Vol. 90, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 12, 1979 Pages 933-939

IDENTIFICATION AND CHARACTERIZATION OF THE cAMP BINDING PROTEINS OF YEAST BY PHOTOAFFINITY LABELING

Charlotte Dery, Stephen Cooper, Michael A. Savageau and Sarah Scanlon

Department of Microbiology and Immunology
The University of Michigan,
Ann Arbor, Michigan 48109

Received August 15,1979

SUMMARY

Contrary to previous reports, we find the molecular weight of the major cAMP-binding protein in Saccharomyces cerevisiae to be 54,000 daltons. A number of lower molecular weight proteins have also been identified and are believed to be breakdown products of the major protein. The binding protein is soluble and specific for cyclic purine nucleotides with a 3':5' configuration.

INTRODUCTION

3':5'-Adenosine monophosphate (cAMP) is implicated in a wide variety of biological phenomena. There is convincing evidence that cAMP exerts its influence by activating specific protein kinases in the cell (1). In hormone-sensitive cells it is believed that the hormone activates production of intracellular cAMP, that this leads to phosphorylation of specific proteins, and that the specific pattern of phosphorylated proteins is ultimately expressed in the biological response of the target cell. A cAMP-dependent protein kinase has been reported in yeast (2,3) but its function remains to be determined.

cAMP-binding proteins in yeast have been described in a number of conflicting reports. Sy and Richter (4) described a cAMP-binding protein and determined its molecular weight to be 24,000 by sucrose density gradient centrifugation. They also mentioned a protein with minor cAMP-binding activity that had a "higher molecular weight". Takai, Yamamura and Nishizuka (2), using gel exclusion chromatography, reported a molecular weight of 28,000 for a cAMP-binding protein that was released from purified protein kinase of yeast. They

0006-291X/79/190933-07$01.00/0

further suggested that the binding protein is comprised of two subunits, each with a molecular weight of 14,000. Hixson and Krebs (3) used affinity chromatography to purify, from yeast, a cAMP-binding protein having a molecular weight of 50,000 by polyacrylamide gel electrophoresis.

We have used photoaffinity labeling to identify and characterize the cAMP-binding proteins of yeast. The dominant protein has a molecular weight of 54,000 daltons and specifically binds cAMP.

MATERIALS AND METHODS

Preparation of cell-free extracts: Cultures of Saccharomyces cerevisiae (strain c 246 4A) were grown at 25°C in YPD media (yeast extract (1%), peptone (2%) and dextrose (2%)) while shaking at 175 rpm. Cells were harvested by centrifugation, resuspended in Buffer A (10 mM Tris-HCl, 1 mM EDTA, 6 mM mercaptoethanol, pH 7.4) and disrupted in a French Press Cell at 18,000 psi. Unbroken cells and other debris were removed by centrifugation at 10,000 x g for 10 minutes; the supernatant was frozen in separate aliquots at -70°C. Protein concentrations were determined by the dye binding method using a protein assay kit (Bio-Rad).

Photoaffinity labeling: The cAMP-binding proteins were labeled in the cold under subdued light. $8\text{-}N_3\text{-}[^{32}P]$ cAMP (6.2×10^{-8} M final concentration; 27-56 Ci/mmole; purchased from ICN) was added to 0.5 ml of the extract diluted to 2.0 mg/ml. The reaction mixture was incubated for 20 minutes and then irradiated from a distance of 4-6 cm for 5 minutes with a Mineralite short wave ultra-violet lamp (UVS-54). After irradiation the proteins were precipitated with trichloracetic acid at a final concentration of 10%. The precipitates were collected by centrifugation and prepared for electrophoretic analysis.

Electrophoresis and Autoradiographic analysis: The precipitates were resuspended in Buffer B (62 mM Tris-HCl, 2.3% sodium dodecyl sulfate, 5% mercaptoethanol, pH 6.8) to achieve a protein concentration of 5 mg/ml and boiled for 1 minute. The protein (.02 ml) was then analyzed by electrophoresis on a 10% polyacrylamide gel according to the method of Laemmli (5). Following electrophoresis, the gels were stained with Coomassie blue, destained (7.5% acetic acid), dryed on Whatman 3MM filter paper, and autoradiographed. Autoradiograms were scanned with a Joyce-Loeble densitometer.

