FEMS Microbiology Letters 18 (1983) 93–97
Published by Elsevier Biomedical Press

Degradation of a cAMP-binding protein of *Dictyostelium discoideum* by an endogenous protease

Stephen Cooper, Sarah Scanlon and Anne Ferguson

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.

Received 12 November 1982
Accepted 20 December 1982

1. INTRODUCTION

When cells of *Dictyostelium discoideum* are starved they aggregate to form multicellular organisms that eventually can differentiate into fruiting bodies of spores and stalk cells. cAMP plays a dual role in this process: it is the chemotactic agent that allows cells on a moist surface to aggregate to a common point to form the multicellular organism [1], and it is also involved in the process of differentiation. Pulsatile additions of cAMP stimulate cell differentiation [2,3]. High concentrations of cAMP can induce stalk cell formation [4,5] and, under certain conditions, spore cell production can be induced by cAMP [6,7]. Cyclic AMP has also been shown to induce differentiation in the multicellular aggregate [8]. Further evidence of the involvement of cAMP in differentiation is given by the finding that addition of cAMP to cells affects the expression of developmentally regulated proteins [9] and the expression of specific genes [10].

Receptors for cAMP have been identified by many workers. Membrane-associated cAMP-binding proteins have been reported [11–14], and soluble cAMP-binding proteins have also been identified [14–19]. There has been a report that the soluble cAMP-binding proteins are associated with protein kinase activity [16] but this report has not been confirmed [17,18]. Wallace and Frazier [14] reported that the soluble cAMP-binding proteins are of M_r 26 000, 33 000, and 36 000, but Cooper et al. [19] have reported that the M_r are higher, in the 36 000–40 000 range.

Here we describe our studies indicating that the endogenous cAMP-binding protein of M_r 40 000 is extremely sensitive to endogenous protease activity, and the lower M_r binding proteins are produced during the process of freezing the cells. When fresh extracts are used, only the 40 000 M_r binding protein is observed. Tos-LysCH_2Cl, an inhibitor of proteinase I of *Dictyostelium* [20,21] inhibits this degradative reaction.

2. MATERIALS AND METHODS

2.1. Materials

Tos-LysCH_2Cl was obtained from Sigma. 8-Azidoadenosine 3′,5′-cyclic monophosphate ([^{32}P]-8-N_3-cAMP) was purchased from ICN. Nitrocellulose membranes for the tritiated cAMP binding assay were obtained from Schleicher and Schuell.

2.2. Conditions of growth and development

D. discoideum strain AX3 was grown axenically in HL5 broth with shaking to a density of 2–5 × 10^6 cells/ml. The cells were harvested by centrifugation, washed twice with KK2 buffer (20 mM

KH_2PO_4/K_2HPO_4 (pH 6.1)/2 mM $MgSO_4$), suspended in KK2 buffer at a concentration of 10^7 cells/ml, and shaken at not over 90 rev./min in a New Brunswick rotary shaker. Starvation on water-agar or KK2 agar was also used. After development the cells were harvested and centrifuged in a Microfuge tube and the pellets were frozen at −70°C or used directly.

2.3. *Preparation of extracts*

To frozen or fresh pellets, 1.0 ml of TME buffer (0.01 M Tris–HCl (pH 7.4)/1 mM EDTA/5 mM 2-mercaptoethanol) was added, and the cells were lysed in a Branson sonicator with a microtip. The sonicates were immediately centrifuged for 2 min in a Microfuge and the supernatants were used directly for photoaffinity labeling. All of the above preparative steps were done at 0–4°C.

