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Differential cellular distribution of cAMP-dependent protein kinase during development of *Dictyostelium discoideum*

(prespore/prestalk/pattern formation/cell–cell contact)

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ABSTRACT **It was shown previously by us that cAMP-dependent protein kinase activity in the cellular slime mold *Dictyostelium discoideum* increased during the early stages of development. Results from other laboratories showed that during the subsequent stage of cell differentiation and positioning, the accumulation of a number of prespore mRNAs and proteins (but not prestalk mRNAs and proteins) was dependent upon cAMP. The present communication describes the cellular distribution of the cAMP-dependent protein kinase at that stage of development. Pseudoplasmodia were disrupted, and prespore cells were separated from prestalk cells by sedimentation through a Percoll gradient. Prespore cells had approximately 4-5 times as much of both the catalytic and regulatory subunits of the cAMP-dependent protein kinase as did the prestalk cells. That the increase of cAMP-dependent protein kinase during development reflected *de novo* synthesis of the enzyme in both prespore and prestalk cells was demonstrated on the basis of [^{3}H]leucine incorporation into the regulatory subunit. The findings are consistent with a role of the cAMP-dependent protein kinase in mediating the effects of cAMP on the synthesis of prespore-specific mRNAs and proteins at the stage at which cAMP appears to be required for the cell type-specific syntheses.**

The cellular slime mold *Dictyostelium discoideum* is a favorite organism for the study of cellular differentiation and morphogenesis. Cyclic AMP plays a central role in the development of the organism. The amoebae, when deprived of nutrients, eventually give rise to fruiting bodies consisting of three differentiated types of cells—spores supported by a column of nonviable stalk cells resting on a basal disk also composed of nonviable cells.

Cellular differentiation is apparent once the pseudoplasmodium (slug) has been formed; amoebae in the anterior fourth of the slug eventually will become stalk cells, while the amoebae in the posterior portion are predestined to become primarily spores; a small population of "anterior-like" cells, which are associated with the posterior part of the slug, will form the basal disk during culmination. The anterior and posterior cells can be distinguished by morphological and biochemical criteria including the occurrence of specific mRNAs, proteins, and subcellular organelles (1).

The implementation of the first stage of the developmental program from the onset of starvation to the formation of loose aggregates does not require cell–cell contact and is stimulated by exogenous cAMP (2). This stage of development is characterized by the appearance of new mRNAs and proteins, which are formed presumably in all amoebae up to aggregation. During aggregation (prior to tip formation), another class of mRNAs and proteins appears, which also probably is found in all aggregating amoebae; but, at a later stage of development, its occurrence is confined to the anterior part of the slug (3-5). A third family of mRNAs and proteins first appears concomitant with the formation of tips on the aggregates. The synthesis of this latter class of macromolecules is confined to the posterior part of the pseudoplasmodium and, typically, many of the species are found eventually in the spores. The onset of the synthesis of the prespore mRNAs and proteins apparently requires tight cell–cell contact; they are lost if the tipped aggregates are dispersed and the amoebae are maintained as single cells in suspension. Exogenous cAMP added to amoebae developing in suspension does not initiate the synthesis of these species; however, the addition of cAMP to amoebae from dispersed tight aggregates prevents the loss of these mRNAs. Furthermore, exogenous cAMP added after the disapperance of those mRNAs suffices, even in the absence of cell–cell contact, to bring about their reaccumulation. It appears that, after the initial establishment of tight cell–cell contacts, cAMP suffices for the accumulation of those mRNAs and proteins (3, 4, 6, 7).

