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Oscillation of ADP-ribosyl cyclase activity during the cell cycle and function of cyclic ADP-ribose in a unicellular organism, *Euglena gracilis*

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Abstract In *Euglena gracilis*, the activity of ADP-ribosyl cyclase, which produces cyclic ADP-ribose, oscillated during the cell cycle in a synchronous culture induced by a light-dark cycle, and a marked increase in the activity was observed in the G2 phase. Similarly, the ADP-ribosyl cyclase activity rose extremely immediately before cell division started, when synchronous cell division was induced by adding cobalamin (which is an essential growth factor and participates in DNA synthesis in this organism) to its deficient culture. Further, cADPR in these cells showed a maximum level immediately before cell division started. A dose-dependent Ca^{2+} release was observed when microsomes were incubated with cADPR.

Key words: ADP-ribosyl cyclase; Cyclic ADP-ribose; Cell cycle; Ca^{2+} release; *Euglena gracilis*

1. Introduction

The temporal rise of the intracellular Ca^{2+} concentration has been well demonstrated to be one of the important factors to regulate cell cycle progression [1–3]. Recently, cyclic ADP-ribose (cADPR), a metabolite of NAD^+, has been reported to have a Ca^{2+}-mobilizing activity in sea urchin egg microsomes [4–6], rat brain microsomes [7], cardiac muscle cells [8], and pancreatic β-cells [9]. ADP-ribosyl cyclase, which converts from NAD^+ to cADPR, has been purified from *Aplysia* ovotestis [10]. In addition, a T-lymphocyte antigen marker, CD38, and its family are reported to have an ADP-ribosyl cyclase activity in mammalian cells [11–13]. Okamoto and his colleagues reported that cADPR regulated insulin secretion by Ca^{2+}-mediated mechanisms in pancreatic β-cells [9]. However, the physiological function of cADPR in cell cycle regulation has not yet been elucidated.

Euglena gracilis, a unicellular protist containing chloroplasts, is a useful model to study cell cycle regulation, because this organism is readily synchronized by an appropriate light-dark cycle [14]. Recently, we proposed that reversible mono-ADP-ribosylation was involved in the regulation of cell cycle progression, since mono-ADP-ribosyltransferase activity mainly increased in the S phase and G2-M transitional phase whereas ADP-ribosylhydrolase activity increased extremely in the S and G0 phases [15,16]. In the present paper, we report that ADP-ribosyl cyclase activity oscillates during the cell cycle in synchronous cultures of *E. gracilis*, and propose that cADPR, the product of the enzyme, participates in the regulation of cell cycle progression in the G2-M transitional phase by Ca^{2+}-mediated mechanisms.

2. Materials and methods

2.1. Organisms and culture

A synchronously dividing culture of *E. gracilis* Z was obtained at 25°C in Cramer-Mayers medium [17], as described previously [16]. Illumination was provided by a cool-light fluorescent lamp (8000 lux), and a 24 h cell cycle could be entrained by imposing a light-dark (14:10) cycles. Cell density was monitored using a hemocytometer, and DNA content was measured according to a published method [18].

E. gracilis SM-ZK, a chloroplast-lacking mutant derived from strain Z by treatment with streptomycin [19], was cultured heterotrophically in Koren-Hutner medium [20] without supplement of Cbl (vitamin B12) at 25°C in the dark for 5 days. Synchronous cell division was induced by addition of cyano-Cbl (10 ng/ml) to the Cbl-deficient culture.