2.4. *Photoaffinity labeling*

The reaction mixture was cell extract in TME buffer and 3.2 nM 8-N_3-cyclic[^{32}P]AMP in a total volume of 0.5 ml. After a 30-min incubation on ice, the extracts were irradiated with UV light from a Scientific Products UV-54 lamp (short wavelength) for 5 min at a distance of 2–3 cm. The incubation and irradiation were carried out in subdued light, although control experiments indicate that exposure to room light from fluorescent lamps did not affect the labeling. After irradiation the extract was treated with ice-cold 10% trichloroacetic acid and centrifuged for 2 min in a Microfuge, and the pellet was taken up in sample buffer (62 mM Tris–HCl (pH 6.8) 2.3% $NaDodSO_4$/5% 2-mercaptoethanol/10% glycerol) and placed in boiling water for 2 min. Electrophoresis was performed (50–100 μg of protein/sample) according to the method of Laemmli as described in [19] using a 10% separating gel. Gels were stained in 0.05% Coomassie blue/25% isopropanol/10% acetic acid overnight, and destained in 10% isopropanol/10% acetic acid for 24 h. After the gels were dried onto Whatman 3 MM chromatography paper, autoradiography was carried out with Kodak XAR-5 film in Dupont intensifying screen cassettes at −70°C.

Hydrolysis of 8-N_3-cAMP was monitored by TLC on polyethyleneimine cellulose developed with 0.2 M lithium chloride. Spots were visualized with UV light and autoradiography.

2.5. *Protein assay*

Protein concentration was determined by using the Bio-Rad dye binding assay (measured at 595 nm).

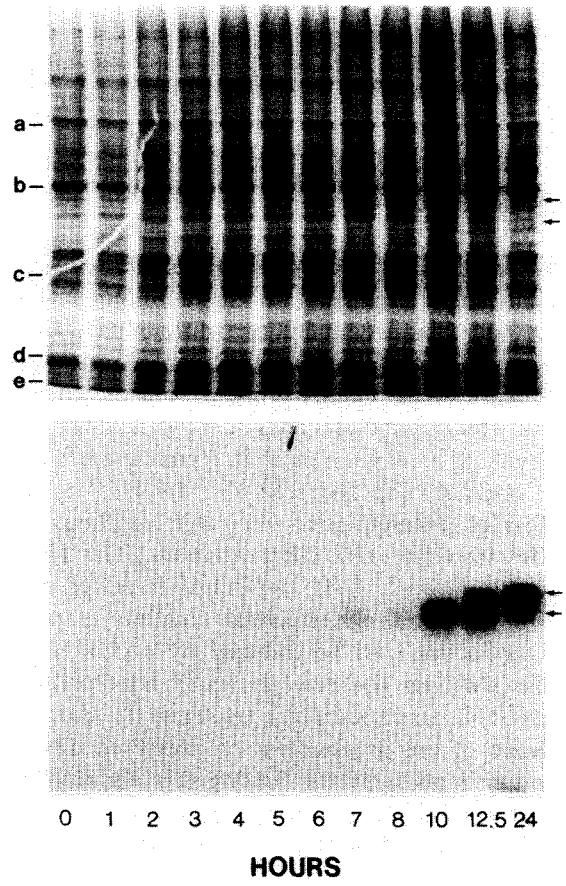

Fig. 1. Time course of appearance of cAMP-binding proteins during development of *D. discoideum* AX3. Cells were starved in KK2 buffer and harvested at the indicated times. Extracts were labeled with azido-cAMP and analyzed by electrophoresis and autoradiography. M_r-markers (a) bovine serum albumin (68000); (b) ovalbumin (42000); (c) phenylmethylsulfonyl fluoride-treated trypsinogen (24000); (d) β-lactoglobulin (18400); and (e) lysozyme (14300). The arrows indicate autoradiographic bands of M_r 40000 and 37000. No labeling of actin is observed.

3. RESULTS

3.1. The photoaffinity assay

Our photoaffinity-labeling assay includes 5 mM 2-mercaptoethanol as a reducing agent. Pannbacker and Bravard [22] have shown that dithiothreitol and other reducing agents inhibit the cAMP-phosphodiesterase of *Dictyostelium*. That our labeling conditions inhibit phosphodiesterase activity is supported by the observation that photoaffinity labeling is completely inhibited by a 100-fold excess of cAMP but not by equivalent amounts of 5'AMP. Further, extracts did not hydrolyze the 8-N_3-cAMP to the 5'AMP derivative as measured by TLC. The cAMP-binding proteins, as measured by photoaffinity labeling, coincide in elution behavior from a DEAE-cellulose column with the cAMP-binding activity measured by using the Millipore filter method with tritiated cAMP. In all of our experiments, we have found a correspondence between the cAMP-binding protein assayed using tritiated cAMP and the binding protein assayed by photoaffinity labeling.