It is generally assumed that the intracellular effects of cAMP in eukaryotes are mediated by cAMP-dependent protein kinases. Accordingly, we have studied this enzyme in *D. discoideum* and described the occurrence and characteristics of the holoenzyme and its subunits in cytosolic extracts of the slime mold (8–11). Other investigators also have described the occurrence of the free subunits of the enzyme (12–16). We found that the cAMP-dependent protein kinase occurs at low levels in vegetative amoebae and that there is a 4- to 5-fold coordinate increase in the amounts of the two subunits, with maximal accumulation during aggregation, prior to the formation of the tips and the prespore mRNAs. Most, if not all, of the increase constitutes *de novo* synthesis. Furthermore, maximal levels of enzyme are achieved in the absence of sustained cell–cell contact. Cyclic AMP-dependent protein kinase levels remain elevated after tipped aggregates are dispersed and amoebae are maintained as single cells in suspension (10, 11).

The three facts that (*i*) the early increase in cAMP-dependent protein kinase does not require cell–cell contact, (*ii*) the level of the enzyme remains high after dispersal of the tipped aggregates, and (*iii*) exogenous cAMP effects the maintenance or reaccumulation of certain prespore mRNAs and proteins after the dispersal of the tipped aggregates are consistent with the hypothesis that the cAMP-dependent protein kinase mediates the effects of cAMP on the synthesis of prespore mRNAs and proteins once tipped aggregates have been formed. In this report, we show that the cAMP-dependent protein kinase occurs in both anterior and posterior cells but that the levels are 4–5 times higher in the posterior, prespore cells than in the anterior cells.

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MATERIALS AND METHODS

Materials. Kemptide was purchased from Boehringer Mannheim; Bacto agar, from Difco; the chromatofocusing resin and buffers and the Percoll, from Pharmacia; and the protease inhibitors, from Sigma. Cappel Laboratories (Cochranville, PA) supplied the fluorescein isothiocyanate-conjugated goat anti-rabbit-IgG antisera. [^{32}P]ATP, [^{3}H]leucine, [^{3}H]UDPgalactose, and ENLIGHTNING were purchased from New England Nuclear; NCS tissue solubilizer was from Amersham and 8-azido[^{32}P]cAMP was from ICN. Other chemicals were reagent grade or better.

Growth and Development of Amoebae. *D. discoideum*, strain Ax3/RC4, was grown axenically to a density of 2–4 × 10^6 amoebae per ml on medium HL5 (17) as described (10). Strain NC4 was grown on SM agar plates in association with *Klebsiella aerogenes* (18). Amoebae were harvested by sedimentation, washed twice in either PDF buffer (50 mM K/Na:phosphate, pH 6.5/20 mM KCl/1.5 mM $MgCl_2$) (19) or in water, and allowed to develop at a density of $\approx 7 \times 10^6$ amoebae per cm^2 on plates containing 2% Bacto agar in PDF buffer for strain Ax3 and in water for strain NC4. In experiments in which the rates of synthesis of the cAMP-dependent protein kinase were to be determined, the washed amoebae were starved at a density of $\approx 10^7$ amoebae per cm^2 on Millipore filters (type AABG) in PDF buffer; the filter rested on a support pad saturated with PDF buffer.

Separation of Prespore and Prestalk Cells by Centrifugation Through a Percoll Gradient. Amoebae were allowed to develop until they reached the slug stage. The procedure for the separation was a modification of that described by Tsang and Bradbury (20). The aggregates were disrupted in 50 mM Tris, pH 7.0/13 mM dimercaptopropanol/0.01% Pronase (BAL/Pronase). The single cells, after passage through a 25-gauge needle, were washed twice in PDF buffer and then centrifuged for 25 min at 20,000 × *g* through gradients of 85% Percoll/15% buffered salts (0.2 M K phosphate, pH 6.0/6.5% NaCl/0.02 M EDTA). The amoebal suspension was resolved into two bands. The upper band or light fraction consisted of anterior and "anterior-like" cells and will be called the prestalk fraction in the present context. The lower band or heavy fraction was composed of posterior, prespore cells. In most experiments, there were 3 times as many cells in the heavy fraction as there were in the light fraction. The amoebae were washed free of the Percoll by repeated centrifugation and resuspension in PDF buffer. Amoebae tested for possible effects of exposure to BAL/Pronase and sedimentation through Percoll on the cAMP-dependent protein kinase were obtained by mixing all cells in a Percoll gradient tube.

