CELL SURFACE LOCALIZATION OF 5'AMP NUCLEOTIDASE IN PRESTALK CELLS OF DICTYOSTELIUM DISCOIDEUM

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SUMMARY

5'AMP nucleotidase activity was localized by electron microscopy in Dictyostelium discoideum during cell differentiation. In addition, the activity was assayed by micro enzymic methods in sections dissected from specific cellular regions of lyophilized individuals. The results of the 2 procedures were in agreement, demonstrating that at the culmination stage of development the activity is strikingly localized in the prestalk cells adjacent to the prespore region. The cytochemically stained reaction product appeared only along the plasma membrane of the cells. As prestalk cells migrate into the stalk sheath and undergo differentiation, the activity is rapidly lost. Examination of stained cells at high magnification revealed the product accumulation to be primarily at the cell surface, suggesting that the enzyme functions extracellularly. Occasionally, cells having the morphological appearance of prestalk cells were found within the prespore region. These cells demonstrated 5'AMP nucleotidase activity at their plasma membrane in sharp contrast with neighbouring prespore cells. The strategic localization of 5'AMP nucleotidase may reflect a mechanism for establishing and maintaining regulatory levels of extracellular 5'AMP and/or adenosine during pattern formation in this model system.

INTRODUCTION

Cellular differentiation in the cellular slime mould Dictyostelium discoideum leads to the formation of a fruiting body which is composed of stalk cells and spore cells. During preliminary migrations the presumptive stalk cells attain an apical position atop the prespore cell mass. Histological examination of the developing fruiting body reveals a sharp disjunction between the 2 morphologically distinct cell types. This line of demarcation is not delineated by a discernible physical structure; instead, the cells at the boundary of the 2 cell types are in close contact. The cells at this interface region do not appear to be specialized as they are morphologically identical to the remainder of their respective cell types. This situation is not unlike many other examples of cell differentiation and specialization found in animal and plant embryos. However, the structural simplicity offered by the presence of only 2 cell types makes the search for the mechanisms underlying cellular specialization in Dictyostelium somewhat more approachable than in more complex systems.

Actual stalk construction commences with synthesis of a cellulosic tube by prestalk cells near the apex. This stalk sheath is composed of 2 layers, the outer with fibrils

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oriented parallel to the stalk axis and the inner with randomly oriented fibrils (George, Hohl & Raper, 1972). The cells of the prestalk region are closely packed and extend radially outward. These cells migrate toward the apex of the sorus and enter the stalk sheath. The sheath elongates apically as the new stalk cells vacuolize, extending the stalk upward. The course of stalk cell differentiation may be followed visually along the path taken by the migrating prestalk cells. Initially, cells are amoeboid, containing intact mitochondria and small autophagic vesicles. As cells move into the stalk sheath, the vacuoles expand and mitochondria begin to degenerate and are engulfed by enlarging autophagic vesicles (George et al. 1972). The swollen cells become polyhedral in shape, form cellulose walls and eventually lose viability as they complete differentiation.

During spore formation the cells become spherical and are lifted en masse up the elongating stalk. The most distinguishing ultrastructural feature of the prespore cells is the appearance of prespore vesicles (Maeda & Takeuchi, 1969; Hohl & Hammamoto, 1969). These heavily walled vesicles, which first appear at the late pseudoplasmodium stage, contain amorphous material which is probably secreted from the cell as the vesicles fuse with the plasma membrane (Hohl & Hammamoto, 1969). Clusters of ribosomes form peripheral layers around the prespore nucleus and mitochondria (Loomis, 1975), and the mitochondria become spheroid, often bearing inclusions. The spore coat is formed of cellulose and protein arranged in three layers and surrounded by mucopolysaccharide (Hemmes, Kojima-Buddenhagen & Hohl, 1972).

The morphogenic changes described above have recently been shown to be accompanied by cell-specific biochemical events (Rutherford, 1976; Wilson & Rutherford, 1978; Armant & Rutherford, 1979). Earlier histochemical studies provided qualitative evidence indicating that certain enzymes, particularly alkaline phosphatase and 5′nucleotidase, appear only in specific cells during culmination (Bonner, Chiquoine & Kolderie, 1955; Krivanek & Krivanek, 1958). The activities of alkaline phosphatase and 5′AMP nucleotidase both increase during the course of development, reaching their maximum at the culmination stage (Lee, Chance, Weeks & Weeks, 1975).

We have purified both activities to a single band on SDS gel electrophoresis and have found that the 2 activities co-purify (unpublished). Thus, the alkaline phosphatase and 5′AMP nucleotidase activities reside in a single protein molecule.

