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A cAMP-BINDING PROTEIN FROM DICTYOSTELIUM DISCOIDEUM REGULATES MAMMALIAN PROTEIN KINASE

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SUMMARY

A cAMP-binding protein isolated from the cytosol of Dictyostelium discoideum was partially purified. The protein binds cAMP maximally at pH 5-7; its K_D for cAMP is in the nanomolar range and the specificity for cAMP is high; the molecular weight is approximately 42,000. This cAMP-binding protein is not found in vegetative amoebae; it appears during the first two hours of development. The protein inhibits the activity of the purified catalytic subunit of bovine cAMP-dependent protein kinase in the absence, but not in the presence, of cAMP. It is suggested that the cAMP-binding protein, isolated from Dictyostelium, is either the regulatory subunit of cAMP-dependent protein kinase or the product of the partial degradation of such a regulatory subunit.

INTRODUCTION

The occurrence in Dictyostelium discoideum of two classes of cAMP-binding proteins has been described. Membranal, cell surface cAMP-binding sites increase greatly in number during early development and form part of the intercellular signaling system based on cAMP (1-5). Intracellular, soluble cAMP-binding proteins have also been described (5-11); their role is unknown, but their cytoplasmic location suggests that these proteins may mediate intracellular functions of cAMP.

In the present communication we describe a cytoplasmic cAMP-binding protein which, when added to the purified catalytic subunit of a mammalian cAMP-dependent protein kinase, inhibits the activity of the latter in the absence, but not the presence, of cAMP. The significance of this finding is that it lends support to an earlier claim (7) of the occurrence of a cAMP-dependent protein kinase in Dictyostelium. For reasons unknown a number of investigators (e.g. 8,9) have failed to substantiate this claim.

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METHODS

Growth and development of amoebae. D. discoideum, strain AX3/RC3, was grown axenically on Medium HL 5, supplemented with 50 mM glucose, harvested, and the amoebae disrupted, as described earlier (11). Briefly, amoebae were harvested when growth had reached a density of 2-4 X 10^6 amoebae/ml, washed twice in ice-cold Mes-LPS buffer (7.6 mM 2-(N-morpholino) enthanesulfonic acid pH 6, 20 mM KCl, 5 mM $MgCl_2$) and either extracted (vegetative amoebae) or resuspended to a density of approximately 1 X 10^7 amoebae/ml in the same Mes-LPS buffer and incubated at 22°C with shaking for 8 hr (developing amoebae).

Assay of binding of cAMP. The binding of cAMP was measured essentially as described earlier (11). The volume of the reaction mixture was 120 µl; it contained 10-200 µg of the sample in 25 mM Mes buffer, pH 7.0, which was 2 mM in dithiothreitol, 2 mM in $MgCl_2$ and 0.4 mM in EGTA; [^{32}P] cAMP was employed for measurement of binding in the nanomolar and [^{3}H] cAMP in the micromolar range. Routinely, the mixtures were kept at 0°C for 60 min and the reaction terminated by the addition of 300 µl of 50 mM Tris-HCl, pH 7.5 containing 6 percent (w/v) charcoal and 2 percent bovine serum albumin. The mixture was immediately vortexed, centrifuged and 200 µl of the supernatant fluid, containing the cAMP-protein complex, removed for the determination of the radioactivity. Procedures for the purification of the nucleotides and corrections for background are described in an earlier publication (11) as is the method for testing the effects of potential inhibitors of the binding of cAMP.

Preparation of extracts and purification of cAMP-binding proteins. Vegetative and developing amoebae harvested after various periods of starvation, were resuspended to approximately 5 X 10^8 cells/ml in 5 mM Mes buffer, pH 7, containing 2 mM dithiothreitol, 5 mM NaCl, 0.75 mM $MgCl_2$ and 1 mM phenylmethylsulfonylfluoride. Amoebal suspensions were stored at -60°C and disrupted by thawing at 4°C. Extracts from developing amoebae were employed for the purification of the cAMP-binding proteins. The starting material was the supernatant fluid, obtained after centrifugation at 100,000 g for 90 min, which was adsorbed onto a DE-52 (Whatman) column. The column was washed with buffer containing 5 mM NaCl (see legend, Fig. 1) and a linear gradient of 5-500 mM NaCl was applied. The eluted material showed a large peak of cAMP-binding activity when assayed at pH 5 and 0.5 µM [^{3}H] cAMP at an NaCl concentration of about 75 mM and a smaller, broad plateau of cAMP-binding activity, which centered at about 125 mM NaCl, when assayed at pH 7 and 0.5 µM [^{3}H] cAMP. Two pools were prepared: Pool I had a ratio of cAMP binding pH 5/pH 7 of about 7 and eluted at 75 mM NaCl; Pool II had a ratio of cAMP binding pH 5/pH 7 of about 1.3 and eluted at 125 mM NaCl. The material from the two pools was concentrated by precipitation with 55 percent saturated $(NH_4)_2SO_4$, dialyzed and passed through Sephacryl S200 columns. The protein derived from DE-52 Pool 1, when assayed at 0.5 µM [^{3}H] cAMP, yielded a peak of cAMP-binding activity with a pH 5/pH 7 ratio of about 10 and a molecular weight of approximately 185,000 (Fig. 1A). DE-52 Pool II, on passage through Sephacryl S200, yielded two major fractions; one corresponded to a molecular weight of about 185,000 with a pH 5/pH 7 ratio of cAMP binding of approximately 10 and a second fraction of molecular weight of about 42,000 with a pH 5/pH 7 ratio of cAMP binding of approximately 1; there was little overlap between the two species (Fig. 1B). As a matter of convenience, the two cAMP-binding fractions are called henceforth "42K cAMP-binding protein" and "185K cAMP-binding protein", respectively.