The effectiveness of the separation of the two types of cells was tested by two independent assays—i.e., the determination of UDP-galactose–polysaccharide transferase activity and immunofluorescent staining with anti-spore antibody. The acceptor for the UDP-galactose–polysaccharide transferase assay was prepared from *Dictyostelium mucoroides* strain IS-2 (provided by C. Town) by a modification of the procedure of Sussman and Osborn (21). The transferase assay was performed as described by Kay (22); [^{3}H]UDP-galactose served as the galactose donor. The immunofluorescence staining was carried out essentially as described by Devine *et al.* (23). The anti-spore antisera were provided by D. Ratner and by K. Devine and W. Loomis. Amoebae were incubated with the antisera, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit-IgG sera. The ratio of fluorescent to nonfluorescent amoebae was determined by scoring on a Zeiss fluorescence microscope.

Determination of Levels of cAMP-Dependent Protein Kinase. Cyclic AMP-dependent protein kinase levels were determined as described (11) in amoebae harvested after growth and obtained from slugs and from the two fractions separated by centrifugation through the Percoll gradient. Briefly, the amoebae were washed in 10 mM 4-morpholinepropanesulfonic acid, pH 7.2/1 mM EDTA and lysed by sonic disintegration in 10 mM 4-morpholinepropanesulfonic acid, pH 7.2/15% sucrose/1 mM EDTA containing the protease inhibitors phenylmethylsulfonyl fluoride at 1 mM, benzamidine at 10 mM, antipain at 200 μg/ml, and chymostatin at 200 μg/ml. The lysate was centrifuged at 30,000 × *g* for 15 min, and the levels of the regulatory and catalytic subunits were then determined in the supernatant fluid. The regulatory subunit was assayed (after passage of an aliquot of the supernatant fluid through a column of Sephadex G-25 so as to remove endogenous nucleotides) by photoaffinity labeling with 8-azido[^{32}P]cAMP at a concentration of 200 nM followed by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. The region of the gel containing the regulatory subunit was excised, and the radioactivity was determined. Every sample was photoaffinity-labeled in triplicate. Appropriate control experiments (11) showed that the labeling was specific for the regulatory subunit. We found earlier that the native molecular weight of the regulatory subunit of the *D. discoideum* cAMP-dependent protein kinase is 41,000 (11). The level of the catalytic subunit was also determined as described (10, 11). An aliquot of the supernatant fluid was passed through a column of PBE 94 chromatofocusing gel (Pharmacia) preequilibrated to pH 7.8. The column was eluted with Polybuffer (pH 5.0). Fractions were assayed for phosphorylating activity with Kemptide as substrate in the presence of 1 μM cAMP. The chromatofocusing procedure separates the catalytic subunit (pI 6.4) from other protein kinases and allows quantitation of its activity.

Measurement of the *de Novo* Synthesis of the Regulatory Subunit. Amoebae were labeled from the onset of starvation until the early slug stage with 150 μCi (1 Ci = 37 GBq) of [^{3}H]leucine per filter ($\approx 10^7$ amoebae per cm^2). The slugs were then disaggregated, and the resulting amoebae were subjected to Percoll gradient centrifugation as described above. The amoebae in the two bands were lysed by sonic disruption, the lysates were centrifuged, and aliquots of the supernatant fractions were analyzed by photoaffinity labeling with 8-azido[^{32}P]cAMP. Other aliquots were subjected to immune precipitation as described (10, 11). The extracts were treated with 20 μl of normal rabbit serum before incubation with 20–50 μl of antiserum against the *D. discoideum* regulatory subunit; a 10:1 ratio of heat-treated, formalized *Staphylococcus aureus* to antiserum was used to precipitate the two complexes. The immune precipitate was then submitted to electrophoresis on a $NaDodSO_4$/polyacrylamide gel. The band corresponding to a molecular weight of 41,000 was excised and solubilized in NCS tissue solubilizer, and the radioactivity was determined. Total [^{3}H]leucine incorporation into protein was measured in samples precipitated with trichloroacetic acid; synthesis of the regulatory subunit is expressed as the percentage of total [^{3}H]leucine incorporated into protein. A second round of immune precipitation with antiserum against the regulatory subunit did not yield additional regulatory subunit. In order to assess the purity of the immunoprecipitated material, replicate aliquots of the first immunoprecipitate were submitted to electrophoresis, the gels were treated with ENLIGHTNING, dried, and exposed to Kodak XAR film at −70°C. Only one band corresponding to the regulatory subunit of molecular weight 41,000 was labeled.