Armant & Rutherford (1979), using quantitative microbiochemical procedures, demonstrated that alkaline phosphatase activity, which is evenly distributed throughout the pseudoplasmodium, becomes highly localized at the culmination stage. The activity was limited to the interface region of the 2 presumptive cell types. Confinement of activity to this significant region of the organism could reflect the involvement of this enzyme in such developmental mechanisms as pattern formation, prestalk-prespore cell communication or establishment of metabolic gradients. The purpose of the present study is to characterize the enzyme by: (1) demonstrating its cell-specific localization quantitatively in sections dissected from culminating individuals with 5′AMP as substrate, (2) resolving whether the activity is expressed in one or both of the specific cell types, and (3) determining its precise ultrastructural localization at culmination by cytochemical methods.
MATERIALS AND METHODS

Spores of *D. discoideum* (NC-4) were germinated in the presence of a food source, *Escherichia coli*. The cells were grown on nutrient agar at 22 °C for 48 h and then were harvested in salt solution (Bonner, 1947). After being centrifuged to separate the myxamoebae and bacteria, slime mould cells were replated on Whatman Number 50 filter paper and were allowed to proceed through their developmental cycle to the culmination stage. The tissue was then either frozen and lyophilized or was fixed in glutaraldehyde. The lyophilized material was stored under vacuum at −76 °C as described earlier (Rutherford, 1976).

On the day an assay was to be performed a lyophilized individual was removed from the filter paper and was dissected under a stereo microscope. Sections were cut with microscalpels, were manipulated with hair tips (Lowry & Passonneau, 1972; Rutherford, 1976), and then were placed in 10-μl depression wells (Falcon microtest 3032). The tissue was assayed for 5′AMP nucleotidase (E.C. 3.1.3.5) by adding 1.0 μl of a reaction mixture containing 2.1 nM [2-3H]5′AMP (Amersham) (25 mCi/mmol) in 100 mM Tris-HCl buffer, pH 9.0. A layer of hexadecane and mineral oil (3:7 v/v) was applied to each well to prevent evaporation of the reagents during the subsequent 2-h incubation at 25 °C. Unreacted 5′AMP was then removed by adding the total reaction mixture volume to 600 μl of a suspension of anion exchange resin (Dowex, AG 1-X2) and centrifuging for 15 min at 500 g. Finally, 300 μl of the supernatant were removed to a scintillation vial, and the radioactivity of the sample was determined (Beckman model LS-230).

A method adapted from Reale & Luciano (1967) was used to localize the enzyme at the ultrastructural level. The tissue was fixed by placing 20–30 individuals supported on a 1-in. (2.54-cm) square of filter paper in 0.01 M cacodylate buffer at pH 7.5. The tissue was then washed by transferring the filter paper to 0.1 M glycylglycine buffer at pH 9.0. The sample was assayed for 5′nucleotidase by incubating 20 min in a reaction mixture consisting of 80 mM CaCl₂, 10 mM 5′AMP and 5 mM MgCl₂ in 40 mM glycylglycine buffer, pH 9.0. Two controls were run concurrently with the complete reaction mixture, one lacking 5′AMP (the substrate) and one in complete reaction mixture buffered with glycglycine at pH 7.0. All samples were then washed in cold distilled water for 3 min, incubated in 50 mM Pb(NO₃)₂ for 2 min, and washed again in glycglycine buffer (pH 9.0). The tissue was postfixed in 1 % osmium tetroxide, dehydrated in an ethanol series, transferred to acetone and embedded in Spurr’s resin. Sections were made using a diamond knife and were stained on the grid with uranyl nitrate and lead citrate. The material was viewed in a JEOL 100 C microscope. All chemicals were purchased from Sigma Chemical Co., St Louis, Mo.

RESULTS

The distribution of 5′AMP nucleotidase was determined quantitatively in prespore and prestalk cells of *D. discoideum* at the culmination stage of development (Fig. 1). The enzyme was highly localized in sections taken from the area delimiting the interface of prestalk and prespore cells. In some individuals the mature stalk cells were assayed (data not shown) and were found to be void of activity. Interestingly, an acid phosphatase has been shown to accumulate specifically in stalk cells (Armant & Rutherford, 1979). Thus, an opposing gradient of acid and alkaline phosphatase activity develops during stalk construction. The gradient occurs over the length of the stalk extending from the completely undifferentiated prestalk cells near the interface region to the fully developed stalk cells at the base.