Assay of protein kinase activity. The catalytic subunit of beef heart cAMP-dependent protein kinase (a gift from Dr. J. Maller) was diluted in Sephacryl S200 Column Buffer (see legend to Fig. 1 for composition) containing 0.5 mg/ml of bovine serum albumin. The assay mixture, in a total volume of 0.1 ml, contained, in addition to the protein kinase, 50 mM Mes buffer, pH 6.5; 2 mM dithiothreitol; 2 mM $MgCl_2$; 0.4 mM EGTA; 10 mM NaF; 0.2 mM [^{32}P] ATP, prepared by the method of Johnson and Walseth (12) and diluted to approximately 500 cpm/pmole, and 20 µM Kemptide (Peninsula Laboratories, Inc.) (13) as substrate for the protein kinase. Unless stated otherwise, the reaction was

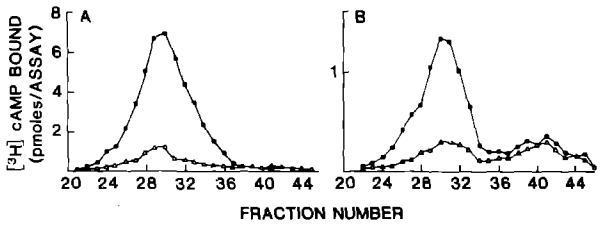


Figure 1. Binding of cAMP by fractions eluted from Sephacryl S200 columns. The columns were eluted with buffer of the following composition: 5 mM Mes, pH 7; 0.1 mM EDTA; 2 mM dithiothreitol; 10 percent glycerol; and 100 mM NaCl. The binding of [^{3}H] cAMP (input of 0.5 μM [^{3}H] cAMP) was determined for aliquots of Sephacryl S200 fractions at pH 5.0 (●—●) and pH 7.0 (Δ——Δ).

A. The column was charged with material eluted from a DE-52 column at about 75 nM NaCl (185K cAMP-binding protein).

B. The column was charged with material eluted from a DE-52 column at about 125 mM NaCl (42K cAMP-binding protein).

initiated by the addition of Kemptide and terminated by the addition of 1 ml of 30 percent acetic acid; the phosphorylated product was separated from the [^{32}P] ATP by the method of Kemp et al. (13). The amount of Kemptide was in excess insofar as at the termination of the reaction less than 10 percent of the substrate had been phosphorylated. Kemptide was used because it does not dissociate the protein kinase into its regulatory and catalytic subunits (13). To examine the inhibition of bovine protein kinase activity by the cAMP-binding protein, the latter was mixed with the catalytic subunit and the mixture kept at 0°C for 10 min prior to the initiation of the phosphorylation reaction. The observed inhibition was not affected by the presence of the components in the assay mixture at 0°C. The effect of cAMP in preventing the inhibition by the cAMP-binding protein was the same, irrespective of whether the cAMP was present for 10 min at 0°C or whether it was added simultaneously with the Kemptide at the start of the phosphorylation reaction. The activity of the catalytic subunit of protein kinase, in the absence of the binding protein, was not affected by cAMP.

RESULTS

Characterization of cAMP-binding. Binding of cAMP was assayed with [^{32}P] cAMP in the nanomolar and with [^{3}H] cAMP in the micromolar range; the results of Scatchard analyses of the data on binding are shown in Table 1. The 42K cAMP binding protein has a K_D in the nanomolar range at both pH 5 and pH 7. The number of 42K cAMP-binding sites per amoeba is the same, irrespective of whether the measurement is made at pH 5 or 7. The 185K cAMP-binding protein preparation shows at pH 7 some high affinity binding (6 nM) which may be due to contamination with the 42K cAMP-binding protein. At pH 5 the number of low affinity cAMP-binding sites (K_D of about 1,700 nM) is 30-80-fold greater than the number of high affinity binding sites.