2.2. Assay of ADP-ribosyl cyclase activity

Cells were harvested by centrifugation at 1000×*g* for 5 min at 4°C, washed with 20 mM HEPES-Tris buffer, pH 7.0, containing 0.34 M glucose, 1 mM $MgCl_2$, 12.5 mM benzamidine, 0.1 mM EDTA, 0.1 mM EGTA, 20 μg/ml soy bean trypsin inhibitor, 100 μM PMSF and 10 μM leupeptin, and disrupted by sonication in the buffer. After centrifugation (1000×*g*, 5 min) at 4°C to remove cell debris, the supernatant (3 mg protein in 1 ml) was incubated with 4 mM [^{32}P]NAD^+ (700 000 cpm/μmol) at 25°C to allow the ADP-ribosyl cyclase reaction. After incubation for 30 min, 0.1 ml of acetone was added to stop the reaction. The mixture was placed on an ice bath for 60 min, and centrifuged at 10 000×*g* for 10 min. Radioactivity of cADPR in the supernatant which was produced during the enzyme reaction was measured by a radio high-performance liquid chromatography system using an Inertsil ODS column (150×4.6 mm, GL Science). The supernatant was applied onto the column, preequilibrated with 20 mM potassium phosphate buffer, pH 6.0, containing 2% methanol, and cADPR was eluted with the same buffer at a flow rate of 0.8 ml/min. In this chromatography, cADPR, NAD^+, adenosine 5′-diphosphoribose and adenosine 5′-monophosphate were eluted at 4, 14, 5 and 9 min, respectively. ADP-ribosyl cyclase activity higher than 10 pmol/min/mg protein was detectable in this assay system. Protein content was determined according to Bradford [21] using bovine serum albumin as a standard.

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Abbreviations: cADPR, cyclic adenosine diphosphoribose or cyclic ADP-ribose; Cbl, cobalamin; PMSF, phenylmethylsulfonyl fluoride

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2.3. Measurement of cADPR

Cells were harvested by centrifugation at 1000×*g* for 5 min at 4°C. After washing with ice-cold water, the cells were immediately frozen in liquid nitrogen and stored at −80°C. The frozen cells were added to an 8% trichloroacetic acid solution to extract cADPR, and the content of cADPR was measured according to a published method [22].

2.4. Measurement of Ca^{2+} release from microsomes

The microsome fraction was prepared from *Euglena* cells according to a published method [7] with some modifications. Cells were harvested, washed with buffer A (20 mM HEPES, 250 mM *N*-methylglucamine, 250 mM potassium gluconate, 1 mM $MgCl_2$, 100 μg/ml soy bean trypsin inhibitor, 100 μM PMSF, 10 μM leupeptin and 2 μM monensin; pH was adjusted to 7.0 with acetic acid), and homogenized by gentle sonication in buffer A at 4°C. After removal of cell debris by centrifugation (1000×*g*, 5 min), the microsome fraction was collected by centrifugation at 100 000×*g* for 40 min, and suspended with buffer A, supplemented with 1 mM ATP, 5 mM phosphocreatine, 5 units/ml creatine phosphokinase and 1 mM NaN_3. The microsome suspension (0.5 mg protein/ml) was incubated with cADPR in the presence of 1 μM fluo-3 at 25°C, and the change in Ca^{2+} concentration was followed as described previously [7].

3. Results and discussion

3.1. Oscillation of ADP-ribosyl cyclase activity during the cell cycle in synchronous cultures

ADP-ribosyl cyclase activity was found in *E. gracilis* Z cultured autotrophically under continuous illumination (0.67 nmol/min/mg protein). This activity is comparable to that reported in mammalian tissues [12,23]. The enzyme activity was found in the microsome fraction in *Euglena*, when subcellular localization was examined according to Takenaka et al. [16]. To examine the involvement of ADP-ribosyl cyclase in cell cycle regulation in *E. gracilis*, cells were synchronized in a light-dark cycle (14 h light and 10 h dark). Under these conditions, mitosis began at the onset of darkness and synchronous cell division was observed during the dark interval

