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IDENTIFICATION AND CHARACTERIZATION OF THE ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE BINDING PROTEINS APPEARING DURING THE DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

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Summary

A photosensitive, radioactive analogue of cyclic adenosine monophosphate, 8-azido-adenosine 3′,5′-[^{32}P]monophosphate (8-N_3-cyclic AMP), was used to label the cyclic AMP binding proteins of *Dictyostelium discoideum*. During development cytosolic proteins appear which are specifically labeled by the photoaffinity agent. The proteins are developmentally regulated since they are only found in starved, developing cells. Unlabeled cyclic AMP competes specifically with the labeled analogue for protein binding sites in contrast to unlabeled 5′-AMP which does not compete. A mutant which develops spores but is deficient in stalk cell production produces a different set of cyclic AMP binding proteins from the parent strain.

Introduction

Adenosine 3′,5′-monophosphate is an important molecule in the life cycle of *Dictyostelium discoideum*. Starved amoebae show a chemotactic response to cyclic AMP pulses by aggregating into multicellular organisms [1]. Recent evidence suggests that cellular differentiation in the multicellular pseudo-plasmodium is dependent on, or induced by, cyclic AMP [2—5]. The molecular mechanisms by which the cell responds to cyclic AMP are uncertain, although it has been suggested that cyclic AMP modulation of cellular events could occur through activation of various protein kinases [6,7].

Previous studies have identified cyclic AMP binding sites on the *Dictyostelium* cell surface [8—10] and in the soluble portion of cell extracts [7,11—13]. These investigations were performed using classical millipore filter binding

assays. In this paper we report the identification and characterization of cyclic AMP binding proteins in *Dictyostelium* using photoaffinity labeling techniques.

Materials and Methods

Organisms. Dictyostelium discoideum, strains V12/M2 and NC4, were obtained from Dr. Julian Gross. Mutant KY19 [14,15] was obtained from Dr. R. Dimond.

Growth of amoebae. D. discoideum amoebae were grown in liquid on *Escherichia coli* B/r [heat killed for 15 min at 80°C; at a concentration 10^{10} cells/ml in buffer 1 (20 mM potassium phosphate, pH 6.1, 2 mM $MgSO_4$)] with shaking at 125 rev./min. Growth at 22°C was monitored with a hemocytometer. Vegetative cells were harvested at $2-4 \cdot 10^6$ cells/ml.

Developmental conditions. Amoebae were washed free of bacteria by four low speed centrifugations and resuspended in buffer 2 [6.7 mM Mops (morpholinopropanesulfonic acid), 20 mM KCl, 5 mM $MgSO_4$, and 3 mg/l streptomycin, pH 6.5] at 10^8cells/ml. Development on agar was initiated by spreading 0.2 ml amoebae on 47 mm petri dishes, or 0.8 ml on 100 mm petri dishes containing 1.5% agar in buffer 2. After the liquid was absorbed by the agar, the dishes were sealed with Parafilm and incubated at 20°C. When cells were allowed to starve and develop in liquid the amoebae were resuspended in buffer 1 at 10^7 cells/ml and shaken at 125 rev./min in an Ehrlenmeyer flask. In addition to agar as a developmental medium, we have employed Millipore filter pads as a substrate and obtained similar results. Development on agar was preferred because agar allowed easier removal of developing organisms. The cells were harvested from the agar with buffer 1 and centrifuged at $15\,000 \times g$ for 2 min. The pellets, from agar or liquid development, were stored at −70°C.

Chemicals. 8-azido-adenosine 3′,5′ [^{32}P]phosphate (8-N_3-cyclic AMP; 56—70 Ci/mmol, purchased from the ICN Pharmaceuticals, Irvine, CA was stored frozen and protected from the light. It was diluted in buffer 3 (10 mM Tris-HCl, 1 mM ethylenedinitrilotetraacetic acid, 5 mM mercaptoethanol, pH 7.4) immediately prior to use. Nucleotides and other reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of extracts Pellets of starved or vegetative cells were suspended in 0.5 ml cold buffer 3 and sonicated in an ice-bath for 30 s (Heat Systems sonicator plus microprobe). All procedures thereafter were carried out at 4°C. Sonicates were labeled directly or centrifuged at $15\,000 \times g$ for 3 min to remove particulate material. No difference in results was noted between clarified and unclarified extracts. Gel filtration of cell extracts on Sephadex G-25 prior to labeling did not alter the labeling pattern.

