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Cyclic AMP Binding Proteins and Cyclic AMP-Dependent Protein Kinase from *Blastocladiella emersonii*

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The stoichiometry of cyclic AMP binding protein to cyclic AMP in sporulating cells of *Blastocladiella emersonii* and the resistance of protein-bound cyclic AMP to enzyme-catalyzed hydrolysis suggest that the distribution of cyclic AMP between free and protein-bound pools is an important factor in cyclic AMP metabolism. Most but not all of the cyclic AMP binding protein in sporulating cells is associated with a cyclic AMP-dependent protein kinase.

In a recent study we showed that cyclic AMP phosphodiesterase activity increases as much as 20-fold during sporulation of *Blastocladiella emersonii* (5). The enzyme, or enzymes, responsible for this activity are essentially specific for cyclic AMP as substrate. Surprisingly, however, the cyclic AMP content of sporulating cells remained unaltered before, during, and after phosphodiesterase activity increased (5). These observations suggest that phosphodiesterase activity in sporulating cells is not rate limiting for hydrolysis.

In this communication we present evidence that the amount of cyclic AMP bound to specific receptor proteins in *Blastocladiella* could be an important factor, along with the rates of synthesis and loss, in determining the basal cyclic AMP level in sporulating cells. Cyclic AMP bound to such proteins from mammalian cells is resistant to enzyme-catalyzed hydrolysis (3, 11). Moreover, there is rough molar parity between levels of cyclic AMP and cyclic AMP binding sites in different tissues (7), such that significant amounts of cyclic AMP are likely to be protein bound in vivo (8, 14, 17). Beavo et al. (1) have accommodated these facts in the hypothesis that the amounts of cyclic AMP binding proteins and their affinity for cyclic AMP in vivo are important parameters in regulating basal cyclic AMP content in animal cells, but this hypothesis remains untested.

To determine whether or not cyclic AMP binding protein could play a role in regulating *Blastocladiella* cyclic AMP content, we have partially characterized the cyclic AMP binding proteins from this organism. We report here the following. (i) Most but not all of the cyclic AMP binding protein from sporulating cells is associated with a cyclic AMP-dependent protein kinase characterized by a strong preference for protamine over histone as phosphoryl acceptor protein. (ii) Cyclic AMP bound to *Blastocladiella* cyclic AMP binding protein is resistant to hydrolysis catalyzed by the *Blastocladiella* cyclic AMP phosphodiesterase that we previously described (5). (iii) In early sporulating cells there is a twofold molar excess of cyclic AMP over cyclic AMP binding sites; however, later in sporulation, cyclic AMP binding activity increases three- to fourfold, such that binding sites are in excess over cyclic AMP. By removing cyclic AMP from the unbound pool available for hydrolysis, this extra binding protein could limit the rate of cyclic AMP hydrolysis, thereby accounting for our observations (5).

MATERIALS AND METHODS

Cells and media. Cells and media were as previously described (5, 13), except that cells were harvested by rapidly filtering cell suspensions (200 to 400 ml, 1×10^6 to 2×10^6 cells per ml) over heavy-duty Nitex cloth (7-μm porosity). The cell cakes thus obtained were stored at −70°C until used.

Preparation of extracts. Frozen cell cakes were suspended in 4 volumes, usually about 2.5 ml, of cold, 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 20% (vol/vol) glycerol (buffer B). The suspension was mixed with an equal volume of acid-washed glass beads (0.5 mm) and agitated for 2 min in a CO_2-cooled Braun homogenizer. The fluid above the beads was removed and replaced with 0.5 ml of buffer, as above. The beads were extracted for 30 s in the homogenizer. The combined fluids were centrifuged in the cold for 20 min at 30,000 × *g*. The 30,000 × *g* supernatant fluid was used for assays.