TABLE I

CYCLIC AMP-BINDING PROTEINS

Fraction	pH	K_D nM	Estimated number of binding sites/cell
185K cAMP-binding protein	5.0	1,700	1,000,000
	7.0	5.7	12,000
42K cAMP-binding protein	5.0	5.7	32,-40,000
	7.0	1.3	

The cAMP concentrations used for the measurements ranged from 0.3 to 12 nM [^{32}P] cAMP at low input and from 25 to 900 nM [^{3}H] cAMP at high input. At all concentrations a blank, representing radioactive cAMP not displaced by excess non-radioactive cAMP, was subtracted. The number of cAMP-binding sites per assay was calculated from the Scatchard plot; the number of cAMP-binding sites per cell was estimated from the number of sites per assay normalized for the recovery of the binding protein during the fractionation procedure.

The data in Table 2 show that the binding of cAMP by the 185K protein is inhibited by AMP and ATP; as reported earlier (11), purified cAMP is bound with an approximately 10-fold higher affinity than are AMP, ADP and ATP. The binding of cAMP by the 42K protein is insensitive to AMP and ATP, but is inhibited by high concentrations of N^6,$O^{2'}$-dibutyryl cAMP and cGMP.

Inhibition of protein kinase activity by the 42K cAMP-binding protein. Increasing amounts of partially purified 42K cAMP-binding protein obtained from developing D. discoideum were added to a constant amount of bovine heart protein kinase catalytic subunit in either the presence of the absence of 100 μM cAMP. The data present in Fig. 2 show that there is a concentration-dependent inhibition of protein kinase activity in the absence, but not in the presence, of cAMP. This observation is consistant with the hypothesis that the 42K cAMP-binding protein functions as the regulatory subunit of a cAMP-dependent protein kinase in D. discoideum. We were able to exclude the unlikely possibility that the inhibition by the 42K preparation was due to its contamination with either a hypothetical, cAMP-inhibited protease or ATPase, since preincuba-

TABLE II

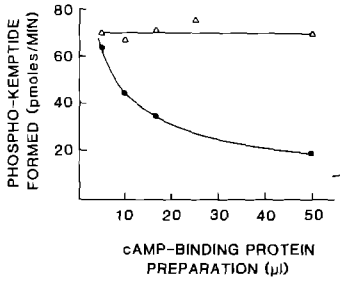
COMPETITION FOR BINDING OF cAMP

Addition	Percent of Residual Binding: 185K cAMP-binding protein	42K cAMP-binding protein
cAMP	(0)	(0)
AMP	6	100
ATP	6	120
cGMP	92	48
$N^6,O^{2'}$-dibutyryl cAMP	91	44

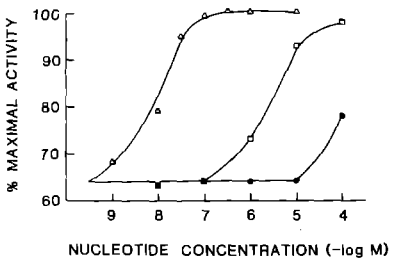
The binding of [^{3}H] cAMP (.5-1.2 μM) was measured in the absence and presence of non-radioactive nucleotides (200-fold excess). The numbers represent the percent of binding of [^{3}H] cAMP with the percentage set at 100 in the absence of non-radioactive nucleotides. In all cases, a "non-specific" binding (i.e. the binding not competed by excess cAMP) of approximately 10 percent has been subtracted. The competing nucleotides were added to the incubation mixtures (containing [^{3}H] cAMP) prior to the addition of the binding protein extract.

tion of the reaction mixture for 10 minutes prior to the addition of the cAMP did not affect the subsequent phosphorylation reaction. The 185K cAMP-binding protein, at the concentrations tested, did not inhibit the catalytic subunit of the bovine protein kinase.

We examined the ability of different concentrations of cAMP, $N^6,O^{2'}$-dibutyryl cAMP and AMP to prevent the inhibition of protein kinase activity by the 42K cAMP-binding protein. The data in Fig. 3 show that, at the concentrations of the two proteins employed in the experiment, the half-maximal concentrations of the nucleotides required to prevent the inhibition were 0.01 μM for cAMP, 2 μM for $N^6,O^{2'}$-dibutyryl cAMP and 200 μM for AMP. The ratios of the concentrations of the three nucleotides required for the blocking of the inhibition of protein kinase activity on the one hand and for the inhibition of the binding of cAMP to the 42K protein on the other hand (estimated from the data in Table II) are similar. Both the phosphorylation and the cAMP-binding assay were performed at 0°C for this comparison.



