REGULATION OF GUANYLATE CYCLASE ACTIVITY DURING CYTODIFFERENTIATION OF BLASTOCLADIELLA EMERSONII*

Philip M. Silverman+

Department of Molecular Biology, Division of Biological Sciences,
Albert Einstein College of Medicine, Bronx, N. Y. 10461

Received March 17, 1976

SUMMARY: Levels of guanylate cyclase activity in extracts of the unicellular eukaryote Blastocladiella emersonii differed by at least 100-fold at different stages of the cell cycle, paralleling changes in the cyclic GMP content of this organism (Proc. Natl. Acad. Sci. U.S.A. 72, 442 (1975)). Extracts of vegetative cells lacked appreciable guanylate cyclase activity, whereas the specific activity of the enzyme in zoospore extracts was 2 nmol cyclic GMP synthesized/min/mg protein at 35°. Guanylate cyclase activity increased at least 50-fold during the period of zoospore formation when cyclic GMP begins to accumulate in vivo. Since actinomycin D or cycloheximide added at the beginning of this period blocked any increase in enzyme activity, it appears that de novo synthesis of guanylate cyclase during sporulation is responsible for the accumulation of cyclic GMP that occurs at that time.

Cyclic GMP[1] synthesis is catalyzed in vitro by guanylate cyclase using GTP as substrate and functioning optimally in the presence of Mn^{2+} ions (1). The enzyme has been isolated from a variety of mammalian and bacterial sources (1-4) and has been extensively purified, though not to homogeneity, from bovine lung (5) and E. coli (4). It is widely assumed that guanylate cyclase activity is an important factor in the regulation of cellular cyclic GMP levels, though in no experimental system can a change in cellular cyclic GMP content be definitely attributed to a change in the level or activity of the enzyme.

This communication shows that there is a direct relation between cyclic GMP accumulation in vivo and the appearance of guanylate cyclase activity in

*These studies were supported by grants GM 11301 and NCI PO1-CA-13330 from the National Institutes of Health and 75-144 from the American Heart Association.

+Established Investigator of the American Heart Association.

[1]The abbreviations used are: cyclic GMP, guanosine 3',5'-monophosphate; cyclic AMP, adenosine 3',5'-monophosphate.

extracts of the unicellular eukaryote Blastocladiella emersonii. Cyclic GMP accumulation in B. emersonii is tightly coupled to cytodifferentiation, occurring at a single stage of the cell cycle, sporulation,[2] when the intracellular level increases as much as 100-fold (6). We proposed that cyclic GMP accumulation requires synthesis of a component absent from vegetative cells. The experiments reported below indicate that this component is guanylate cyclase.

EXPERIMENTAL PROCEDURES

Methods. Unless otherwise specified, zoospores were obtained from single-generation cultures grown on nutrient agar (8). Vegetative cells were grown in liquid glucose-casamino acid medium (6,8) supplemented with glutamate (0.4 mg/ml), harvested by filtration over heavy-duty Nitex cloth (7 μm porosity) and stored as frozen cakes at -70°.

Extracts were prepared by grinding frozen cell cakes to a fine powder in the presence of solid CO_2. Phenylmethylsulfonyl fluoride (10 μl of a 0.15 M solution in isopropanol per wet g of cells) was ground into the frozen powder which was then extracted with 1 ml of buffer A (50 mM Tris-HCl (pH 7.8), 0.1 M KCl, 5 mM 2-mercaptoethanol and 20% (v/v glycerol) per wet g of cells. Zoospore extracts were assayed directly. Extracts of vegetative cells were cleared of unbroken cells and cell wall debris by centrifugation in the cold at 2,000 x g for 10 min. The supernatant fluid was removed and each pellet extracted twice with an equal volume of buffer A. The combined supernatant fractions were used to measure guanylate cyclase activity. Centrifugation of zoospore extracts at forces as high as 10,000 x g had little effect on guanylate cyclase activity in the supernatant fluid.

