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Regulation of adenylyl cyclase from *Blastocladiella emersonii* by guanine nucleotides

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GTPγS stimulates adenylyl cyclase in particulate fractions of *Blastocladiella emersonii* zoospores. Cholera toxin catalyses the ADP-ribosylation of a membrane protein of a molecular weight (46,000) similar to that of the α subunit of G_S found in vertebrate cells. A membrane protein of 46 kDa can also be recognized in Western blots by an antipeptide antiserum (RM/1) raised against the C-terminus of $G\alpha_S$-subunits. These results suggest that a G-protein mediates the regulation of *Blastocladiella* adenylyl cyclase by guanine nucleotides.

G-protein; Adenylyl cyclase; *Blastocladiella emersonii*

1. INTRODUCTION

Zoospore germination in *Blastocladiella emersonii* is accompanied by a rapid rise in intracellular cAMP levels [1] and a concomitant activation of PKA [2], which leads to events that can be mimicked by addition of exogenous cAMP [3]. Zoospore encystment, the first stage of the germination program, can be induced by okadaic acid [4] and occurs in the absence of either RNA or protein synthesis [5], implying that the early events following induction operate at a post-translational level.

The above evidences point out adenylyl cyclase as a key enzyme implicated in the cascade of biochemical events that triggers zoospore germination. The mechanism underlying the regulation of adenylyl cyclase activity in zoospore has not been studied in detail. Only two facts have been described: first, adenylyl cyclase activity is very low in vegetative cells, increasing sharply during sporulation, the morphogenetic transition that precedes zoospore formation [6]; secondly, adenylyl cyclase was described as a Mn^{2+}-dependent enzyme, the activity with Mg^{2+} being negligible [6,7]. This later result resembles those of lymphoma S49 cyc^- cells and corresponds to those of the catalytic subunit of adenylyl cyclase of many tissues [8,9]. There the catalytic component of adenylyl cyclase separated from the guanine-nucleotide-binding protein, utilized Mn-ATP but not Mg-ATP as substrate and showed no response to guanine nucleotides.

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Abbreviations: PMSF, phenyl-methylsulfonyl fluoride; GTPγS, guanosine 5′-*O*-(3-thio-triphosphate); GDPβS, guanosine 5′-(*O*-2-thio)-diphosphate; IBMX, isobutyl-methyl-xanthine.

In this work the existence of a GTP-binding protein in *Blastocladiella* was investigated. We show that in a carefully prepared zoospore particulate fraction, guanine nucleotides regulated adenylyl cyclase. Furthermore, we identified a polypeptide of 46 kDa which is ADP-ribosylated by cholera toxin (CTX) and cross-reacts with an antipeptide antiserum (RM/1), raised against the C-terminus of $G\alpha_S$-subunits.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Chemicals and other reagents were obtained from the following sources: [α-^{32}P]ATP (3,000 Ci/mmol), [2,8-^{3}H]cAMP (3,000 Ci/mmol) and [^{32}P]NAD (1,000 Ci/mmol) from Amersham; RM/1 antiserum from NEN, Dupont. All other reagents were from Sigma.

2.2. *Organism and cultivation*

Maintenance and cultivation of *B. emersonii* cells were performed as previously described [10].

2.3. *Membrane preparation*

All operations were carried out at 4°C. Zoospores or sporulating cells were collected by centrifugation and suspended (10^9 cells/ml) in 50 mM Tris-HCl buffer, pH 8.5, containing 10% glycerol, 0.1 mM EGTA, 1 mM $MgCl_2$, 1 mM benzamidine, 50 μg/ml antipain and 150 μg/ml of PMSF (buffer A). The suspension was homogenized by a brief sonication (20 s, 20 V), unbroken cells and cell walls were removed by centrifugation at 1,000 × *g* for 10 min. The supernatant was spun down at 15,000 × *g* for 20 min. The pellet (P15) washed once and resuspended in 0.4 ml of the same buffer, was considered the crude membrane preparation. For the experiment described in Table I, the supernatant of the 15,000 × *g* centrifugation was recentrifuged at 105,000 × *g* (P105).