Protein kinase and cyclic AMP binding assays. Protein kinase activity was assayed in reaction mixtures (0.1 ml) containing 2 μmol of potassium phosphate buffer (pH 7.0), 2.5 μmol of 2-mercaptoethanol,

1 μmol of $MgSO_4$, 2 μmol of theophylline, 8 μmol of [γ-^{32}P]ATP (150 to 250 cpm/pmol), 40 μg of protamine sulfate as phosphoryl acceptor protein, 40 pmol of cyclic AMP (where appropriate), and 20 μl of appropriate column fractions. After 5 min of incubation at 30°C, 50 μl of each reaction mixture was spotted on a 2.4-cm disk of Whatman 3MM paper. The disks were processed as described (4) to isolate acid-insoluble material and assayed in a liquid scintillation counter. One unit of protein kinase activity is the amount required to catalyze the incorporation of 1 nmol of ^{32}P per min into acid-insoluble material. Cyclic AMP binding activity was assayed in reaction mixtures (0.2 ml) containing 10 μmol of sodium acetate buffer (pH 4.0), 0.4 μmol of theophylline, 60 μg of bovine serum albumin, 20 pmol of cyclic [^{3}H]AMP (9,676 cpm/pmol), and 20 μl of appropriate column fractions. After 60 min of incubation at 4°C, cyclic AMP binding was measured by membrane filtration (Millipore) essentially as described by Gilman (6). The reaction was specific for cyclic AMP, since retention of cyclic [^{3}H]AMP was reduced 10-fold by addition of a 10-fold excess of unlabeled cyclic AMP, but was not affected by the same amount of cyclic GMP. One unit of cyclic AMP binding activity is the amount required to retain 1 pmol of cyclic AMP. Specific activities are expressed as units per milligram of protein.

Materials. All materials were obtained as previously described (5, 13) or from standard commercial sources.

TABLE 1. *Cyclic AMP-dependent protein kinase of B. emersonii*

Additions[a]	Protein kinase activity[b] (pmol of ^{32}P)	
	Vegetative cell	Sporulating cell
None	5.7	5.7
Protamine	17.6	36.7
Mixed histone	18.2	28.5
Cyclic AMP	8.1	8.3
Cyclic AMP + protamine	98.9	292.5
Cyclic AMP + mixed histone	18.3	54.4

[a] Additions, where appropriate, were: 40 μg of protamine sulfate (Sigma Chemical Co.); 40 μg of mixed histone (Worthington Biochemical Corp., code HLY); 0.4 μM cyclic AMP.

[b] Enzyme activity was measured as described in the text, except that precipitation was with 5% trichloroacetic acid and precipitates were collected on Whatman GF/C glass-fiber filters. Reaction mixtures contained either 36 μg of protein from a vegetative cell 30,000 × *g* supernatant fraction or 41 μg from a corresponding sporulating cell fraction. Vegetative cells were obtained after 5 h of growth and sporulating cells 150 min after the onset of sporulation, both as previously described (5). Extracts were prepared as described in the text.

RESULTS

Cyclic AMP-dependent protein kinase and cyclic AMP binding protein of *B. emersonii*. As in other eucaryotic cells, cyclic AMP in *Blastocladiella* regulates cellular activity by activation of cyclic AMP-dependent protein kinase (Table 1). Enzyme activity is present in extracts of both vegetative and sporulating cells, as expected, since cyclic AMP, unlike cyclic GMP, is a constitutive regulatory molecule in *Blastocladiella* synthesized throughout its cell cycle (13). In both extracts maximal activity required cyclic AMP and an endogenous phosphoryl acceptor protein, for which enzyme from both cell types shows a marked preference for protamine over histone (Table 1).

Most, but not all, of the cyclic AMP binding activity in late sporulating cells is associated with a cyclic AMP-dependent protein kinase, as shown by diethylaminoethyl-cellulose chromatography (Fig. 1). With protamine as phosphoryl acceptor, a single peak of cyclic AMP-dependent protein kinase activity eluted from the column at a mean NaCl concentration of 0.2 M. No cyclic AMP-dependent or cyclic nucleotide-independent protein kinase activity, with either protamine or histone as phosphoryl acceptor protein, eluted at higher or lower salt concentrations or in the flow-through fractions. In contrast, cyclic AMP binding activity eluted as a major component at the same position as protein kinase activity and as a leading shoulder at about 0.12 M NaCl. The latter eluted at a position essentially devoid of protein kinase activity. This activity may reflect a cyclic AMP binding subunit of protein kinase synthesized during sporulation in excess of catalytic subunit (see below). This explanation is consistent with the subunit structure of cyclic AMP-dependent protein kinases from a variety of sources (9), including other fungal cells (16). Noncoordinate synthesis of the two protein kinase subunits in mammalian tissues has not previously been reported, though yeast cells evidently contain significant levels of cyclic AMP binding activity not associated with cyclic AMP-dependent protein kinase (15, 16). It is also possible that cyclic AMP-cyclic AMP binding protein complexes have a direct regulatory role in fungal cells whether or not the proteins also regulate protein kinase activity.