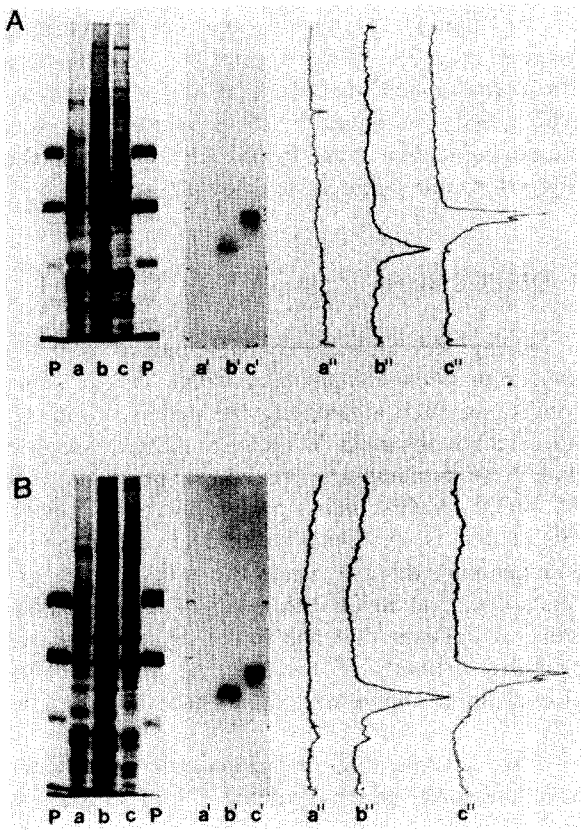

Fig. 2. Conversion of binding proteins before and after photoaffinity labeling. (A) A 1-h starved sample and a 14-h starved sample were analyzed before and after mixing as described in Fig. 1. The (a) slots contained the 1-h sample, the (b) slots the mixture and the (c) slots the 14-h sample. At the left is the Coomassie Blue stained material, in the center the corresponding autoradiograms, and at the right densitometer tracings of the autoradiograms; (B) the sample extracts as in (A) above were used. The 1-h and 14-h starved material was labeled with 8-N_3-[^{32}P]cAMP as described, and then equal aliquots were mixed and incubated for 30 min at 0°C. The samples were then precipitated and analyzed by gel electrophoresis and autoradiography. The slots were labeled in the same way as in (A) above.

3.2. Development of cAMP-binding proteins

When cells starved for 24 h in liquid, on filter pads or on agar, are assayed for cAMP-binding proteins by using the photoaffinity labeling method, a sharp band is seen at the app. M_r of 40000 on NaDodSO$_4$/polyacrylamide gels. In vegetative cells and at early times of development, no cAMP-binding proteins are observed. At later times (approximately 9–11 h) a low-M_r protein (M_r 37000) can be observed. Examples of the appearance of the cAMP-binding proteins during development are presented in Fig. 1. These experiments were done with cells which were frozen prior to sonication and labeling with 8-N_3-cAMP. When fresh extracts were used, no lower M_r binding proteins were observed. This was true even for the earliest times that the cAMP-binding proteins were observed. A similar observation has been made in [24].