Photoaffinity labeling procedure. Photoaffinity labeling of undialyzed extract was performed as described previously [16]. Extracts (clarified by centrifugation unless noted otherwise) were preincubated in the absence of light for 15—25 min with 0.1 μM 8-N_3-cyclic AMP, then subjected to ultraviolet irradiation with a laboratory lamp (Mineralite short wave ultraviolet lamp, UVS-54, with filter removed) at a distance of 6 cm for 5 min. Following irradiation, the proteins were precipitated by adding trichloroacetic acid to a concentration of 10%. If the protein concentration of the extract was low, 100 μg of lyso-

zyme were added as protein carrier to ensure complete precipitation. The precipitates were collected by centrifugation in an Eppendorf microfuge. Specificity for cyclic nucleotide-protein binding was characterized by contrasting the ability of excess unlabeled cyclic nucleotide (0.1 mM) to diminish covalent bond formation as compared to the corresponding 5′-nucleotide (e.g. cyclic AMP vs. 5′-AMP) as reported previously [16]. Specificity was suggested when the addition of cold cyclic nucleotide diminished the protein binding but addition of 5′-nucleotide had no effect.

Gel electrophoresis and autoradiography. The pellets from the photoaffinity labeling procedure were reduced and denatured by boiling for 1 min in buffer 4 (62 mM Tris-HCl (pH 6.8), 10/ glycerol, 2.3% sodium dodecyl sulfate (SDS), 5% mercaptoethanol) and the reduced products were subjected to gel electrophoresis in SDS using a 10% slab gel according to the method of Laemmli [17]. Gels were stained with Coomassie brilliant blue in 50% trichloroacetic acid and destained in 7.5% acetic acid.

An apparent characteristic of gel analysis of *Dictyostelium* protein is that although gel patterns are reproducible the protein bands appear more diffuse than those obtained from *E. coli* or other sources. We are unable to give an explanation for this phenomenon at this time. Autoradiography of the gels was carried out by exposing dried gels to Kodak X-Omat R film for varying periods of time.

Results

Photoaffinity labeling of the proteins of Dictyostelium discoideum V12

Fig. 1 shows the stained gel and the autoradiogram of V12 extracts which were labeled with either 8-N_3-cyclic AMP alone or in the presence of a 1000-fold excess of cyclic AMP, 5′-AMP or cyclic GMP. Two proteins were labeled and have molecular weights of 39 000 and 38 000 as determined by comparison with molecular weight standards. The labeling is completely inhibited by either cyclic AMP or cyclic GMP (both present at 1000 times the concentration of the azido-cyclic AMP) but not by 5′-AMP (also at 1000-fold the 8-N_3-cyclic AMP concentration). At lower concentrations of cyclic AMP and cyclic GMP (10-fold excess) only cyclic AMP gave observable inhibition suggesting that the binding protein is specific for cyclic AMP but as some cross reaction with cyclic GMP. Further proof of the specificity of labeling is suggested by the observation that, of the large number of proteins stained by Coomassie brilliant blue, only two labeled bands were observed.

The fact that saturation of protein binding occurred at concentrations of 8-N_3-cyclic AMP of 10^{-8} M and that the approximate K_d is $5 \cdot 10^{-9}$ M suggested tight ligand binding. The K_d was estimated by determining that concentration of the azido compound which would give half maximal labeling as determined by quantitative analysis of the autoradiographs. Brenner [18] has reported that intracellular cyclic AMP concentrations in *D. discoideum* are of the order of 10^{-6} M, suggesting that intracellular cyclic AMP concentrations are sufficient to substantially affect the internal cyclic AMP binding proteins we have observed.