The guanylate cyclase assay measures the formation of cyclic [^{32}P] GMP separated from [α-^{32}P] GTP substrate by chromatography over neutral alumina in 0.6 M Tris-HCl, pH 7.5 (9). Reaction mixtures contained in 0.2 ml of buffer A: 0.4 μmol $MnCl_2$, 0.1 μmol [α-^{32}P] GTP (30-50 cpm/pmol), 0.12 μmol cyclic GMP, 1 μmol phosphoenolpyruvate (Na^+ salt), 9 μg pyruvate kinase and extract protein as indicated in individual experiments. Reactions were terminated after 5 min at 35° by addition of 20 μl of 1 M EDTA (Na^+ salt, pH 8), followed by 10^5 cpm of cyclic [^{3}H] GMP to estimate recovery. Each reaction mixture was immediately layered on top of a column (0.7 x 2.5 cm) of neutral alumina in 60 mM Tris-HCl (pH 7.5). After the sample ran into the column, the surface was rinsed with 0.2 ml of 60 mM Tris buffer and the column washed with 4 ml of 0.6 M Tris buffer. Samples (4.4 ml) were thoroughly mixed and 1 ml counted in a liquid scintillation spectrometer. Recoveries of cyclic [^{3}H] GMP were 80-90%.

Protein was measured as described by Lowry et al. (10), using bovine serum albumin as standard.

---

[2]Proliferation of B. emersonii requires the sequential appearance of two functionally and morphologically distinct cell types: the motile, uninucleate zoospore and the sessile, multinucleate vegetative cell. The transitions between these two cell types are germination (zoospore to vegetative cell) and sporulation (vegetative cell to zoospores). The entire cell cycle has recently been reviewed (7).

Descending paper chromatography of the reaction product was carried out on Whatman 3 MM paper in two solvent systems: 95% ethanol, 1 M ammonium acetate (7:3 by volume) and saturated ammonium sulfate, 1 M sodium acetate and isopropanol (80:18:2 by volume). Before chromatography the reaction product eluted from an alumina column was separated from Tris buffer by adsorption to AG1 (Cl^-) anion exchange resin and eluted with 0.5 M HCl (9). Fractions containing >94% of the product were pooled and lyophilized to dryness. The residue was dissolved in 0.45 ml of water and neutralized to pH 7 with 15 µl of 1 M Tris base. Aliquots (125 µl containing 25,000 cpm) were chromatographed after addition of 50 nmol each of cyclic GMP, cyclic GMP, 5'-GMP and guanosine as absorbance markers. The dried chromatograms were cut into 1 cm strips for counting. Recovery of radioactivity was >95%. Enzymatic hydrolysis of the ^{32}P-labeled reaction product isolated as described above was carried out on 0.1 ml aliquots after addition of 1 µmol of $MgCl_2$ and 2 µg of purified bovine heart cyclic nucleotide phosphodiesterase (5.4 µmol cyclic GMP hydrolyzed/min/mg protein at 35°). Incubation was for 10 min at 35°, after which the reaction was terminated by boiling. Control incubations lacked enzyme. Aliquots of each sample were chromatographed in the ethanol-ammonium acetate solvent system. Regions of the chromatograms containing cyclic GMP and 5'-GMP were cut out and counted.

Materials. [α-^{32}P] GTP (10-20 Ci/mmol) was purchased from the New England Nuclear Corp. Bovine heart cyclic nucleotide phosphodiesterase purified 300-fold was generously provided by Dr. Rafael Rangel of this Institution. Pyruvate kinase (390 U/mg) from rabbit skeletal muscle was obtained as an ammonium sulfate suspension from the Sigma Chemical Co. The protein was dissolved immediately before use by dilution with an equal volume of 60 mM Tris-HCl buffer, pH 7.5. Sodium phosphoenolpyruvate was from the Sigma Chemical Co. Other materials were as previously described (6,8), or from standard commercial sources.

RESULTS

Zoospore Guanylate Cyclase. Zoospore extracts catalyzed the incorporation of radioactivity from [α-^{32}P] GTP into a product not retained by neutral alumina (Table I). The reaction was strongly dependent on Mn^{2+} ions, a characteristic of the guanylate cyclase reaction (1). In the presence of Mg^{2+} ions, Mn^{2+} stimulated the reaction only about 2-fold, but in these conditions only 60% of the product proved to be cyclic GMP by chromatographic and enzymatic analysis (see below). Accumulation of product also required excess unlabeled cyclic GMP (Table I), which could not be replaced by cyclic AMP and probably acted to protect cyclic [^{32}P] GMP from hydrolysis catalyzed by a labile cyclic GMP-specific zoospore phosphodiesterase recently described (11). A GTP-regenerating system stimulated incorporation only slightly (Table I); in the absence of Mg^{2+} ions GTPase activity, although detectable, is not extensive in B. emersonii extracts over short intervals.[3]

[3] P. Silverman, unpublished result.