2.4. *Adenylyl cyclase assay*

Adenylyl cyclase activity was assayed in a reaction mixture containing 50 mM Tris-HCl, pH 8.5, 0.1 mg/ml BSA, 25 mM creatine phosphate, 1.2 units of creatine phosphokinase, 2 mM 2-mercaptoethanol, 0.2 mM IBMX, 0.5 mM [α-^{32}P]ATP (10–40 cpm/pmol), and 1 mM

[^{3}H]cAMP (20,000 cpm). Divalent cations and effectors were added as described in the text and figure legends. The reaction was initiated by addition of crude particulate extracts (20 μg of protein) to a final volume of 60 μl and the tubes were immediately transferred to a 25°C water bath. The reaction was stopped by addition of 0.2 ml of 0.5 N HCl and [^{32}P]cAMP produced was determined as previously described [11]. The reaction was linear up to 50 μg of protein and 40 min at 25° C.

Protein was determined by the method of Lowry et al. [12].

2.5. *Immunological detection of G protein α-subunits*

P15 fraction (50 μg protein) were subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with an antibody (RM/1) raised in rabbit against the C-terminus of a $G\alpha_s$-subunit (RMHLRQYELL) [13]. Bound antibody was detected with ^{125}I-labeled protein A.

2.6. *Toxin treatment*

For CTX catalysed ADP-ribosylation, the reaction mixture (60 μl) contained 0.3 M potassium phosphate buffer, pH 7.0, 1 mM ATP, 10 mM thymidine, 1 mM EDTA, 0.1 mM GTP, 10 mM $MgCl_2$, 60 μM [^{32}P]NAD (2 20 × 10^6 cpm) and 0.6 μg of CTX that had been preactivated with 25 mM DTT for 30 min at 37°C. P15 fractions (10–50 μg) were incubated with the reaction mixture for 45 min at 32°C. The reactions were stopped by addition of 15 μl of 100% TCA. The suspension was allowed to stand for 20 min at 4°C, and centrifuged for 20 min at 12,000 × g. The protein pellet was washed with ethanol/ether (1:1) and subjected to 10% SDS-PAGE, stained with Coomassie brillant blue, destained, dried and autoradiographed.

3. RESULTS AND DISCUSSION

3.1. *Guanine nucleotide stimulation of adenylyl cyclase*

In order to obtain Mg^{2+}-dependent adenylyl cyclase activity, zoospores were lysed in the presence of protease inhibitors, submitted to differential centrifugation and the enzyme activity of the different fractions measured using either Mg-ATP or Mn-ATP as substrate. As shown in Table I, 15% and 40% of the total Mn-ATP adenylyl cyclase activity were found in P15 and P105 fractions, respectively. In addition, Table I also shows that stimulation of adenylyl cyclase by GTPγS occurred only in the P15, while in the P105, as previously described [6,7], the nucleotide did not stimulate the enzyme activity.

The dose–response curve for stimulation of adenylyl cyclase by GTPγS in P15 is shown in Fig. 1. Half-maximum stimulation occurred at about 1 μM. GDPβS did not activate and strongly inhibited the effects of GTPγS, indicating that the activation was specific for guanine nucleotide triphosphates (data not shown). As in other systems [14,15], GTPγS appeared to alter the V_{max} of the enzyme from 0.150 to 0.250 nmol cAMP/min·mg protein) rather than affecting the K_m for Mg- ATP (0.59 mM), when measured in the presence of 2.5 mM excess Mg^{2+} (data not shown).

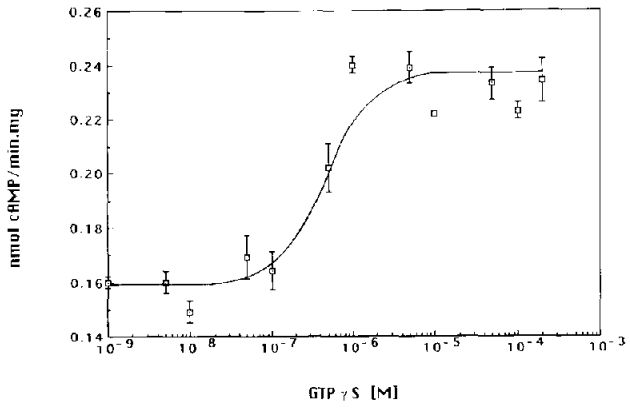

Fig. 1. Adenylyl cyclase stimulation by GTPγS in P15 fraction. Adenylyl cyclase activity was measured in the presence of 3 mM $MgCl_2$ and different concentrations of GTPγS. Error bars in this and other figures indicate the range of triplicate determinations.