Although the sections shown in Fig. 1 contain only 20–30 cells, we could not determine whether the 5′AMP nucleotidase was localized in prestalk or prespore cells. We, therefore, used cytochemical localization of 5′AMP nucleotidase by electron
microscopy to determine more precisely with which cell type the enzyme is associated. We first attempted the ultrastructural localization using individuals which had been lyophilized as described above for the quantitative assays. They were then prepared for cytochemical examination as described for fresh tissue. The lyophilized individuals were used so as to compare directly the results obtained by the quantitative and ultrastructural techniques. In addition, we wanted to determine whether lyophilized tissue could be satisfactorily used for ultrastructural localization of enzyme activity. Fig. 2 shows that lyophilization disrupts much of the cell’s ultrastructure, but allows the gross morphology to be observed relatively unchanged. The micrograph includes the region in which prespore and prestalk cells adjoin and a portion of the stalk bordered by the stalk sheath. The lyophilization has not altered the characteristic morphology of the 2 cell types. That is, the prespore cells were round and well separated while the prestalk cells were amoeboid and closely packed. Heavy deposition of reaction product was found in the prestalk region while only occasional grains are seen in the stalk and prespore regions. Deposition of the reaction product was greatest near the line of demarcation between prestalk and prespore cells with the density diminishing toward the apex. Thus, the activity of 5′AMP nucleotidase is limited to the prestalk cells found nearest the prespore region.

To study the ultrastructural localization of 5′AMP nucleotidase, the cytochemical assay was repeated using the more classical method of glutaraldehyde-fixation of fresh tissue. Figs. 3 and 4 show that the distribution of reaction product is limited to the cell membrane of prestalk cells. No activity was found in either prespore cells
or in stalk cells. It was readily apparent from Figs. 3 and 4 and similar sections that
the activity was limited to the regions of cell-to-cell contact. For example, in Fig. 4
there was markedly less deposition of reaction product on the cell surface which was
in contact with the stalk sheath, while the same cell shows heavy deposition at the
area of cell contact. Likewise, no activity was present on the prestalk cell surfaces facing
the external surface of the individual. In a study of the preimplantation mouse
embryo, Mulnard & Huygens (1978) similarly found that alkaline phosphatase is
confined to plasma membranes in which cells are in contact. The outside plasma

Fig. 2. Cytochemical demonstration of 5'AMP nucleotidase activity in lyophilized
tissue. The reaction product is densest in the prestalk (ps) region adjacent to the
prespore mass, while absent from the prespore (psp) and stalk (s) cells.
Fig. 3. Reaction product is present at the periphery of the prestalk cells but is absent in prespore cells.

Fig. 4. Reaction product is present at the periphery of the prestalk cells (arrow a) and absent from the surface of the stalk cells (arrow b). The stalk is separated from the other cells by a sheath (ss).

Fig. 5. Control preparation in the absence of enzyme substrate. The reaction mixture lacked 5′AMP.

Fig. 6. Control preparation assayed at pH 7.0.
Figs. 7, 8. Fig. 7. Localization of 5'AMP nucleotidase reaction product on the outer surface of the plasma membrane (pm). Shown are the areas of contact between prestalk cells. The reaction product is in the area between adjacent cells and appears to be on the surface of the membrane. Fig. 8. Reaction product on the surface of a prestalk-like cell within the prespore mass. Within groups of prespore cells, cells were often found that demonstrated prestalk morphology as well as 5'AMP nucleotidase activity on their surface.
membranes, as well as those facing the blastocoel, remained devoid of reaction product.

Appropriate controls were run to confirm the validity of the cytochemical assay for 5′AMP nucleotidase. Fig. 5 demonstrates the result of incubating tissue in a reaction mixture lacking the substrate, 5′AMP. Relatively few grains are present on either side of the stalk sheath. Similar results were obtained upon incubation in complete reaction mixture, but at pH 7.0 rather than the normal pH 9.0 (Fig. 6). We have found that when assayed in vitro using cell free extracts, 5′AMP nucleotidase activity at pH 7.0 is only 5 to 10% of the activity assayed at pH 9.0.

We further examined the sections at higher magnification (×95,000) in order to determine precisely the cellular location of the reaction product. Fig. 7 shows that the electron-opaque granules are associated with the outer surface of the plasma membrane. This is particularly obvious in regions in which the cells are not in close contact (see Fig. 7) where the precipitate remains associated with the membrane.

Occasionally, cells which possessed the morphological characteristics of a prestalk cell were found within the prespore mass. These prestalk-like cells were characterized by an irregular cell outline, unlike the more globular shape of the prespore cells. The cytoplasm of the prestalk-like cells had a granularity quite different from those surrounding prespore cells but similar to that of the prestalk cells. In addition the prestalk-like cells do not contain the prespore vesicles which are characteristic of prespore cells. When stained for 5′AMP nucleotidase activity, these prestalk-like cells showed heavy deposition of reaction product on the plasma membrane (Fig. 8). The amount of precipitate in these cells was equivalent to the most heavily stained prestalk cells. It appears that these are prestalk cells, perhaps still in the process of sorting out, which express 5′AMP nucleotidase activity even though they are displaced from their normal position in the organism.