RESULTS

Cyclic AMP-Dependent Protein Kinase in Prestalk and Prespore Cells. The efficiency of the separation of prestalk from

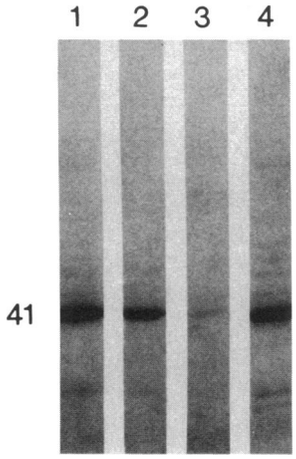

FIG. 1. 8-Azido[^{32}P]cAMP photoaffinity labeling of *D. discoideum* (strain Ax3) extracts from different types of cells. Amoebae were harvested at the slug stage and treated as described. Lanes: 1, extract from slugs (27 μg of protein); 2, extract from BAL/Pronase-treated cells (14 μg of protein); 3, extract from prestalk cells (13 μg of protein); 4, extract from prespore cells (27 μg of protein). Size is shown in kDa.

prespore cells was assessed by two criteria. The first was the activity of the enzyme, UDP-galactose–polysaccharide transferase; the enzyme is spore-specific and first appears during the formation of the tips on the aggregates (21, 22). The second criterion for the purity of the respective centrifugal fractions was based on the immunofluorescent staining of the amoebae with highly specific antibody directed against prespore cells, followed by reaction with fluorescein isothiocyanate-conjugated goat anti-rabbit-IgG serum (24). The range of contamination of the prestalk fraction by prespore cells, determined in a number of experiments, was 11–20%, similar to the level of contamination found by other workers (22, 24). The cAMP-dependent protein kinase activities of the prestalk cells reported here have been corrected for the contamination. In most experiments, a ratio of prespore cells to prestalk cells of 3:1 was observed.

Fig. 1 shows an autoradiogram of extracts obtained from slugs, prestalk cells, and prespore cells of strain Ax3, respectively, photoaffinity labeled with 8-azido[^{32}P]cAMP. The levels of both the regulatory and of the catalytic subunits were 4- to 5-fold higher in extracts of prespore cells than in those of prestalk cells (Table 1). The results have been normalized for the concentration of protein in the respective fractions (11); a similar ratio of cAMP-dependent protein kinase subunit levels was obtained when levels were normalized for DNA content or cell number. The 4- to 5-fold ratio was obtained in five experiments with the axenic strain Ax3 and in one experiment with slugs of wild-type strain NC4, which had developed on water-agar.

Table 1. cAMP-dependent protein kinase in prestalk and prespore cells (strain Ax3)

Cell type	Regulatory subunit, fmol/mg of protein	Catalytic subunit, pmol·min^{-1}/mg of protein
Vegetative	39	40
Slug	131	139
BAL/Pronase-treated	153	148
Prestalk	43	42
Prespore	178	206
Prespore/prestalk	4.1	4.9

Regulatory subunits are expressed as fmol of 8-azido[^{32}P]cAMP bound per mg of protein. Catalytic subunits are stated as pmol of Kemptide phosphorylated per min per mg of protein. (Note that, although the numerical values of the activities of the regulatory and catalytic subunits are similar, they are expressed in different units.)

