Vol. 95, No. 2, 1980
July 31, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 701-706

CYCLIC AMP-DEPENDENT PROTEIN KINASE OF NEUROSPORA CRASSA

Patricia A. Powers and Martin L. Pall*

Program in Genetics and Program in Biochemistry and Biophysics
Washington State University, Pullman, WA 99164 (U.S.A.)

Received June 16,1980

SUMMARY

Neurospora crassa was surveyed for cyclic AMP-dependent protein kinase activity. Two peaks (I and II) of protein kinase activity were demonstrated by DEAE-cellulose chromatography of wild type Neurospora extracts. Peak I was stimulated by cyclic AMP,eluted below 60 mM NaCl and had high activity using histone H2B as substrate. Peak II eluted at 200-250 mM NaCl; its activity was not cyclic AMP stimulated and was highest with dephosphorylated casein as a substrate. Cyclic AMP binding to a protein associated with the protein kinase is specifically inhibited by certain cyclic AMP analogs.

INTRODUCTION

Among lower eukaryotes, Neurospora crassa is a uniquely attractive organism for studies of cyclic AMP function. Features providing such attractiveness include the crisp-1 and frost adenylate cyclase deficient mutants [1,2] and the apparent control of endogenous cyclic AMP levels by the electrical potential across the plasma membrane [3,4].

The in vitro properties of both adenylate cyclase and cyclic AMP phosphodiesterase have been described [5,6]. However, no studies have been reported of possible cyclic AMP receptors in Neurospora. Such receptors might be similar to the cyclic AMP binding proteins devoid of protein kinase activity of bacteria or to the cyclic AMP-dependent protein kinases of animal cells. Some investigators have suggested that cyclic AMP action in Neurospora may be similar to that in mammalian cells [7] while others have proposed similarities to cyclic AMP action in Escherichia coli [8,9].

The presence and some properties of a cyclic AMP-dependent protein kinase of Neurospora are described below.

*To whom correspondence should be addressed.

0006-291X/80/140701-06$01.00/0

MATERIALS AND METHODS

An inoculum of 1 x 10^5 wild type (St. L. 74A) Neurospora crassa or crisp-1, B123 conidia/ml was grown at 29-30°C in one liter of liquid medium in a 2800 ml Fernbach flask containing 1.5% (w/v) sucrose and Vogel's medium N [10]. The culture was shaken at 250 rpm, harvested at late logarithmic phase, at 24 hours, and frozen at -20°C.

Neurospora mycelia were ground in a frozen mortar with an equal weight of acid washed sand for 10 min. Two volumes (w/v) of 5 mM Tris, pH 7.5, 2 mM EDTA, 2 mM NaF (Buffer A) containing 0.5 mM phenyl methyl sulfonyl fluoride and 0.5 mM pepstatin A were added and the homogenate was incubated at 4°C for ten min. while stirring slowly. The homogenate was centrifuged at 10,000 x G for five min. Two mls of the supernatant containing 8 to 25 mg protein/ml were applied to a DEAE cellulose (Whatman DE-52) column (15 x .9 cm) previously equilibrated with Buffer A (See ref. 11). The columns were washed with 50 mls of Buffer A and the proteins were eluted at 6 ml/hr with a linear gradient consisting of 18 mls of buffer A and 18 mls of 0.4 NaCl in Buffer A containing 10% glycerol. One ml fractions were collected. All steps were carried out at 4°C.

Protein kinase activity was determined in a total volume of 70 µl containing 16.5 mM Na_3PO_4, pH 6.8, 12 mM Mg acetate, 25 µg histone H2B, 22 µM ATP including 12.5 nCi (γ-^{32}P) ATP, 20 µl protein solution, and where appropriate, 10 µM cyclic AMP. The reaction was initiated by the addition of ATP and was allowed to proceed at 30°C for 10 min. The reaction was stopped and the precipitate washed as described by Walsh et al. (12).

The (γ-^{32}P) ATP was synthesized by a modification of the method of Glynn and Chappell [13]. The incubation mixture contained 60 µmoles of ATP in a ten ml volume. The incubation was terminated by the addition of one ml of 1 N HCl followed by the addition of 250 mg Norite. This suspension was filtered through two 0.45 µ millipore filters, and the charcoal was washed with 30 mls of 0.15 M ammonium hydroxide in 50% ethanol to extract the ATP, and the ATP solution was dried by rotary evaporation.