3.3. In vitro degradation of the high M_r cAMP-binding protein

We investigated the possibility that the low-M_r binding proteins were produced as breakdown products of the high-M_r protein. We systematically mixed extracts of vegetative cells or extracts of cells starved for < 3 h with extracts from cells that had been starved for a longer time. We found that there was a specific degradation of the high-M_r

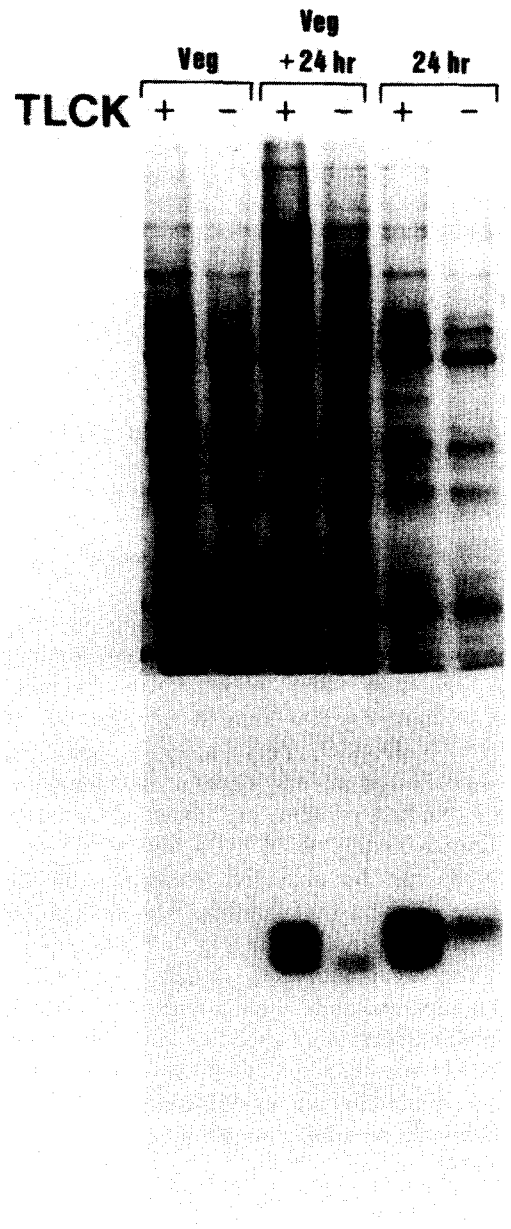

Fig. 3. Effect of Tos-LysCH_2Cl on the conversion of high-M_r cAMP-binding protein to low-M_r binding protein by extracts of vegetative cells. Extracts prepared by sonication with (lanes 1, 3, and 5) or without (lanes 2, 4, and 6) 1 mM Tos-LysCh_2Cl were mixed or left unmixed for 30 min after adding 8-N_3-cAMP. The samples were then irradiated and analyzed by electrophoresis and autoradiography. Lanes: 1 and 2, vegetative cells; 3 and 4, vegetative/24-h cells; 5 and 6, 24-h cells.

protein to a lower M_r protein. When an extract was first labeled with 8-N_3-cAMP and then mixed with the early extracts the same degradation was observed (Fig. 2). The breakdown is stoichiometric in that the same amount of label appears in the low-M_r band is found in the undegraded high-M_r band. It thus appears that most of the material produced by digestion with the vegetative extracts is still able to bind and be labeled with 8-N_3-cAMP.

Addition of Tos-LysCH_2Cl inhibits breakdown of the high-M_r protein to the low-M_r binding protein (Fig. 3). When vegetative extracts were chromatographed on DEAE-cellulose, the proteolytic activity (measured by the protease assay) was eluted coincident with the ability to cleave the high-M_r binding protein to a low-M_r species.

4. DISCUSSION

During development cAMP-binding proteins appear in *Dictyostelium discoideum*. Under some conditions, such as freezing the pellets, lower M_r proteins are observed. We have presented evidence that these proteins are breakdown products of a M_r 40 000 cAMP-binding protein. As noted below, this protein is very sensitive to degradation by an endogenous protease. Since the use of fresh extracts does not reveal the lower M_r binding proteins, it appears that the lower M_r proteins are probably artifacts of the freezing process. This also explains the results of Wallace and Frazier [14].