The possibility that the binding proteins are phosphodiesterase was con-

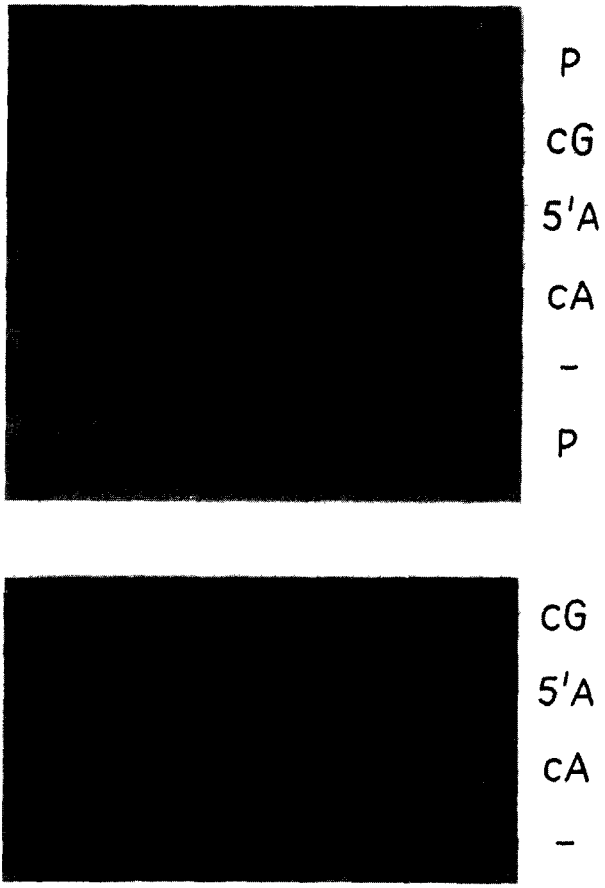

Fig. 1. Specificity of 8-N_3-cyclic AMP binding in *D. discoideum*. Aggregates of *D. discoideum* V12 were harvested from agar plates in the maxi-finger stage [19] at 16 h. Extracts were prepared and labeled with 8-N_3-cyclic AMP as described in Materials and Methods. As indicated, the four samples differ in the presence or absence of 0.1 mM nonradioactive cyclic AMP (cA) 5'-AMP (5'A), or cyclic GMP (cG). The Coomassie blue stained gel is on the top and the autoradiogram is below. The molecular weight standards on the stained gel (P) are *c*-galactosidase (130 000), phosphorylase *A* (92 500); human serum albumin (66 000), ovalbumin (43 000), and soybean trypsin inhibitor (21 500).

sidered and rejected because the observation that the binding is specific for cyclic AMP compared to cyclic GMP, and because the conditions of the reaction are chosen to minimize phosphodiesterase activity [9,10].

Intracellular location of the cyclic AMP-binding proteins

Extracts of *D. discoideum* were centrifuged at high and low speeds and the supernatants and pellets were analyzed for the cyclic AMP binding. As seen in Fig. 2, there is essentially no observable binding activity in the pelleted material. The small amount of binding activity observable in the 100 000 × *g* pellet may be due to slight contamination of this pellet with supernatant material. The pellet was not washed and the pelleted binding activity appears to have the same electrophoretic mobility as the major supernatant binding activity. Quantitative measurements by Millipore filter assay indicate that all of the binding