(2)



(3)

Figure 2. The effect of 42K cAMP-binding protein preparation on the phosphorylation catalyzed by the catalytic subunit of purified bovine protein kinase. A fixed amount of protein kinase catalytic subunit was mixed with different volumes of the 42K cAMP-binding protein preparation in the presence (Δ—Δ) and the absence (●—●) of 100 µM cAMP and the mixture kept for 10 min at 0°C. The reaction was then started by the addition of Kemptide and allowed to proceed for 4 min at 30°C.

Figure 3. The effect of nucleotides on the inhibition of protein kinase activity by the 42K cAMP-binding protein. Fixed amounts of protein kinase catalytic subunit, 42K cAMP-binding protein and increasing concentrations of the three nucleotides were kept for 10 min at 0°C. The phosphorylation reaction was started by the addition of the Kemptide and allowed to proceed at 0°C for 40 min. (The activity of the protein kinase catalytic subunit alone, 13.5 pmole of phospho-Kemptide formed/min, was the same as that obtained in the presence of the cAMP-binding protein and a saturating concentration of cAMP). In this experiment protein kinase activity was inhibited 37 percent by the 42K cAMP-binding protein. The nucleotides were cAMP (Δ—Δ), $N^6,O^{2'}$-dibutyryl cAMP (□—□) and AMP (●—●).

Additional evidence for the identity of the 42K cAMP-binding protein and the inhibitor of protein kinase activity. 42K cAMP-binding protein, partially purified by passage through DE-52 and Sephacryl S200, was submitted to Sephadex G100 chromatography and fractions of the eluate tested for their ablity to inhibit the activity of the catalytic subunit of bovine protein kinase as well as for their capacity to bind cAMP. The data in Fig. 4 show that both the cAMP-binding activity and the material which inhibits the catalytic subunit of the protein kinase, in a manner reversible by cAMP, have a broad Mr with a maximum of about 42,000.

DISCUSSION

The findings presented here suggest strongly that the 42K cAMP-binding protein functions as the regulatory subunit of cAMP-dependent protein kinase also in D. discoideum. Unlike the 185K cAMP-binding protein which is found during vegetative growth as well as development (11), the 42K protein does not occur in vegetative

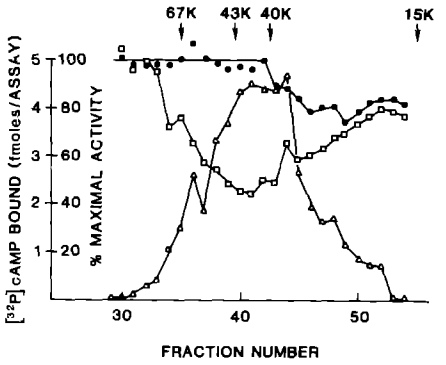


Figure 4. Size of cAMP-binding protein and of inhibitor of bovine protein kinase. The 42K cAMP-binding protein obtained from the Sephacryl S200 column was chromatographed on a Sephadex G100 column; the buffer was as described in the legend to Fig. 1 except that the NaCl concentration was 50 mM, and 0.5 mg/ml bovine serum albumin was added. Fractions were assayed for the binding of [^{32}P] cAMP (Δ Δ) as described. Aliquots of the same fractions were also mixed with a constant amount of the catalytic subunit of bovine protein kinase in the presence (●—●) and absence (□—□) of 100 μM cAMP and the phosphorylation of Kemptide determined as described in Methods; incubation was for 30 min at 30°C. The protein standards were bovine serum albumin, 67,000; ovalbumin, 43,000; bovine protein kinase catalytic subunit, 39,000; and cytochrome C, 15,000.

amoebae, is formed rapidly during the first two hours and then, at a reduced rate, throughout development (data not shown). The molecular weight of the 42K protein is lower than that of the regulatory subunits of protein kinases I and II of mammalian and yeast origin (for review see ref. 14). It is conceivable that the molecular weight of the native regulatory subunit of the D. discoideum protein kinase is approximately 42,000; alternatively, limited proteolysis may have given rise to the 42K species which has retained, however, both the abilty to bind cAMP and to inhibit the activity of the purified catalytic subunit of bovine heart protein kinase. Other investigators (5,9,10) employing different techniques also noted cAMP-binding proteins of unknown function with a molecular weight of approximately 40,000.

ACKNOWLEDGMENTS

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