3.2. *Thermal inactivation*

In order to investigate whether a guanine nucleotide-binding regulatory protein (G-protein) was involved in stimulation of the catalytic component of adenylyl cyclase, we tested for differences in susceptibility of these proteins to thermal inactivation. Fig. 2 shows that adenylyl cyclase activity in the P15 fraction responsive to Mg^{2+} plus GTPγS was considerably more sensitive to a 20 min pretreatment at 30°C than the adenylyl cyclase active in the presence of Mg^{2+}. The presence of Mg-GTP during preincubation prevented this inactiva-

Table I

Distribution of Mn-ATP and GTPγS-stimulated, Mg^{2+}-dependent adenylyl cyclase in fractions from *Blastocladiella* zoospores extracts

Fraction	Specific activity (nmol cAMP/min·mg)					
	Protein (mg)	Mg^{2+}	Mg^{2+} GTPγS	Mn^{2+}	± GTPγS	Total Mn^{2+} activity (%)
Homogenate	6.4	0.149	0.188	0.345	1.26	100
1000 × g sediment	0.9	0.017	0.027	0.064	1.59	2.6
15,000 × g sediment	0.8	0.115	0.226	0.423	1.97	14.9
100,000 × g sediment	1.3	0.152	0.133	0.685	0.88	40.3
100,000 × g supernatant	2.9	0.122	0.098	0.172	0.80	22.6

The zoospores were broken and several fractions obtained as described in section 2. Adenylyl cyclase activity was measured in the presence of divalent cations and guanine nucleotide as indicated: Mg^{2+}, 3.0 mM $MgCl_2$; Mn^{2+}, 3.0 mM $MnCl_2$, and 100 μM GTPγS.

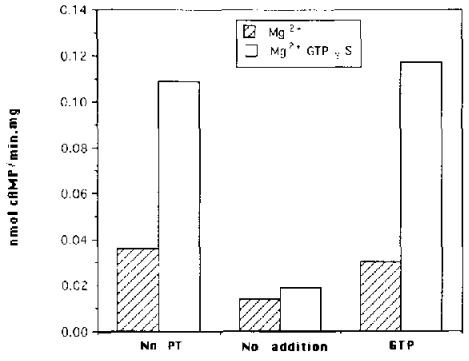

Fig. 2. Effect of preincubation at 30°C on adenylyl cyclase, in the presence and absence of GTP. P15 fractions were first incubated at 30°C for 20 min in buffer A containing 10 mM $MgCl_2$ in the absence (No addition) or presence (GTP) of 100 μM GTP. Adenylyl cyclase was then assayed at 25°C for 10 min in the presence of 3 mM $MgCl_2$ plus 100 μM GTPγS (open boxes) or only 3 mM $MgCl_2$ (filled boxes). Non-pretreated samples (No PT) were held at 4°C in the same buffer prior to assay.

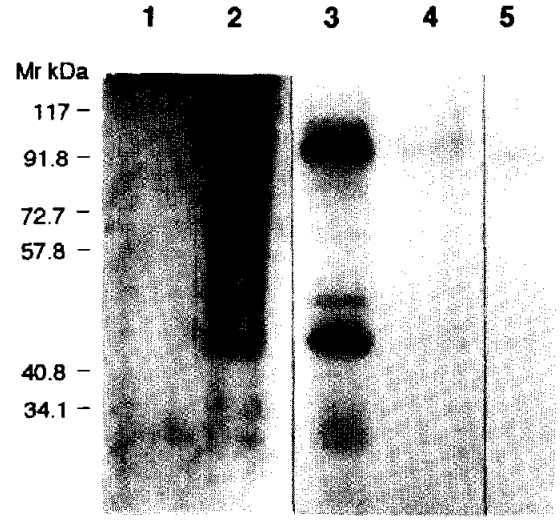

Fig. 3. Immunoblots and ADP-ribosylated proteins of *Blastocladiella* P15 fraction. P15 fractions (50 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with RM/1 antiserum (lane 2) or preimmune serum (lane 1). 75 μg of P15 fractions were incubated with [^{32}P]NAD in the presence (lane 3) or absence of CTX (lane 4). Lane 5 is CTX-catalyzed ADP-ribosylation performed in the presence of 0.8 mM NAD.

tion, suggesting stabilization of a component necessary for guanine nucleotide activation of adenylyl cyclase activity. Gpp(NH)p similarly stabilizes the mammalian G-protein against thermal denaturation; however, in contrast to *Blastocladiella*, in mammalian systems the catalytic subunit is more thermolabile than the regulatory component [8].

