurally to prevent a loss of morphology during the change in tip secretory function, or may provide limited channels for P-acrasin diffusion thus allowing the completion of the aggregation process in the presence of a diminishing P-acrasin source.

Calcofluor staining has also revealed the pattern and timing of stalk cell differentiation. The first stalk cells become detectable during mid-aggregation, when tip differentiation has just become evident, and continue to grow towards the substratum as the cell mass increases in size. In several cases this growing stalk was observed in close proximity to the main cell mass. The fluorescence of the stalk cells is due to the presence of brightly stained particles in the cytoplasm of the prespore cells (c). A, × 24; B, × 110; C, × 250.

Fig. 6. Immunofluorescence staining patterns of pseudoplasmodia of *D. discoideum* and *P. pallidum*. Slugs of *D. discoideum* reveal a clear prespore fluorescent region and a region devoid of fluorescence (A). *P. pallidum* pseudoplasmodia show a fairly uniform fluorescence that diminishes slightly and gradually towards the slug apex and is completely lost by the differentiated stalk cells (B). The fluorescence of the *P. pallidum* sorogen is due to the presence of brightly stained particles in the cytoplasm of the prespore cells (C). A, × 24; B, × 110; C, × 250.
apposition to the cellulose girdle rather than progressing through the centre of the cell mass. Thus a third, alternative function for the cellulose girdle might be in guiding stalk penetration of the main cell mass.

The early appearance of stalk cell differentiation is signalled by the differential neutral-red staining patterns observed at this time. The tips of mid-aggregates and later stages were distinctly redder than the red basal portions, and this brightness was due to the presence of large numbers of cells with big red granules in their cytoplasm. Bonner (1952) first reported that neutral red gave preferential staining of prestalk regions in *D. discoideum*. This was later verified by Francis & O'Day (1971) who showed that cells of the prestalk region could be defined by their content of large red granules while cells of the prespore zone had smaller granules or none. Recent ultrastructural data reveal that the neutral red is specifically accumulated in the autophagic vacuoles of prestalk cells (J. Bluemink, A. Durston and F. Vork, unpublished results). *P. pallidum* thus shows a similar pattern of neutral-red staining to *D. discoideum* and this pattern appears to be due to staining of prestalk cell autophagic vacuoles. The prestalk region of *P. pallidum* defined here with neutral red has previously been revealed by histochemical staining methods (O'Day & Francis, 1973).

Immunofluorescent staining of prespore cells of *P. pallidum* shows that the cell masses at all developmental stages studied possess a generally uniform fluorescence. Only at the apical tip and in completed stalk cells could a decrease in fluorescence be detected. Immunofluorescence staining of *D. discoideum* slugs reveals a large, distinct prespore region and a smaller anterior prestalk area (Takeuchi, 1963). This method specifically stains prespore vesicles (PV) of *Dictyostelium* (Takeuchi, 1972). Hohl et al. (1977) have shown that essentially all of the cells of pseudoplasmodia of *P. pallidum* contain large numbers of PVs, except for cells in the apical tip which show a declining number of PVs. Thus our immunofluorescence pattern correlates with the ultrastructural data of Hohl et al. (1977). Our results also show that the wall of *Polytholytium* spores contains some of the same components as those of *Dictyostelium* and that the prespore cells have already differentiated by mid-aggregation.

The fact that both the neutral-red staining and histochemical methods for stalk cells (O'Day & Francis, 1971) show a larger prestalk zone than PV number or immunofluorescence indicates that the biochemical transition of prespore cells into prestalk cells begins long before the cells actually lose all of their prespore characteristics (i.e. PVs). It also suggests that the presence of PVs or the immunofluorescent staining of cells is not an exact criterion for defining that a cell is, at any one moment, determined as a prespore cell (i.e. prestalk cells possess PVs). The inability of this and a previous (O'Day & Francis, 1973) study to define a prestalk boundary during side branch formation may be related to the subtle difference in the mode of differentiation, or may be due to the small number of cells undergoing the differentiation into stalk cells in these cell masses. Hohl et al. (1977) have reported that the pattern of PV loss during side branch formation is similar to that occurring at the apical tip of sorogens so it is likely that the second alternative is the most reasonable.
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