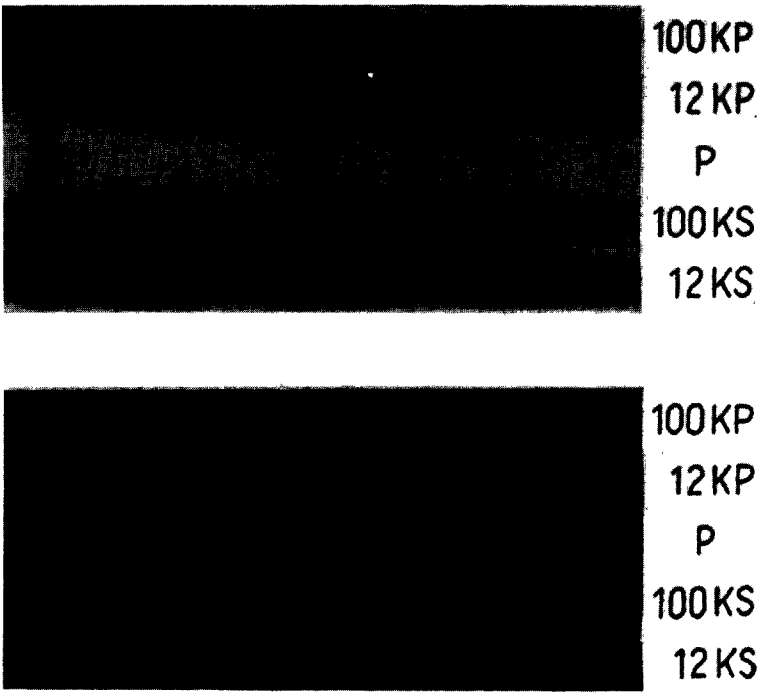

Fig. 2. Photoaffinity labeling by 8-N_3-cyclic AMP of soluble cell fractions of *D. discoideum*. Starved V12 cells harvested from agar plates were sonicated and the sonicate centrifuged at 12 000 × *g* for 10 min. The supernatant was then centrifuged at 100 000 × *g* for 60 min. The first pellet (12 KP) and second pellet (100 KP) and their respective supernatants (12 KS and 100 KS) were labeled with 8-N_3-cyclic AMP. The stained gel is on the top and the autoradiogram below. P is the set of standard proteins.

activity of the sonicate is found in the 100 000 × *g* supernatant whereas approximately one half of the measurable protein was removed from the supernatant. Attempts to label intact cells were unsuccessful, suggesting that labeled proteins described here are not external cell surface components, but are found in the interior of the cell, and in a soluble form.

Development of cyclic AMP-binding proteins

Cells of strains V12, KY19, and NC4 were harvested at various times of development and their cell extracts were analyzed by photoaffinity labeling. As seen in Fig. 3, lower panel, no labeled proteins were detected in vegetative cells, however, labeled proteins appeared and increased dramatically during development. The binding proteins observed in strain NC4 have a higher apparent molecular weight than the proteins in strain V12, the measured molecular weight of the NC4 proteins being 42 000 and 39 000. Additionally, strain NC4 showed changes in the relative amounts of the binding proteins with developmental time. All bands had similar cyclic nucleotide specificities.

Fig. 3, upper panel, is an autoradiogram with a 10-fold increased exposure compared to the lower panel. The same major protein bands seen in the lower panel are overexposed in the upper panel. The longer exposure revealed additional minor bands. No minor band seen in the upper panel of Fig. 3 represents greater than 0.1% of a major labeled protein. Some of these minor bands could