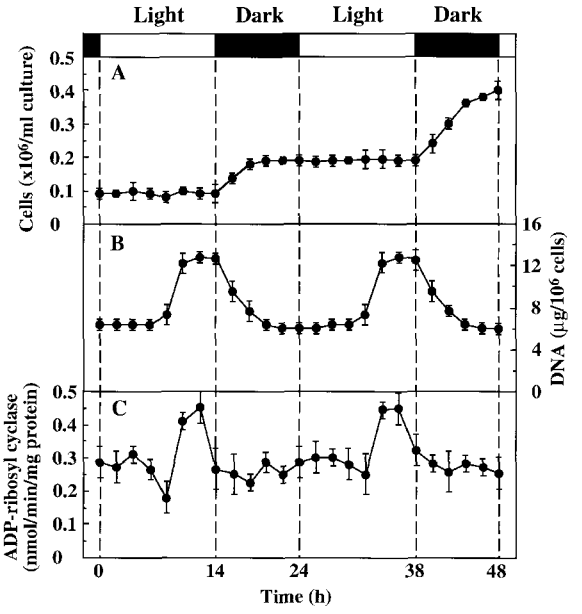

Fig. 1. Oscillation of ADP-ribosyl cyclase activity during the cell cycle in a synchronous culture induced by a light-dark cycle. *E. gracilis* Z was synchronized by a light-dark cycle (14 h light and 10 h dark), and cell number (A), DNA content (B) and ADP-ribosyl cyclase activity (C) were followed during the synchronous culture as described in Section 2. Data are presented as the mean of four experiments (±S.E.).

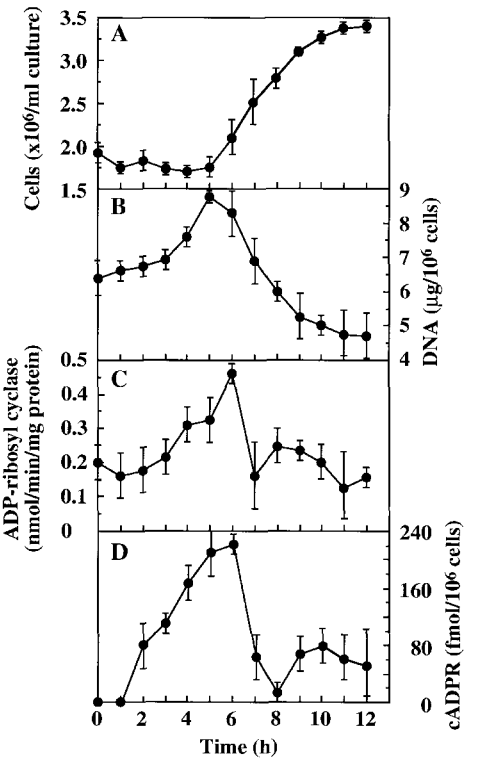

Fig. 2. Changes in ADP-ribosyl cyclase activity and cADPR level in synchronous cell division induced by adding Cbl to its deficient culture. *E. gracilis* SM-ZK was cultured heterotrophically in a Cbl-deficient medium for 5 days to arrest cell growth, and synchronous cell division was induced by addition of cyano-Cbl (10 ng/ml). After the addition of cyano-Cbl, cell number (A), DNA content (B), ADP-ribosyl cyclase activity (C) and cADPR level (D) were followed as described in Section 2. Data are presented as the mean of four experiments (±S.E.).

(Fig. 1A,B), as has been reported previously [14]. The ADP-ribosyl cyclase activity in these cells oscillated during the cell cycle, and a marked increase in the activity was observed in the G2 phase (Fig. 1C).

Euglena requires Cbl (vitamin B12) as an essential growth factor and is easily rendered Cbl-deficient [24], because adenosyl-Cbl is needed as a coenzyme in deoxyribonucleotide reductase which participates in DNA synthesis [25]. *E. gracilis* SM-ZK was cultured heterotrophically in a Cbl-deficient medium for 5 days to arrest cell growth, and a sufficient amount of Cbl was added into the culture. After the addition of Cbl, DNA replication was completed in 5 h, and synchronous cell division occurred during 6–10 h (Fig. 2A,B). These results are consistent with those reported by Johnston and Carell [24]. The ADP-ribosyl cyclase activity in these cells rose extremely just before the cell division started (Fig. 2C). Further, the level of cADPR, which is the product of the enzyme, in these cells showed a maximum in this period (Fig. 2D). These results indicate that ADP-ribosyl cyclase activity oscillates during the cell cycle in *E. gracilis*, and that the level of its product, cADPR, increases markedly in the G2 phase. In addition, it appears that the oscillation of the ADP-ribosyl cyclase activity is responsible for controlling cell division and/or cell cycle progression in this organism.