Millipore filter-binding assay: The cAMP-binding activity of the crude extracts was measured quantitatively by the method of Gilman (6), with the following modifications. $[^3H]$cAMP (27 Ci/mmole, Amersham, Inc.) was added to 0.2 ml of the crude extract (2 mg/ml) to yield a final concentration of 5.2×10^{-7} M. This mixture was incubated for 30 minutes at 0°C, and filtered on a 0.45 µm Millipore filter. The filter was then washed with 25 ml of Buffer C (0.2 M KPO_4, pH 6.4), dried, and counted in a Beckman scintillation counter using Liquifluor (New England Nuclear).

RESULTS

Photoaffinity labeling of cAMP-binding proteins in yeast: Photoaffinity labeling of cAMP-binding proteins using $8\text{-}N_3\text{-}[^{32}P]$ cAMP has been used success-

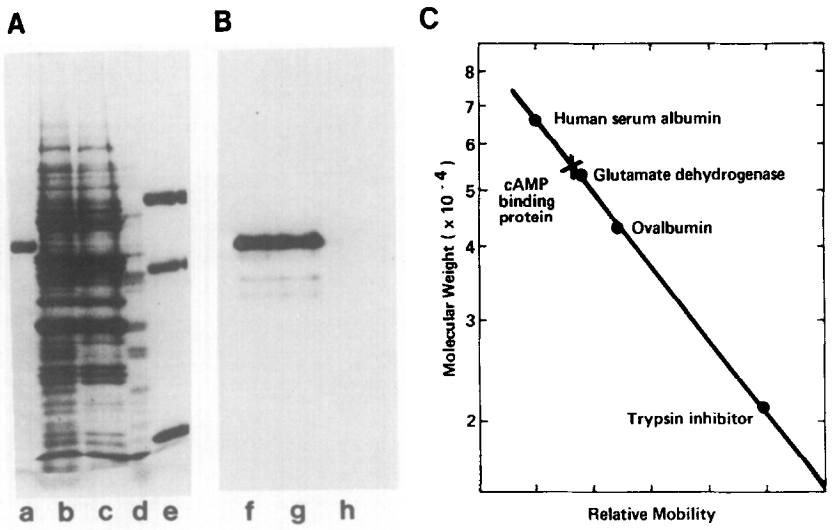

Fig. 1. Characterization of the cAMP-binding proteins of yeast. A. Proteins subjected to gel electrophoresis in SDS and stained with Coomassie blue: (a) glutamate dehydrogenase, 53,000 daltons; (b) total yeast extract; (c) supernatant and (d) pellet after centrifugation of the total extract at 100,000 x g for 60 minutes; and (e) human serum albumin, 66,000 daltons, ovalbumin, 43,000 daltons, and trypsin inhibitor, 21,000 daltons. B. Autoradiogram of the gel to detect the proteins labeled with 8-N_3-[^{32}P]cAMP: (f) total yeast extract; (g) supernatant and (h) pellet after centrifugation. C. Determination of the apparent molecular weight of the major cAMP-binding protein. The X indicates the cAMP binding protein.

fully by several investigators (7-12). This method covalently labels the cAMP-binding proteins, and thus allows the identification of a multiplicity of binding proteins. Other methods may tend to show the dominant binding protein to the exclusion of minor binding activities.

Extracts of yeast were prepared, labeled with 8-N_3-[^{32}P]cAMP and the proteins separated as described in the MATERIALS AND METHODS. The results are shown in Figure 1. As seen in Figure 1, slot f, there is a major labeled protein with a molecular weight of 54,000 daltons (Figure 1C). A number of minor bands which represent lower molecular weight proteins are also observed. The relationship of these minor bands to the major cAMP-binding protein will be discussed below.