If we assume that the proteolytic activity we have measured is the Proteinase I of Gustafson and Thon [21], it is interesting to compare the relative activity of this protease on the cAMP-binding proteins compared with the activity on other enzymes. Gustafson and Thon reported that, with 30 min of incubation at 25°C, purified Proteinase I at 48 μg/ml inactivates 65% of UDPglucose pyrophosphorylase activity. This enzyme was the most sensitive of 11 enzymes studied. If Proteinase I is present in crude extracts at 1.0% (w/w) then this is equivalent to 65% inactivation of UDPglucose pyrophosphorylase with 4.8 mg/ml of crude extract. We find that adding extract at 1 mg/ml leads to complete conversion, at 0°C, of

all of the high-M_r cAMP-binding protein to low-M_r binding protein without any observable change in the protein pattern shown by Coomassie Blue staining of polyacrylamide gels. If the Proteinase I was present at 0.1% of the crude extract, this would be equivalent to adding 48 mg/ml of extract to achieve less breakdown at a higher temperature than we observe when using cAMP-binding protein as a substrate. This analysis suggests that the high-M_r binding protein is much more sensitive to the protease than other *Dictyostelium* proteins.

ACKNOWLEDGEMENTS

This investigation was supported by grant DCM-77-14883 from the National Science Foundation, grants from the University of Michigan Cancer Committee and the Phoenix Memorial Fund of the University of Michigan, and Grant 5 R01 GM28208 from the National Institutes of Health.

REFERENCES

[1] Loomis, W.F. (1975) *Dictyostelium discoideum,* a Developmental System. Academic Press, New York.
[2] Darmon, M., Brachet, P. and Pereira da Silva, L. (1975) Proc. Natl. Acad. Sci. USA 72, 3163–3166.
[3] Gerisch, G., Fromm, H., Huesgen, A. and Wick, U. (1975) Nature 255, 547–549.
[4] Bonner, J.T. (1970) Proc. Natl. Acad. Sci. USA 65, 110–113.
[5] Town, C.D., Gross, J.E. and Kay, R.R. (1976) Dev. Biol. 63, 412–420.
[6] Kay, R.R., Garrod, D. and Tilly, R. (1978) Nature 271, 58–60.
[7] Kay, R.R. (1979) J. Embryol. Exp. Morphol. 52, 171–182.
[8] Feit, I.N., Fournier, G.A., Needleman, R.D. and Underwood, M.A. (1978) Science 200, 439–441.
[9] Sampson, J., Town, C. and Gross, J. (1978) Dev. Biol. 76, 54–64.
[10] Landfear, S.M. and Lodish, H.F. (1980) Proc. Natl. Acad. Sci. USA 77, 1044–1048.
[11] Malchow, D. and Gerisch, G. (1974) Proc. Natl. Acad. Sci. USA 71, 2423–2427.
[12] Henderson, E. (1975) J. Biol. Chem. 250, 4730–4735.
[13] Green, A.A. and Newell, P.C. (1975) Cell 6, 129–136.
[14] Wallace, L.J. and Frazier, W.A. (1979) Proc. Natl. Acad. Sci. USA 76, 4250–4254.
[15] Malkinson, A.M., Kwasniak, J. and Ashworth, J.B. (1973) Biochem. J. 133, 601–603.
[16] Sampson, J. (1977) Cell 11, 173–180.
[17] Veron, M. and Patte, J.-C. (1978) Dev. Biol. 63, 370–376.
[18] Rahmsdorf, H.J. and Gerisch, G. (1978) Cell Differ. 7, 249–257.
[19] Cooper, S., Chambers, D.A. and Scanlon, S. (1980) Biochim. Biophys. Acta 629, 235–242.
[20] Fong, D. and Bonner, J.T. (1979) Proc. Natl. Acad. Sci. USA 76, 6481–6485.
[21] Gustafson, G.L. and Thon, L.A. (1979) J. Biol. Chem. 254, 12471–12478.
[22] Pannbacker, R.G. and Bravard, L.J. (1972) Science 175, 1014–1015.
[23] Fong, D. and Rutherford, C.L. (1978) J. Bact. 134, 524–527.
[24] Leichtling, B.H., Majerfeld, I.H., Coffman, D.H. and Rickenberg, H.V. (1982) Biochem. Biophys. Res. Commun. 105, 949–955.