The ratio of protein kinase to cyclic AMP binding activity across the main peak was constant at about 0.4 nmol of ^{32}P transferred per min per pmol of cyclic AMP bound (Fig. 1, inset), indicating co-chromatography of both activities as a single molecular species. This value may be compared with reported values of 0.15 nmol of ^{32}P transferred per min per pmol of cyclic AMP bound for the purified type I cyclic

AMP-dependent protein kinase from rabbit skeletal muscle (histone substrate) (2), with 0.09 nmol of ^{32}P per min per pmol of cyclic AMP bound for the type II enzyme from bovine cardiac muscle (protamine substrate) (12), and with values of about 0.5 nmol of ^{32}P per min per pmol of cyclic AMP bound in cruder fractions from various rabbit tissues (7).

Effect of cyclic AMP binding protein on enzyme-catalyzed hydrolysis of cyclic AMP. Reconstitution experiments using *Blastocladiella* cyclic AMP binding protein and cyclic AMP phosphodiesterase show that cyclic AMP bound to the fungal proteins is resistant to enzyme-catalyzed hydrolysis (Table 2). The binding protein used in these experiments was a 0.1 to 0.3 M NaCl eluate from a diethylaminoethyl-cellulose column, which contained a low level of cyclic AMP phosphodiesterase activity. This activity, however, was evident only in the presence of a divalent cation (e.g., Mg^{2+}); without addition of a divalent cation, cyclic AMP bound to receptor protein was completely stable for up to 20 min, even at 30°C (Table 2). In the presence of Mg^{2+} about 75% of the total cyclic AMP was hydrolyzed within 10 min at 30°C. The remaining 25% corresponded to the proportion of bound cyclic AMP, and, in fact, very little bound cyclic AMP was hydrolyzed within 10 min at 30°C, and less than half by 20 min (Table 2). Hence, it appears that bound cyclic AMP is resistant to enzyme-catalyzed hydrolysis, at least at the low phosphodiesterase level used in this experiment. We have also carried out the experiment in the presence of endogenously added *Blastocladiella* cyclic AMP phosphodiesterase to restore the ratio of phosphodiesterase to binding protein that prevails in vivo (Table 2). In the absence of binding protein, this amount of phosphodiesterase is sufficient to hydrolyze all of the cyclic AMP in the reaction mixture in less than 2 min. Even with this large amount of phosphodiesterase, the half-life of bound cyclic AMP is approximately 10 min (Table 2), probably re-