The cyclic AMP binding activity was assayed by the Gill and Walton procedure [14] except that the reaction buffer consisted of 50 mM Bis Tris pH 7.5, 20 mM $MgCl_2$, 16 mM theophylline, 50 mM Na acetate and 10 nM ^{3}H cyclic AMP. Samples were incubated at 0°C for 30 minutes. The radioactivity of the sample was determined by using a scintillation counter and scintillation fluid described earlier [15].

The radioactivity of the aqueous samples was determined by dissolving one to one and one-half volumes of radioactive fluid in ten volumes of Triton X-100 scintillation fluid containing two volumes of 0.4% (w/v) PPO and 0.01% (w/v) dimethyl POPOP dissolved in toluene to one volume of Triton X-100. Protein was determined by the method of Lowry et al. [16] using bovine serum albumin as the standard. Dephosphorylation of casein was done by the method of Reimann et al. [17].

All biochemical reagents were obtained from the Sigma Chemical Company, with the exception of cyclic AMP from ICN Pharmaceuticals, Inc., histone H2B (f2b) from Worthington Biochemical Corporation, ^{32}P-labeled inorganic phosphate and ^{3}H-labeled cyclic AMP from ICN Pharmaceuticals, Inc. and New England Nuclear, and DEAE-cellulose (DE-52) from Whatman Chemical Company.

RESULTS AND DISCUSSION

Neurospora crassa demonstrates two protein kinase activities upon the elution of the 10,000 x G supernatant from DEAE-cellulose. One peak of cyclic