Table I

Properties of the Guanylate Cyclase Reaction

Reaction mixture	Cyclic [^{32}P] GMP
	(nmol/5 min)
Complete	6.7
Omit extract	<0.1
Omit $MnCl_2$	0.3
Omit cyclic GMP	0.3
Omit cyclic GMP, add 70 nmol cyclic AMP	0.3
Omit phosphoenolpyruvate, pyruvate kinase	6.1

The complete reaction mixture is described in Methods and contained 740 μg of zoospore protein.

Direct analysis of the ^{32}P-labeled product by paper chromatography in two solvent systems confirmed its identity as cyclic GMP. The single product, accounting for ≥93% of the radioactivity applied, cochromatographed with cyclic GMP in both systems and was clearly distinct from cyclic AMP. Moreover, brief incubation with purified cyclic nucleotide phosphodiesterase quantitatively converted the product to 5'-GMP (data not shown).

The reaction was linear for at least 20 min at levels of protein up to 200 μg protein and for at least 5 min up to 750 μg. The specific activity of the enzyme in zoospore extracts was about 2 nmol cyclic GMP formed/min/mg protein at 35°.

Absence of Guanylate Cyclase Activity in Vegetative Cell Extracts. Vegetative cell extracts had virtually no guanylate cyclase activity when assayed over a wide range of protein concentration (Table II). Mixing experiments with zoospore extracts did not provide evidence for latent vegetative cell guanylate cyclase dependent for its activity on diffusible zoospore components, or for vegetative cell inhibitors of cyclic GMP synthesis or accumulation (Table III).

Table II

Absence of Guanylate Cyclase Activity in Vegetative Cell Extracts

Source of extract	Cyclic [^{32}P] GMP synthesis
	(nmol/5 min)
Vegetative cell (70-550 μg)	≤0.06
Zoospore (315 μg)	3.7
Zoospore (315 μg) plus vegetative cell (70-550 μg)	3.3-2.8

Activity in vegetative cell extracts could not be elicited by Triton X-100 (0.1 to 1% by volume).

Guanylate Cyclase Activity in Sporulating Cell Extracts. Cyclic GMP accumulation in B. emersonii occurs only after a lag period of about 75-90 min extending from the onset of sporulation until the time that the first cells form discharge papilla (6), a characteristic structure of sporulating cells useful as a developmental marker to evaluate the progress and synchrony of sporulation (12). Similarly, the specific activity of guanylate cyclase increased at least 50-fold within the interval of sporulation when the cells formed discharge papilla, usually lasting 45-60 min (Table III). The extract of progeny zoospores isolated after the end of sporulation was at least three times more active than the most active sporulating cell extract (Table III), a difference attributable either to further increase in enzyme activity during late sporulation or to selective incorporation of guanylate cyclase, as compared to total protein, into zoospores as the result of morphogenetic events occurring during zoospore formation (7).

Cycloheximide (10 μg/ml) or actinomycin D (25 μg/ml) added 60 min after the onset of sporulation and 30 min before any cells formed a discharge papillum completely blocked any increase in guanylate cyclase activity over the next 120 min (data not shown).

Table III

Guanylate Cyclase Activity in Sporulating Cell Extracts

Source of extract	Cyclic [^{32}P] GMP synthesis
	(nmol/min/mg protein)
Sporulating cells No discharge papilla	0.01
Sporulating cells 10% discharge papilla	0.04
Sporulating cells 90% discharge papilla	0.57
Progeny zoospores	1.87