Cellular location of cAMP-binding proteins: The crude extract from the French press was subjected to centrifugation and the pellet and supernatant analyzed as shown in Figure 1. The binding proteins are found in the

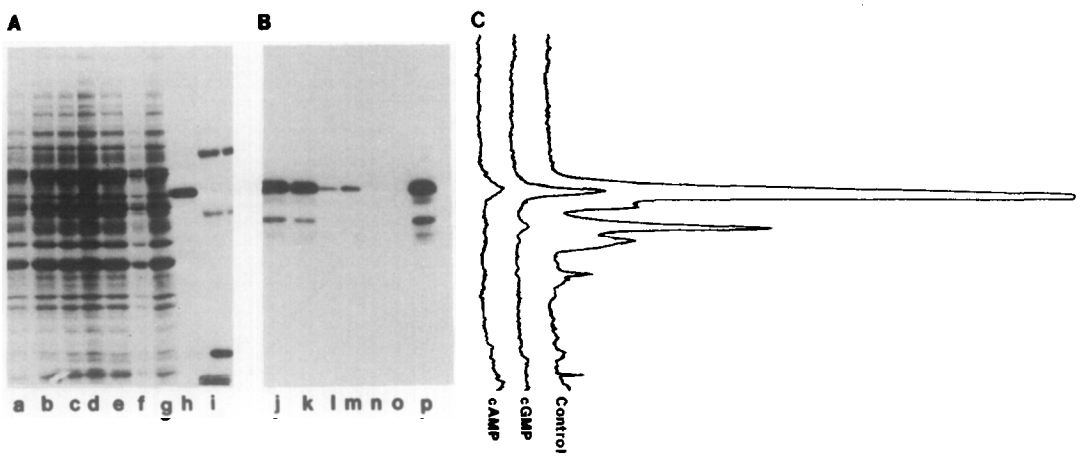

Fig. 2. Determination of the specificity of the binding proteins using unlabeled nucleotides as inhibitors. Proteins were photoaffinity labeled as described in Materials and Methods. The labeling mixture contained $8\text{-}N_3\text{-}[^{32}P]$cAMP at a concentration of 6.2×10^{-8} M plus unlabeled nucleotides as described below. A. Proteins subjected to gel electrophoresis in SDS and stained with Coomassie blue: (a-f) represent the addition of unlabeled cGMP at concentrations of 6.2×10^{-7} M, 6.2×10^{-6} M, and 6.2×10^{-5} M and unlabeled cAMP at concentrations of 6.2×10^{-7} M, 6.2×10^{-6} M, 6.2×10^{-5} M, respectively. (g) represents the control, with no unlabeled nucleotide added. Standard proteins are; (h) glutamate dehydrogenase, 53,000 and (i) human serum albumin, 66,000, ovalbumin, 43,000, and trypsin inhibitor, 21,000. B. Autoradiogram of the gel: (j-p) corresponds to (a-g). C. Densitometer tracings of the autoradiograms for the control (g), 6.2×10^{-5} M cGMP (c) and 6.2×10^{-5} M cAMP (f).

supernatant (slot g) following high-speed centrifugation (100,000 x g for 60 minutes) whereas the pellet (slot h) has no significant binding activity.

Specificity of Binding: The specificity of binding with $8\text{-}N_3\text{-}[^{32}P]$cAMP was analyzed by measuring binding in the presence of unlabeled nucleotides. At high concentrations (1,000 times the azido-cAMP concentration) both 3':5'cAMP and 3':5'cGMP inhibited binding. There was no observable inhibition with 5'AMP, 5'GMP, 5'ADP, 5'ATP, 3'AMP, 2':3'cAMP, or 2':3'cGMP. More quantitative measurements of the inhibition by 3':5'cGMP and 3':5'cAMP are shown in Figure 2. At low concentrations of cGMP there is essentially no inhibition while at these same concentrations, cAMP is a potent inhibitor. The densitometer tracings of Fig. 2 show that cAMP at high concentrations reduces the binding by greater than 95%, whereas cGMP (at the same concentration) only reduces the binding by 84%. In studies using the Millipore filter binding assay, the results are similar in all cases except for ATP which showed an apparent

inhibition. This may be due to the production of cAMP by adenylate cyclase in the crude extract. The binding activity was determined to be 2.24 pmole cAMP bound per mg protein.