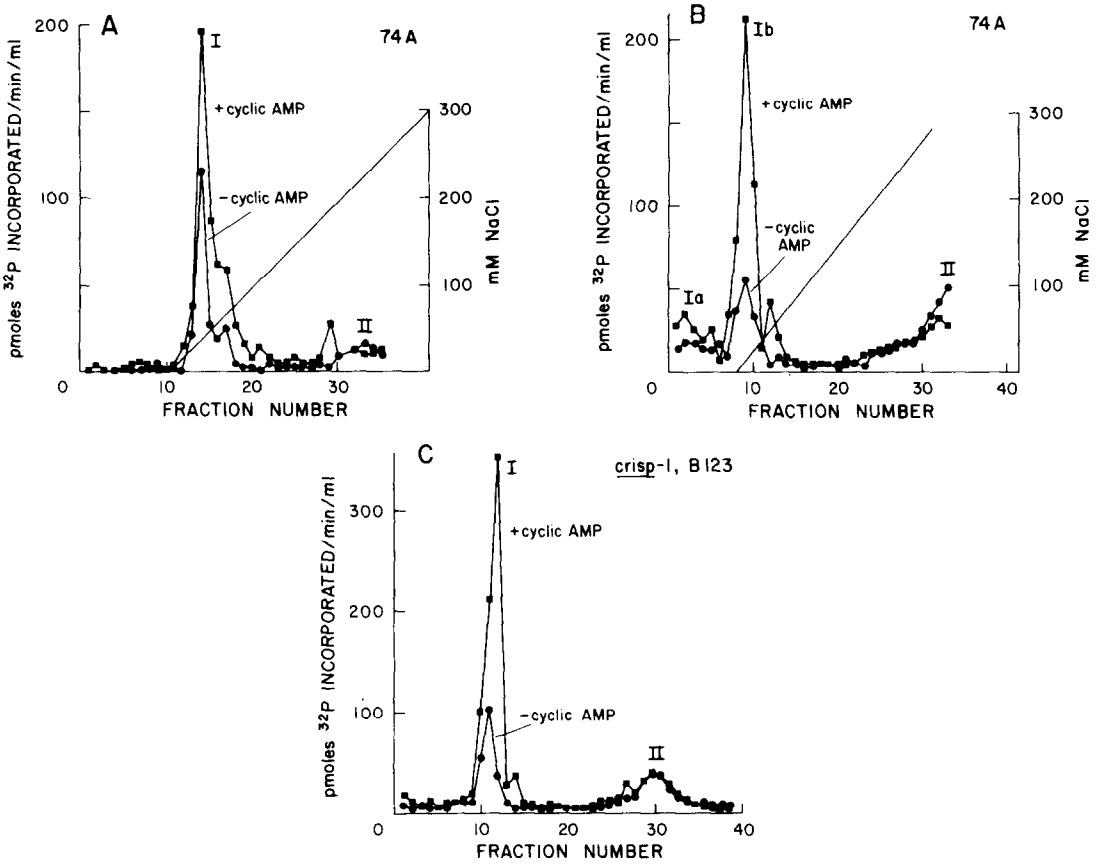

Fig. 1. Protein Kinase Activity from Neurospora. The protein kinase activity eluted from DEAE cellulose is shown when assayed with and without 10 μM cyclic AMP. Extracts from wild type 74A (a and b) and cr-1, B123 (c) were used. The NaCl elution gradients are also shown in a and b.

AMP-dependent protein kinase activity was detected (Fig. 1). The peak I was highly stimulated by cyclic AMP and was eluted at less than 60 mM NaCl. In some experiments, peak I appeared two peaks (Ia and Ib), both showing cyclic AMP dependence and both eluting at low salt concentrations (Fig. 1b). Peak II, which elutes at 250 mM NaCl does not appear to be stimulated by cyclic AMP. Both peaks of protein kinase occurred in wild type and a crisp-1, adenylate cyclase mutant (Fig. 1). The results suggest that although the crisp-1 mutant is cyclic AMP deficient, the receptor for cyclic AMP is intact in the mutant.

TABLE 1
SUBSTRATE SPECIFICITY OF PEAK I AND II NEUROSPORA PROTEIN KINASES

	pmole ^{32}P incorporated/min/ml		
	Peak I		Peak II
Protein substrate	+cAMP	-cAMP	-cAMP
Histone H2B	146.2	17.7	20.7
Mixed histone, Sigma Type (II-A)	12.8	2.7	7.0
Phosvitin	3.73	1.3	30.8
Dephosphorylated casein	12.5	3.6	75.9
Protamine sulfate	36.8	3.3	13.6
Histone H2B and 4 units of bovine heart protein kinase inhibitor	70.5	----	----

Enzyme activity was assayed under standard conditions except that 25 μg Histone H2B or 480 μg of other acceptor proteins were added.

Little protein kinase activity was found in the flow through or wash fractions. It was necessary to prepare the homogenate in low ionic strength buffer to detect cyclic AMP-stimulated protein kinase activity possibly because, as reported with animal cells, the regulatory and catalytic subunits are dissociated in high salt [11].

As shown in Table I, the peak I cyclic AMP-dependent protein kinase will utilize histone H2B as a substrate, but mixed histones, bovine serum albumin, phosvitin and protamine sulfate were less suitable substrates for the enzyme. The cyclic AMP-independent protein kinase of peak II demonstrates a preference for dephosphorylated casein as a substrate over the other substrates tried.

A heat stable inhibitor protein which acts directly on the free catalytic subunit of the mammalian cyclic AMP-dependent protein kinase to form an inhibitor-catalytic subunit complex was discovered by Walsh and coworkers [18, 19]. Addition of 4 units of protein kinase inhibitor to 50 μg of protein of peak I (Table I) inhibited over 50% of normal kinase activity.

A cyclic AMP binding activity was also found in extracts of Neurospora. Various nucleotides and adenosine were used to measure the specificity of the cyclic AMP binding activity (Table II). The cyclic AMP binding activity has properties similar to those of mammalian cyclic AMP-dependent protein kinases [20], including a high specificity for cyclic AMP and and substantial apparent

TABLE II
SPECIFICITY OF CYCLIC AMP BINDING ACTIVITY

Addition	(fraction of binding with no addition)
None	(1.00)
Cyclic AMP	0.097
Cyclic GMP	0.59
8-bromo cyclic AMP	0.80
N^6-monobutyryl cyclic AMP	0.80
$O^{2'}$-monobutyryl cyclic AMP	0.91
2'deoxy cyclic AMP	0.94
5'AMP	0.91
Adenosine	1.05

^{3}H cyclic AMP binding assays were run using the crude extract with 10 nM ^{3}H-cyclic AMP; each addition was used at a concentration of 100 nM. The values represent the mean of duplicate determinations.

affinity for cyclic GMP, N^6-monobutyryl cyclic AMP and 8-bromo cyclic AMP, and virtually no affinity for $O^{2'}$-monobutyryl cyclic AMP, 2'deoxycyclic AMP, adenosine and 5'AMP (Table II). The four nucleotides with substantial affinity were also the same nucleotides which stimulated the growth of crisp-1, cyclic AMP deficient mutants [21] as expected if the binding activity represents the cyclic AMP receptor.