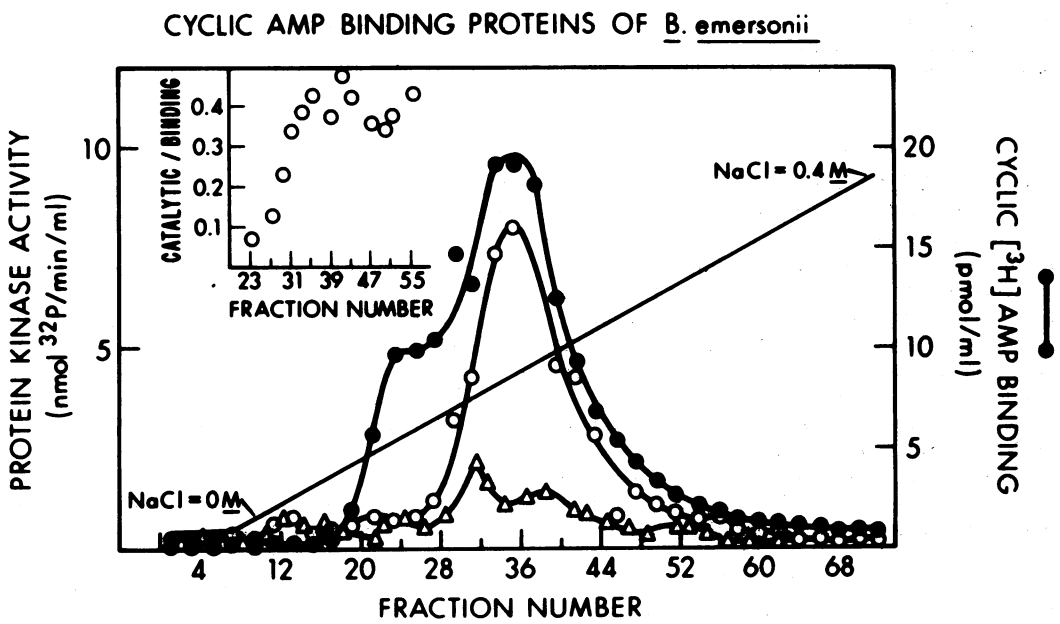

FIG. 1. *Cyclic AMP binding proteins and cyclic AMP-dependent protein kinase of B. emersonii. Sporulating cells (2.6 g, harvested 150 min after the onset of sporulation) were suspended in 9 ml of cold buffer B containing 0.37 M phenylmethylsulfonylfluoride. A 30,000 × g extract was prepared as described in the text. The 30,000 × g supernatant fluid (8 ml, containing 62.4 mg of protein, 504 U of cyclic AMP-dependent protein kinase, and 440 U of cyclic AMP binding activity) was applied to a column (2.4 by 4 cm) of diethylaminoethyl-cellulose (Whatman DE23) previously equilibrated with buffer B. After the unadsorbed protein was eluted with buffer B (44 ml), protein kinase and cyclic AMP binding protein were eluted with a 220-ml linear gradient of NaCl (0 to 0.5 M) in buffer B. Alternating fractions (3.1 ml) were assayed for protein kinase or cyclic AMP binding activity. Recovery of protein kinase activity was 322 U and, of cyclic AMP binding activity, 1,023 U. No activity was eluted with the unadsorbed protein. (●) Cyclic AMP binding; (○) protein kinase activity in the presence of cyclic AMP; (△) protein kinase activity in the absence of cyclic AMP. Inset: ratio of protein kinase to cyclic AMP binding activity (nanomoles of ^{32}P per minute per picomole of cyclic AMP bound) in the different column fractions (see the text).*

TABLE 2. *Effect of cyclic AMP binding protein on enzyme-catalyzed hydrolysis of cyclic AMP*

Additions[a]	Hydrolysis[b]				
	% Total cyclic AMP after:		% Bound cyclic AMP after:		
	2 min	10 min	1 min	10 min	20 min
Binding protein					<5
Binding protein + $MgCl_2$[c]	51.3	75.2	<5	15.4	33.0
PDE[d] + $MgCl_2$	100				
PDE + binding protein + $MgCl_2$	68.1	89.4	<5	55.3	81.7

[a] Reaction mixtures (0.1 ml) contained 2 μmol of potassium phosphate buffer (pH 7.0), 11.7 pmol of cyclic [^{3}H]AMP (12,000 to 17,000 cpm/pmol), and, where appropriate, 2.73 U of cyclic AMP binding protein (diethylaminoethyl-cellulose fraction, see text). After 30 min of incubation at 4°C to allow the binding reaction to reach a yield, $MgCl_2$ (1 μmol) and/or cyclic AMP phosphodiesterase (2.7 U of peak II enzyme; ref. 5) were added, and the reaction mixtures were incubated at 30°C for the indicated times.

[b] Total cyclic AMP hydrolysis was measured as previously described (5). Hydrolysis of bound cyclic AMP was taken to be equal to the reduction in cyclic [^{3}H]AMP retained by a nitrocellulose filter.

[c] The binding-protein preparation used in this experiment contained approximately 0.001 U of cyclic AMP phosphodiesterase activity per unit of binding activity. Phosphodiesterase activity shows an absolute requirement for a divalent cation, supplied in this case by Mg^{2+}.

[d] PDE, Cyclic AMP phosphodiesterase.

flecting the dissociation rate of cyclic AMP from the cyclic AMP-protein complex.