Table 2. Synthesis of the regulatory subunit (strain Ax3)

Cell type	Total [^{3}H]Leu incorporation, cpm/mg of protein	[^{3}H]Leu in regulatory subunit: cpm per gel slice	[^{3}H]Leu in regulatory subunit: cpm/mg of protein	% of protein synthesis
Slug	907,000	1185	117	0.013
Prestalk	975,000	405	57	0.005
Prespore	848,000	1468	126	0.015

All indications are that the observed values of catalytic and regulatory subunit activities are a true measure of native subunit levels unaffected by experimental manipulation (10, 11). Furthermore, the relevant control experiment (see *Materials and Methods*) indicates that neither BAL/Pronase treatment nor subsequent sedimentation through the Percoll gradient had an effect on the measured activities of the catalytic and regulatory subunits; only the regulatory subunit of molecular weight 41,000 (and no proteolytic fragments) was observed. Two-dimensional gel electrophoresis (25) showed that photoaffinity labeling with 8-azido[^{32}P]cAMP of extracts from prestalk and prespore cells gave rise in each extract to the same two radioactive spots that were observed in slugs (11). Experiments in which extracts from prestalk cells and prespore cells were mixed showed that extracts from prestalk cells contained neither inhibitors of regulatory or catalytic subunit activities nor any unique prestalk cell-specific proteolytic activity. It should be noted also that the two subunit-specific levels are higher in prespore cells than they are in the whole slug, which is composed of both prespore and prestalk cells.

Synthesis of cAMP-Dependent Protein Kinase. We showed earlier that the regulatory subunit and probably also the catalytic subunit of the cyclic AMP-dependent protein kinase are synthesized *de novo* during the aggregation stage of development. Because the specific activities of the cAMP-dependent protein kinase subunits in the vegetative and the prestalk cells are similar, the possibility was considered that during development *de novo* synthesis of the enzyme occurred exclusively in the prespore cells. In order to examine this possibility, amoebae were labeled with [^{3}H]leucine from the onset of starvation until the slug stage, and then prestalk and prespore cells were separated as described above. The ratio of prespore to prestalk cells, the contamination of the prestalk fraction with prespore cells, and the occurrence of the regulatory subunit (as measured by photoaffinity-labeling with $8N_3$-[^{32}P]cAMP) in the two fractions were found to be similar to those values reported above. The amount of [^{3}H]leucine in the immunoprecipitated regulatory subunit of each cell type was a measure of *de novo* synthesis. The amount of newly synthesized regulatory subunit (normalized to total leucine incorporation) was 3-fold higher in the prespore cells than in the prestalk cells, but there was clearly some newly synthesized regulatory subunit in the prestalk cells (Table 2). Identical results were obtained in two experiments. The difference in the rates of synthesis of the regulatory subunit was similar to the difference in the cAMP-dependent protein kinase activities of the two types of cells.

DISCUSSION

It appears that cell–cell contact is required for the initial synthesis of certain prespore mRNAs and proteins in the development of *D. discoideum* but that, once they are formed, cAMP suffices for their maintenance or their synthesis; there is evidence that cAMP plays a role in both the stabili-