When special care is taken to collect Neurospora samples rapidly and avoid proteolysis, the cyclic AMP binding activity extracted cochromatographs with the first protein kinase peak and the binding peptide has an apparent molecular weight of between 45,000 and 50,000 (Trevillyan and Pall, unpublished data) using the method of Haley (22). We are currently trying to purify the protein kinase and binding activity in order to compare its properties with those of the mammalian cyclic AMP-dependent protein kinase.

The Neurospora cyclic AMP dependent protein kinase studied here shows several similarities to the mammalian cyclic AMP-dependent protein kinase I, including high activity with histone H2B, elution from DEAE cellulose at low salt, nucleotide binding specificity, inhibition by bovine protein kinase inhibitor and apparent molecular weight of the cyclic AMP-binding subunit.

ACKNOWLEDGEMENTS

We are indebted to Dr. Diane Russell for many helpful suggestions and for sharing unpublished data and procedures. We thank Drs. Alice Schroeder, Ralph

Yount, and Steve Norton for helpful discussion and Mr. Douglas Ferro for excellent technical assistance. This work was supported by Public Health Service Grant GM 24368.

REFERENCES

1. Terenzi, H.F., Flawia, M.M., Tellez-Inon, M.T. and Torres, N.H. (1976). J. Bacteriol. 126, 91-99.
2. Scott, W.A. (1976). Proc. Natl. Acad. Sci. (USA) 73, 2995-2999.
3. Pall, M.L. (1977). J. Biol. Chem. 252, 7146-7150.
4. Trevillyan, J.M. and Pall, M.L. (1979). J. Bacteriol. 138, 397-403.
5. Flawia, M.M. and Torres, N.H. (1972). J. Biol. Chem. 247, 6873-6879.
6. Scott, W.A. and Solomon, B. (1973). Biochim. Biophys. Res. Commun. 53, 1024-1030.
7. Tellez-Iñon, M.T. and Torres, H.N. (1970). Proc. Natl. Acad. Sci. (USA) 66, 459-463.
8. Mishra, N.C. (1976). Naturwissenchaften 63, 485-486.
9. Terenzi, H.F., Jorge, J.A., Roselino, J.E. and Migliorini, R.H. (1979). Arch. Microbiol. 123, 251-258.
10. Vogel, H.J. (1964). American Naturalist 98, 435-466.
11. Haddox, M.K., Roeske, W.K. and Russell, D.H. (1979). Biochim. Biophys. Acta 585, 527-534.
12. Walsh, D.A., Perkins, J.P., Brostrom, C.D., Ho, E.S. and Krebs, E.G. (1971). J. Biol. Chem. 246, 1968-1976.
13. Glynn, I.M. and Chappell, J.B. (1964). Biochem. J. 90, 147-149.
14. Gill, G.N. and Walton, G.M. (1974). Methods in Enzymology, Vol. 38 (Hardman, J.G. and O'Malley, B.W., Eds.) pp. 376-382, Academic Press, New York.
15. Murad, F., Manganiello, V. and Vaughan, M. (1971). Proc. Natl. Acad. Sci. (USA) 68, 736-739.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.
17. Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1971). J. Biol. Chem. 246, 1986-1995.
18. Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fisher, E.H. and Krebs, E.G. (1971). J. Biol. Chem. 246, 1977-1985.
19. Ashby, C.D. and Walsh, D.A. (1973). J. Biol. Chem. 248, 1255-1261.
20. Meyer, R.B., Jr. and Miller, J.P. (1974). Life Science 14, 1019-1040.
21. Rosenberg, G. and Pall, M.L. (1979). J. Bacteriol. 137, 1140-1144.
22. Haley, B.E. (1975). Biochemistry 14, 3852-3857.