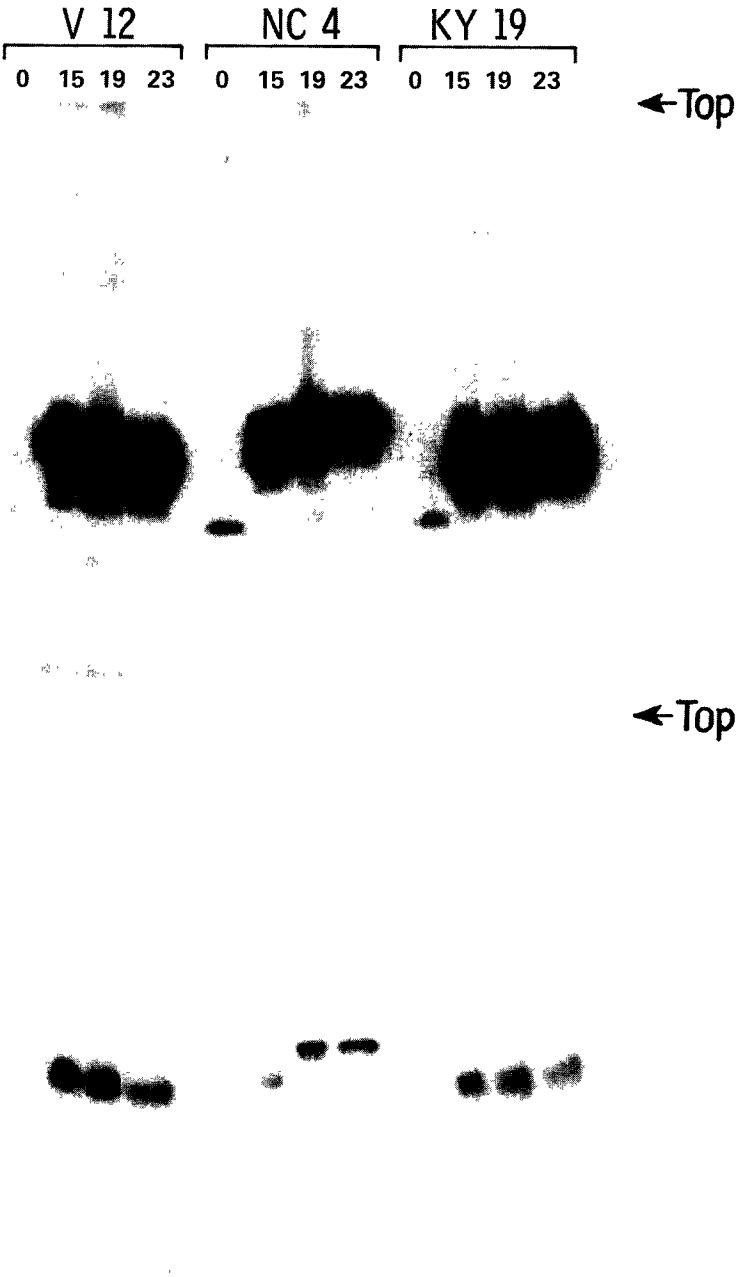

Fig. 3. Comparison of cyclic AMP binding proteins in different strains of *D. discoideum* during development. Two wild-type strains of *D. discoideum* were analyzed in the vegetative (unstarved) and starved states as well as a mutant of strain NC4 referred to as KY19. Each channel contained 200 μg of protein. The upper and lower autoradiograms are of the same gel and differ only in the time of autoradiography. The numbers indicate the hours of development of the cells on millipore pads, and 0 h indicates vegetative cells harvested immediately from the pads. The upper gel has 10-fold greater exposure.

represent proteolytic degradation products of the major protein or be functional in their own right. Of greater potential interest are the bands which are of higher molecular weight since their appearance cannot be explained by proteolytic degradation. The physiological significance of these bands is currently under investigation.

Cyclic AMP binding proteins in the mutant KY19

KY19 is a developmental mutant of NC4 which has been characterized as 'stalkless' [14] in that it makes only a rudimentary stalk and the spore heads rest on the developmental substrate. Studies of KY19 by Loomis et al. [15] revealed no deficiency in the synthesis of a number of developmentally regulated enzymes. Fig. 3 shows the absence in strain KY19 of the higher molecular weight binding protein appearing in strain NC4. This observation, to our knowledge, represents the first distinct biochemical alteration to be found in mutant KY19.

Discussion

The utility of the photoaffinity labeling method using 8-N_3-cyclic AMP to identify cyclic AMP receptor proteins has been demonstrated by several investigators [20—25]. We have utilized this method to examine the profile of cyclic AMP binding proteins during the life cycle of *D. discoideum*. The proteins that we have observed may not represent the total catalog of cyclic AMP binding proteins in this organism. Some proteins may be lost during the processing of the cells and others may be masked in such a way as to preclude photoaffinity labeling. However, the criteria for cyclic nucleotide specificity strongly suggested that the proteins we have described are specific cyclic nucleotide binding proteins. Competition experiments indicated they have a much greater affinity for cyclic AMP than cyclic GMP.

The cyclic AMP binding proteins described herein have the characteristics of developmental proteins. they are absent in unstarved, vegetative cells, but appear in the soluble portion of the cell following starvation and the induction of development. Hahn et al. [26] used photoaffinity labeling to study the cyclic AMP binding proteins of intact cells and identified two proteins (molecular weights 48 000 and 33 000) which appear on the surface of starved, but not vegetative, *D. discoideum*. We have been unable to find these cell surface proteins. Possible explanations for this discrepancy could lie in different experimental conditions and protocols. These cannot be compared at this time because the abstract describing the surface cyclic AMP binding activity [26] did not present the detailed experimental conditions.