zation of these developmental mRNAs and in their enhanced synthesis (3, 4, 26). That the major increase in cAMP-dependent protein kinase occurs prior to the formation of tipped aggregates (i.e., prior to the appearance of the prespore-specific mRNAs and proteins) and that elevated levels of the enzyme persist after the disruption of the aggregates (11) are consistent with a role for the cAMP-dependent protein kinase in mediating the effects of cAMP on the synthesis and persistence of prespore-specific mRNAs and proteins. This hypothesis is strengthened considerably by our finding that the increase in both the catalytic and the regulatory subunits of the cAMP-dependent protein kinase occurs primarily in the prespore cells. Little is known about the nature of the requirement for cell–cell contact for development. It is possible that one of the effects of cell–cell contact is the emergence of a new substrate(s) for the cAMP-dependent protein kinase; such a substrate would have to survive the dispersal of aggregates. The stimulatory effects of added cAMP then would imply a decrease in endogenous cAMP subsequent to disaggregation. Although it is tempting to attribute a causal role to the protein kinase and to cAMP in the synthesis of spore-specific mRNAs, such a role remains hypothetical until the substrates of the cAMP-dependent protein kinase have been identified and until the mechanisms by which the cAMP-dependent protein kinase affects the transcription and the stabilization of prespore-specific mRNAs have been elucidated.

A large number of differentiation-specific mRNAs and proteins have been associated with either the prespore or the prestalk cells of the slug. These entities express and maintain the specific functions and positions of the differentiating cell types, but they probably do not initiate the bifurcation of the two types of cells. The cAMP-dependent protein kinase appears to be the earliest known link in the chain of events that leads to the accumulation of the prespore-specific functions subsequent to the formation of sustained cell–cell contacts. The mechanism by which the kinase functions at this stage of development remains to be explored; studies of the mechanism by which the kinase is accumulated preferentially in the prespore cells may elucidate the initial events of the bifurcation.

Our earlier findings (11) showed that the initial burst of the *de novo* synthesis of the cyclic AMP-dependent protein kinase was over by the time the amoebae had aggregated; a plateau in the level of the enzyme was observed at that stage of development. It cannot be stated at this point whether there is a net increase in cAMP-dependent protein kinase in all cells at very early stages of development or only in those cells destined to become prespore cells (there is *de novo* synthesis of the enzyme in both types of cells; see Table 2).

Rutherford *et al.* (27) showed that during culmination there is an unequal distribution of several enzymes (and intermediates of metabolism) in prespore and prestalk cells. These proteins may well play a role in the functions of the two types of cells during culmination; it is unlikely that they initiate culmination. We observed a second burst of synthesis of cAMP-dependent protein kinase at culmination (11); therefore, it is quite possible that the cAMP-dependent protein kinase plays a role in the initiation of culmination. Tsang and Bradbury (20) and Ratner and Borth (24) reported that their Percoll gradient techniques were unsuitable for the separation of the cell types isolated from culminating structures. We are developing a procedure for the separation of the amoebae at that stage of development.

The increase in the amount of cAMP-dependent protein kinase during development, reported here and in our earlier communication (11), raises certain questions. In principle, the activity of the cAMP-dependent protein kinase can be modulated over a wide range merely by changes in cAMP levels in the absence of any increase in the amount of the enzyme. Is it possible that the newly synthesized enzyme has a function different from that of the preexisting one? No differences in the physical nature of the catalytic subunit have been detected; the pI on chromatofocusing columns stays the same irrespective of the stage of development at which the enzyme is isolated. By the same token, the regulatory subunit behaves in an identical manner upon two-dimensional gel electrophoresis at all stages of development and irrespective of whether it is isolated from prestalk or prespore cells (unpublished results). Differences in subcellular location of the cAMP-dependent protein kinases at different stages of development are not excluded. Alternatively, is it possible that the relevance of the increase in the amount of the cAMP-dependent protein kinase lies primarily in the increase of the regulatory subunit? It is conceivable that the regulatory subunit has a function additional to that of the control of the activity of the catalytic subunit. Such a role, perhaps analogous to that of the catabolite gene activator protein of *Escherichia coli*, would presumably be stoichiometric rather than catalytic; hence, an increase in the amount of the protein would be required for an increase in activity, and then the increase would be physiologically significant.

Differences in the distribution of cAMP-dependent protein kinase have been observed in terminally differentiated tissues and during the development of homogeneous populations of tissue cultured cells (28–30). This report provides evidence for an unequal cellular distribution of the enzyme during a developmental process that leads to two differentiated types of cells.

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