3.3. *Identification of Gα protein subunit*

The apparent similarity of *Blastocladiella* and vertebrate adenylyl cyclase system led us to investigate the presence of G proteins in zoospore P15 fraction. Using an antipeptide antiserum (RM/1), directed against the C-terminus of Gα-subunits [13], we identified a polypeptide of 46 kDa on immunoblots of *Blastocladiella* membrane proteins (Fig. 3, lane 2). Since ADP-ribosylation by exogenous toxin [16] is a special characteristic of GTP-binding proteins, we elected to determine whether a cholera toxin substrate could be identified in zoospores. As shown in fig. 3 (lane 3) cholera toxin catalysed the labelling of a protein band in the P15 fraction that also migrated as a 46 kDa protein on SDS-PAGE. This labeling was totally inhibited by the addition of 0.8 mM NAD to the reaction mixture (lane 5). Thus, both RM/1 antiserum and cholera toxin identified a 46 kDa protein that can represent a Gα-like subunit of *Blastocladiella*.

3.4. *Effect of guanine nucleotide on adenylyl cyclase activity during sporulation*

The response of the adenylyl cyclase activity to various effectors as cells underwent sporulation, is shown in Fig. 4. Cells harvested from growing cultures showed no significant enzymatic activity when measured with either Mg-ATP or Mn-ATP as substrate. Activity was detected after 2–3 h of starvation and was maximal after 4 h, in the zoospore stage. The ability of the guanine nucleotide GTPγS to stimulate adenylyl cyclase activity was also observed to increase over the course of differentiation, being more pronounced in the zoospore. This results suggested that the Gα-protein, like the catalytic subunit, was developmentally regulated and agree with the finding that zoospore differentiation occurs in the last hour of sporulation [17].

Our results represent the first demonstration of the presence of a cholera toxin ADP-ribosylated protein in *Blastocladiella* of similar molecular weight ($M_r \approx 46{,}000$) to the α subunit of G_S found in vertebrate cells. Furthermore, these data demonstrate the presence

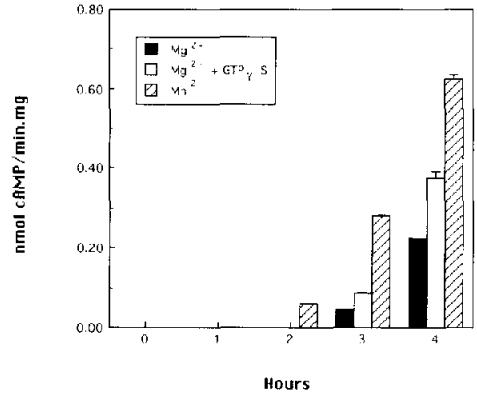

Fig. 4. Changes in adenylyl cyclase activity during sporulation. Cells previously grown for 14 h at 19°C in nutrient medium [10] were harvested, resuspended in sporulation solution (1 mM Tris-maleate, pH 6.8, 1 mM $CaCl_2$) and incubated at 27°C. Zoospores were fully released after 4 h. At the indicated times, samples were harvested and the adenylyl cyclase activity of the P15 fraction was assayed in the presence of 3 mM $MnCl_2$ (dashed boxes), 3 mM $MgCl_2$ (closed boxes) and 3 mM $MgCl_2$ plus 100 μM GTPγS (open boxes).

of an adenylyl cyclase in this aquatic fungus that can be activated in vitro by a guanine nucleotide. The failure of previous attempts to find such an activity [6,7] may have been due to lability of the regulatory component, the specific localization of the enzyme responsive to guanine nucleotides in the $15{,}000 \times g$ particulate fraction, the absence of protease inhibitors in the lysis buffer or a combination of these. The similarity of *Blastocladiella* and vertebrate adenylyl cyclase system provides the basis for further studies on the role of this enzyme in the control of zoospore germination.

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