Cyclic AMP binding capacity of sporulating cells. The preceding experiments show that in *Blastocladiella*, as in mammalian cells (3, 11), the dissociation of cyclic AMP bound to specific receptor proteins could be rate limiting in hydrolysis. To determine how much cyclic AMP could be protein bound in vivo, we measured the cyclic AMP binding capacity of extracts obtained from cells harvested at different times during sporulation. The data (Table 3) are expressed in picomoles of cyclic AMP binding per milligram of protein in the 30,000 × *g* supernatant fraction, which contained >80% of the total cyclic AMP binding activity of the cell. From our previously published value of 1.1 pmol of cyclic AMP per μg of DNA (5), and from our determinations of 221 μg of DNA per g (wet weight) of cells and 26.4 mg of protein soluble at 30,000 × *g* per g (wet weight) of cells, the cyclic AMP content of sporulating cells was calculated to be 9.2 pmol/mg of protein in the 30,000 × *g* supernatant fraction. Hence, over the interval of sporulation when cyclic AMP phosphodiesterase increases and total cyclic AMP content is unaltered, the cyclic AMP binding capacity of sporulating cells increases three- to fourfold, from an amount about half the cyclic AMP content to an amount twice as great. Two points regarding this experiment should be noted: (i) our measurements and those of Murphy and Lovett (10) show no significant change in the protein content of sporulating cells over the interval shown in Table 3; hence the changes observed are in the level of binding activity; and (ii) the results cannot be attributed to endogenous cyclic AMP bound to receptor protein from cells harvested at the early times because binding activities were only slightly altered after incubation of undiluted extracts at pH 7.0 and 30°C for 20 min in the presence of 10 mM $MgCl_2$. As shown (Table 2), bound cyclic AMP dissociates under these conditions and would have been hydrolyzed immediately by endogenous cyclic AMP phosphodiesterase in the extracts. Since the binding assay measures a stoichiometric rather than a catalytic property of the protein, the data likely reflect an increase in the amount of cyclic AMP binding protein during sporulation.

TABLE 3. *Cyclic AMP binding activity in sporulating cells*

Age (min) of sporulating cells for extract[a]	Binding activity[b]	
	− Incubation	+ Incubation[c]
30	5.1	5.4
60	5.7	6.4
90	6.9	5.9
120	9.8	11.0
150	16.7	18.8

[a] Cells harvested at different times during sporulation were obtained as previously described (5), and 30,000 × *g* supernatant fractions were prepared as described in the text.

[b] Picomoles of cyclic AMP binding per milligram of protein.

[c] Incubation was for 20 min at 30°C in buffer B containing 10 mM $MgCl_2$ (see the text).

DISCUSSION

Beavo et al. (1) suggested that a substantial amount of cyclic AMP in unstimulated tissue is protein bound and therefore resistant to phos-

phodiesterase-catalyzed hydrolysis. We have found that cyclic [^{3}H]AMP bound to *B. emersonii* cyclic AMP binding protein is hydrolyzed at a much slower rate, if at all, by cyclic AMP phosphodiesterase. Our data indicate that the rate-limiting step for hydrolysis of bound cyclic AMP is dissociation of the cyclic AMP-protein complex. On the basis of these results, we suggest that cyclic AMP binding protein synthesized during sporulation draws free cyclic AMP into a protein-bound pool. To explain our observation that total cyclic AMP in the cell is unaltered, we further suggest that elevated phosphodiesterase activity (5) maintains the rate of cyclic AMP hydrolysis at a constant level as the pool of cyclic AMP available for hydrolysis is drained. The net result is a redistribution of cyclic AMP in the cell, but no change in the total content.

This hypothesis requires no alteration in any other component involved in cyclic AMP metabolism or in the distribution of these components among different, physically separate subcellular compartments. However, it provides only a qualitative explanation for our results. We do not know either the intracellular affinity of cyclic AMP binding proteins for this ligand at different stages of sporulation or the relation between this important parameter and the intracellular concentrations of these components, nor do we know the intracellular rates of cyclic AMP synthesis, hydrolysis, or excretion. Nevertheless, our data are consistent with the hypothesis that the distribution of cyclic AMP between free and protein-bound pools is an important factor in cyclic AMP metabolism.

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