The discovery that a mutant of *D. discoideum* NC4, strain KY19, is altered in the production of at least one cyclic AMP binding protein may be significant in the study of the physiological role of cyclic AMP in *Dictyostelium* development. Analysis of a number of developmentally regulated enzymes in this mutant [15] showed that all enzymes were present in both mutant and wild-type parental strains. The striking alteration in cyclic AMP binding proteins is, therefore, the first biochemical alteration observed in this mutant. Since KY19 produces a normal complement of spores but has no normal stalk

cells, our working hypothesis is that the high molecular weight cyclic AMP binding protein detected in NC4 may be associated with the differentiation of stalk cells.

The specific biochemical events associated with the cyclic AMP binding proteins are unknown at this time. Sampson [7] reported that *D discoideum* V12, starved in liquid for 1—2 h, contained two different cyclic AMP-activated protein kinases, and that these proteins could account for his observed cyclic AMP binding. However, Veron and Patte [12] and Rahmsdorf and Gerisch [13] have suggested that cyclic AMP binding and protein kinase activity are not obligatorily associated and, in fact, could be separated by various techniques. The discovery of a *Dictyostelium* mutant altered in cyclic AMP binding protein, which aggregates normally in response to cyclic AMP but shows altered differentiation, may allow the definition of a more precise role for cyclic AMP binding proteins in *Dictyostelium* development.

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References

1 Konijn, T M , Barkley, D A , Chang, Y.Y and Bonner, J T (1968) Am Nat 102, 225—233
2 Bonner, J.T (1970) Proc Natl Acad Sci U S A 65, 110—113
3 Town, C D , Gross, J D and Kay, R R (1976) Nature 262, 717—719
4 Chai, W K. (1975) Dev Biol 44, 239—252
5 Feit, I N , Fournier, G A , Needleman, R D and Underwood, M.A (1978) Science 200, 439—441
6 Kuo, J F and Greengard, P (1969) Proc Natl Acad Sci U S A. 64, 1349—1355
7 Sampson, J (1977) Cell 11, 173—180
8 Malchow, D and Gerisch, G (1974) Proc Natl Acad Sci U S A 71, 2423—2427
9 Green, A and Newell, P (1975) Cell 6, 129—136
10 Henderson, E J (1975) J Biol Chem 250, 4730—4736
11 Malkinson, A M , Kwasniak, J and Ashworth, J M (1973) Biochem J 133, 601—603
12 Veron, M and Patte, J.C (1978) Dev Biol 63, 370—376
13 Rahmsdorf, H J and Gerish, G (1978) Cell Differ 7, 249—257
14 Loomis, W F. (1970) J Bacteriol 103, 375—381
15 Loomis, W F , White, S and Dimond, R L (1976) Dev Biol 53, 171—177
16 Friedman, D.L and Chambers, D A (1978) Proc Natl Acad Sci U S A 75, 5286—5290
17 Laemmli, U K. (1970) Nature 227, 680—685
18 Brenner, M (1978) Dev Biol 64, 210—223
19 Soll, D R (1979) Science 203, 840—849
20 Haley, B E (1979) Biochemistry 14, 3852—3857
21 Haley, B.E. and Hoffman, J.R (1974) Proc Natl Acad Sci U S A 71, 3367—3371
22 Pomerantz, A H , Rudolph, S.A., Haley, B E and Greengard, P (1975) Biochemistry 14, 3858—3862
23 Walter, U , Uno, I , Liu, A Y and Greengard, P (1977) J. Biol Chem. 252, 6494—6500
24 Chambers, D.A. and Stubbs, J D (1976) Proc. 10th Int Congr Biochem. p 525
25 Dreyfuss, G , Schwartz, K J. and Blout, E R (1978) Proc Natl Acad Sci U.S A 75, 5926—5930
26 Hahn, G L , Metz, K , George, R P and Haley, B (1977) J Cell Biol 75, 41a