Minor cAMP-binding proteins: As noted above, in addition to the major binding protein of molecular weight 54,000, there are a number of minor binding proteins of lower molecular weight which range from 51,000 to 35,000 daltons. Although they may be present and functional in the intact cell, we suggest that they are proteolytic products of the major binding protein which retain the binding site for cAMP. This conclusion is consistent with the following evidence: 1) Variability is observed in the lower molecular weight binding proteins in different preparations, 2) although approximately half of the total protein is above the 54,000 molecular weight range, as seen in Coomassie blue stained gels, all of the binding proteins are equal to or less than 54,000 daltons. 3) the presence of proteases in yeast is extensively documented (13), and 4) Hixson and Krebs (personal communication) find proteolytic enzymes in crude extracts of yeast that are able to destroy the cAMP-binding protein. Treatment of the labeled proteins with 10 times the normal concentrations of sodium dodecyl sulfate and mercaptoethanol and increasing the boiling time to 10 minutes did not lead to any loss of the 54,000 molecular weight protein. This indicates that the 54,000 dalton protein is not made up of dissociable subunits.

cAMP-Binding Proteins in Different Cell Types: The cAMP-binding proteins were detected in other yeast strains including different mating types, mutants of these mating types, and diploid strains. All exhibit the major 54,000 daltons binding protein (data not shown).

DISCUSSION

These studies on photoaffinity labeling of the cAMP-binding proteins of yeast have clarified the physical properties of the binding protein. Early studies reported very low molecular weights for the binding protein. The results of Sy and Richter (8) in which a molecular weight of 24,000 was

assigned to the binding protein may have been due to the non-globular shape of the protein leading to an underestimate of its molecular weight. Similarly, gel exclusion chromatography as used by Takai, et al., (2) may have led to underestimates of the molecular weight. Retardation of the binding protein may have resulted from adherance to the Sephadex. A more likely explanation of these results, however, is the presence of proteases that break down the binding protein to yield products that still bind cAMP. Pringle has presented extensive evidence that even highly purified yeast proteins have proteolytic contaminants (13). The proteins of lower molecular weight that are reported here may or may not be functional, but we feel that extensive handling of extracts as required by purification procedures undoubtedly will lead to extensive breakdown of the binding protein.

The function of the cAMP-binding protein in yeast appears to be analogous to that of mammals. For example, both yeast and rat liver cAMP-binding protein can inhibit homologous or heterologous protein kinase (2). There may also be structural similarities, as a molecular weight of 54,000 daltons has been reported for the regulatory subunit of cAMP-dependent protein kinase in bovine heart (10). Since yeast can be easily manipulated, both physiologically and genetically, it may be an excellent model system for studies of cAMP-dependent protein kinase.

Acknowledgments: The authors would like to thank Stacy Stachler for typing the manuscript. This work was supported in part by grants from The National Science Foundation (DCM 77-14883 to S.C., PCM 7807635 to M.A.S.) and The University of Michigan Cancer Research Institute to M.A.S. and S.C. and Phoenix Memorial Grant (The University of Michigan) to S.C.

REFERENCES

1. Greengard, P. (1978). Science 199:146-152.
2. Takai, Y., Yamamura, H., and Nishizuka, Y. (1974). J. Biol. Chem. 249:530-535.
3. Hixson, C.S., and Krebs, E. (1979). International Congress of Biochemistry Abstracts p. 259.
4. Sy, J., and Richter, D. (1972). Biochemistry 11:2784-2787.
5. Laemmli, V.K. (1970. Nature 227:680-685.
6. Gilman, A.G. (1970). Proc. Nat. Acad. Sci. U.S.A., 67:305-312.
7. Haley, B.E. (1979). Biochemistry 14:3852-3857.
8. Haley, B.E., and Hoffman, J.F. (1974). Proc. Nat. Acad. Sci. U.S.A. 71:3367-3371.

9. Pomerantz, A.H., Rudolph, S.A., Haley, B.E., and Greengard, P. (1975). Biochemistry 14:3858-3862.
10. Walter, V., and Greengard, P. (1978). J. Cyclic Nuc. Res. 4:437-444.
11. Freidman, D.L., and Chambers, D.A. (1978). Proc. Nat. Acad. Sci. U.S.A. 75:5286-5290.
12. Cooper, S., Chambers, D.A., and Scanlon, S. (1979). (Submitted for publication.)
13. Pringle, J.R. (1975). Methods in Cell Biology, XII:149-184.