**DISCUSSION**

By a cytochemical assay we have found 5′AMP nucleotidase to be active only in prestalk cells during the culmination stage. No activity is present in prespore cells, and as prestalk cells mature into stalk tissue, the activity is lost. The validity of this finding was substantiated biochemically through the microassay of the various cell types dissected from lyophilized tissue. Thus, at the culmination stage of development adenosine production and 5′AMP degradation are confined to the least mature prestalk cells. In addition, prestalk-like cells containing 5′AMP nucleotidase on their cell surfaces were frequently found within the prespore mass. These findings bring to light several intriguing questions regarding the role of this enzyme during development. Is the expression of 5′AMP nucleotidase on the prestalk cell surface a response to positional information? Is the activity, found only in displaced prestalk-like and in normally positioned prestalk cells adjacent to the prespore area, induced by the prespore cells? Alternatively, the 5′AMP nucleotidase may be an expression of an early event in the differentiation of stalk cells. Although low levels of activity are detectable in both undifferentiated amoebae and in fully developed stalk cells, the
enzyme accumulates only in prestalk cells at the culmination stage. Perhaps the enzyme plays a role in segregation of the 2 cell types or in maintaining their integrity once sorting out has occurred.

A realistic model for the function of this enzyme in development is beyond the limits of the available data. However, some information about the enzyme has accumulated, both from our own work and from the literature. We found in the present report that the enzyme is located ultrastructurally at the plasma membrane. Previous cell fractionation studies of 5'AMP nucleotidase are in agreement with this result (Lee et al. 1975; McMahon, Miller & Long, 1977). The accumulation of reaction product, inorganic phosphate, in the intercellular space between prestalk cells indicates that the active site of 5'AMP nucleotidase is situated on the exterior surface of cells. Such an orientation suggests that the enzyme performs an extracellular function rather than a strict metabolic one. That is, it may regulate the extracellular concentrations of 5'AMP and adenosine which in turn may be regulators of cell function and differentiation (Fox & Kelley, 1978). A concentration gradient of either molecule would result from the localization of the enzyme in prestalk cells, assuming the presence of a corresponding source or sink elsewhere in the organism. A distant source of 5'AMP derived from cAMP degradation has already been demonstrated by the localization of cAMP phosphodiesterase in the mature stalk (Brown & Rutherford, in preparation). No phosphodiesterase activity was found in spore cells while stalk cells showed an increasing gradient of activity toward the base of the stalk.

The possibility of the existence of a cAMP gradient during differentiation of the 2 cell types is particularly interesting in view of the rapidly accumulating evidence that cAMP is not only the chemotactic signal for aggregation, but is involved in differentiation of stalk and spore cells. It is well documented that myxamoebae are chemotactic towards cAMP during the early aggregation stage of development (Bonner et al. 1969). cAMP may also direct some of the cell movements leading to sorocarp construction (Durston & Vork, 1979). Matsukuma & Durston (1979) have shown that only the prestalk cells of the pseudoplasmodium retain an attraction for cAMP signals. Prestalk cells migrate from the prestalk area into the stalk sheath during culmination, perhaps under the direction of localized cAMP signals. The localization of 5'AMP nucleotidase and the resulting degradation of 5'AMP may affect the distribution of extracellular cAMP by maintaining a gradient of cAMP in the developing sorocarp. If the stalk-specific phosphodiesterase were active in vivo, an extracellular pool of 5'AMP could accumulate. The presence of a 5'AMP sink in the undifferentiated prestalk cells could establish extracellular gradients of 5'AMP and adenosine. Adenosine has been shown to increase the intracellular cAMP concentration in several tissue types including lymphocytes, brain, vagus nerve, pig skin, bone, and platelet cells (Fox & Kelley, 1978). It was found to decrease cAMP levels in other cell types such as fat, kidney cortex, liver, and ascites tumour cells. Further, adenosine can have other more diverse metabolic consequences. It has been shown to block pyrimidine synthesis by inhibiting orotate phosphoribosyltransferase, reduce the intracellular concentration of PP-ribose-P, inhibit DNA, RNA and protein synthesis, increase adenine nucleotide levels and inhibit protein kinases. Adenosine also has
various cytotoxic and morphological effects on cells (Fox & Kelley, 1978). Thus, the role played by adenosine in *Dictyostelium*, as well as in other systems, is open to speculation at this point and deserving of further work. We predict that cell-specific extracellular levels of both adenosine and 5'AMP are to be expected due to the localization of 5'AMP nucleotidase activity at culmination. The actual metabolic effect of differential levels of these molecules and their resulting morphological significance remain to be explored. The localization of 5'AMP nucleotidase activity may help maintain a gradient of extracellular modulators throughout the organism during morphogenesis. Such gradients may regulate the movement and differentiation of prespore and prestalk cells directly or indirectly via a secondary molecule such as cAMP.

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REFERENCES


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