3.2. cADPR-induced Ca^{2+}-release from Euglena microsomes

It has been well demonstrated that cADPR induces Ca^{2+}

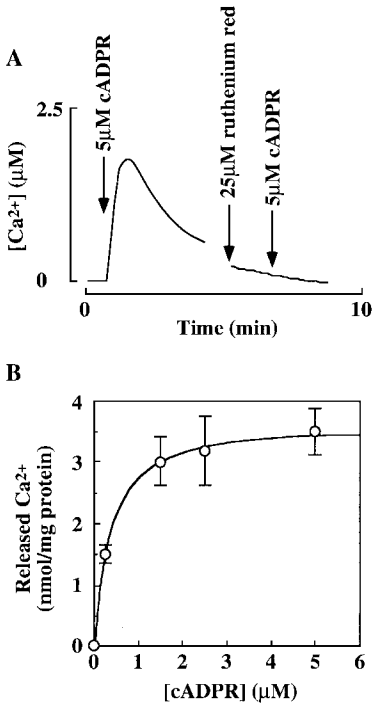

Fig. 3. Ca^{2+} release induced by cADPR. Microsomes, prepared from *E. gracilis* SM-ZK cultured heterotrophically in the dark, were suspended in a buffer containing fluo-3 at 0.5 mg protein/ml, and an increase in Ca^{2+} concentration in the suspension was followed after addition of cADPR. Details are given under Section 2. cADPR elicited a rapid Ca^{2+} release from the microsomes (A, left). Ruthenium red completely inhibited the cADPR-induced Ca^{2+} release (A, right). The cADPR-induced Ca^{2+} release showed a dose-dependent profile (B). In B, data are presented as the mean of four experiments (±S.E.).

release from an intracellular Ca^{2+} store through the ryanodine receptor in various types of cells [7–9]. To elucidate the function of cADPR in *E. gracilis*, cADPR was incubated with *Euglena* microsomes. As shown in Fig. 3A, cADPR elicited a Ca^{2+} release from the microsomes (3.4 nmol/mg microsome protein). This cADPR-induced Ca^{2+} release showed a concentration-dependent profile, and a half-maximum effect was achieved at a concentration around 0.3 μM (Fig. 3B). This dose dependence is almost the same as those reported in other types of cells [7,8,26]. Ruthenium red completely inhibited the cADPR-induced Ca^{2+} release in *Euglena* microsomes (Fig. 3A), as well as in sea urchin eggs [4]. Neither adenosine 5′-diphosphoribose, the non-cyclic analogue of cADPR, nor NAD^+ exhibited any activity to release Ca^{2+} from *Euglena* microsomes (data not shown).

These results indicate that cADPR has the ability to induce Ca^{2+} release from an intracellular Ca^{2+} store in a unicellular organism, *E. gracilis*, as well as mammalian cells [7–9] and sea urchin eggs [4]. On the basis of our present data (Fig. 2D) and of previous reports showing cell volume and cell structure [27,28], the concentration of cADPR in cytosol is thought to be higher than 0.2 μM in the G2 phase in *E. gracilis*. It is thus reasonable to postulate that Ca^{2+} release from an intracellular Ca^{2+} store is induced by the action of cADPR in this phase. We attempted to examine the alteration of Ca^{2+} concentration in intact cells during cell cycle progression by using fluo-3 acetoxymethylester; however, unfortunately, it was impossible due to self-fluorescence of the cells (*Euglena* cells, as well as other photosynthetic organisms, have many kinds of pigments even in the chloroplast-lacking mutant grown in the dark). In sea urchin eggs, it is proposed that a calmodulin-dependent protein kinase plays an important role in the progression from the G2 to M phase [29]. In addition, it is reported that budding yeast is arrested before the M phase if the expression of the calmodulin gene is depressed [30]. Since calmodulin has been found in *E. gracilis* [31], our data presented in this paper suggest that in this unicellular organism cADPR participates in the regulation of cell cycle progression from the G2 to the M phase by Ca^{2+}-dependent mechanisms in which calmodulin is involved as a mediator.

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