Vegetative cells (8L, 1.7 x 10^5 cells/ml) were grown in liquid growth medium at 28° for 6.5 hours (4-6 nuclei/cell), concentrated to about 100 ml by filtration, suspended in 2 L of buffered $CaCl_2$ to initiate sporulation (6) and incubated at 28° with stirring and aeration in two 1 L water-jacketed spinner flasks. The progress and synchrony of sporulation were evaluated by phase contrast microscopy to determine the fraction of cells with a visible discharge papillum or the appearance of progeny zoospores (12). At the indicated stages of sporulation 500 ml aliquots of the culture were filtered over Nitex cloth and the cell cakes (0.5-0.7 wet g) frozen at -70° until extracted. At 240 min after starvation, when >90% of the cells had released zoospores, the remaining 500 ml aliquot was filtered over Sargent No. 500 paper to remove cell wall debris. Zoospores in the filtrate (1.4 x 10^6/ml) were collected by centrifugation at 2,000 x g for 5 min, the medium was removed by aspiration and the zoospore pellets were stored at -70° until extracted. Extracts of sporulating cells were prepared as described in Methods for vegetative cells. The amounts of protein isolated from sporulating cells were between 17 and 23 mg/wet g of cells. The zoospores were frozen and thawed twice in 0.5 ml of buffer A. In all cases, guanylate cyclase assays were carried out at three levels over a 4-fold range of protein (50 to 200 μg).

DISCUSSION

On the basis of the above results, it can be proposed that guanylate cyclase, absent from vegetative cells, is synthesized de novo during an intermediate stage of sporulation and is responsible for the accumulation of cyclic GMP that occurs at that time (6). First, the specific activity of the enzyme in extracts of vegetative cells, which do not carry out net cyclic GMP synthesis

(6), was less than 1% of that in extracts of zoospores, which maintain comparatively high levels of cyclic GMP (6). Second, the appearance of guanylate cyclase activity during zoospore formation correlated well with the onset of intracellular cyclic GMP accumulation at a comparable stage of differentiation (6). Finally, cyclic GMP (6) and guanylate cyclase accumulation *in vivo* were both blocked by cycloheximide or actinomycin D. Since guanylate cyclase appears not to be a constitutive component of *B. emersonii*, it seems likely that cyclic GMP itself functions in this organism uniquely during zoospore formation or in the maintenance or function of zoospores.

One fact not fully explained by our present results is that cyclic GMP accumulation ceases abruptly before the end of sporulation (6), whereas zoospore extracts contain very high levels of guanylate cyclase activity. Cyclic GMP accumulation may be limited *in vivo* by the action of cyclic GMP phosphodiesterase, recently detected in zoospore extracts by Vale *et al.* (11). This enzyme appears to be very labile (11) and was not detected in crude extracts used in our previous study (6), though we have since confirmed the observation of Vale *et al.* (11).[4] It is also possible that zoospore guanylate cyclase activity is regulated *in vivo*. Analysis of the cyclic GMP levels in zoospores isolated and maintained under different conditions, along with further studies of zoospore guanylate cyclase, may provide some indication as to the nature of the mechanisms that regulate the cyclic GMP level in these cells.

ACKNOWLEDGEMENT

Ms. Pravina Mehta provided excellent technical assistance.

REFERENCES

1. Hardman, J. G., Beavo, J. A., Gray, J. P., Chrisman, T. D., Patterson, W. D. and Sutherland, E. W. (1971) Ann. N. Y. Acad. Sci. 185, 27-35.
2. Clark, V. L. and Bernlohr, R. W. (1972) Biochem. Biophys. Res. Commun. 46, 1570-1575.
3. Sun, I. C., Shapiro, L. and Rosen, O. M. (1974) Biochem. Biophys. Res. Commun. 61, 193-203.

[4] M. Lei and P. Silverman, unpublished observation.

4. Macchia, V., Varrone, S., Weissbach, H., Miller, D. C. and Pastan, I. (1975) J. Biol. Chem. 250, 6214-6217.
5. White, A. A. and Zenser, T. (1974) Methods in Enzymol. 38c, 192-195.
6. Silverman, P. M. and Epstein, P. M. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 442-446.
7. Lovett, J. S. (1975) Bacteriol. Rev. 39, 345-404.
8. Silverman, P. M., Huh, M. and Sun, L. (1974) Dev. Biol. 40, 59-70.
9. Mao, C. C. and Guidotti, A. (1974) Anal. Biochem. 59, 63-68.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. C. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
11. Vale, M. R., Gomes, S. L. and Maia, J. C. C. (1975) FEBS Letters 56, 332-336.
12. Murphy, N. M., Sr. and Lovett, J. S. (1966) Dev. Biol. 14, 68-95.