Culture Age, Intracellular Ca^{2+} Concentration, and Protein Phosphorylation in Encystment-Induced *Colpoda cucullus*

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Abstract In *Colpoda cucullus*, intracellular Ca^{2+} mediates the encystment induction and protein phosphorylation that occur just prior to morphogenetic transformation into the resting form. When rapidly growing cells were stimulated to encyst, encystment was not readily induced, and the protein phosphorylation level was lower. On the other hand, in post-growing cells stimulated to encyst, the encystment rate and protein phosphorylation level were elevated. These results suggest that protein phosphorylation is closely linked to encystment induction. Why, then, are the protein phosphorylation level and encystment rate difficult to elevate in the rapidly growing cells? Fura 2 ratiometry showed that the intracellular Ca^{2+} concentration ($\text{F}_{340}/\text{F}_{380}$ ratio) was raised in rapidly growing cells as well as in post-growing cells when the cells were stimulated to encyst. It is presumed that the Ca^{2+} -mediated signal transduction pathways for protein phosphorylation and encystment may be triggered in rapidly growing cells, but downstream certain steps may be suppressed by certain intracellular components.

Keywords *Colpoda cucullus* · Ca^{2+} · Phosphorylation · Encystment induction · Phos-tag · Fura 2

Introduction

Resting cyst formation (encystment) of the unicellular eukaryote *Colpoda cucullus* is efficiently and synchronously induced by cell-to-cell mechanical stimulation due

to the overpopulation of vegetative cells [1] in the presence of external Ca^{2+} [2–4]. Based on the finding that encystment of *C. cucullus* is suppressed by the elimination of either external Ca^{2+} by the addition of ethylene glycol tetraacetic acid (EGTA) or intracellular Ca^{2+} via the introduction of ethylene bis (oxy-2, 1-phenylenenitrilo) tetraacetic acid into the cell interior, it has previously been suggested that the Ca^{2+} inflow promoted by cell-to-cell stimulation may activate the signaling pathways for encystment induction [3]. Recently it has been reported that in *C. cucullus*, the phosphorylation level in several proteins is enhanced within 1 h after the onset of encystment induction [5, 6], and such protein phosphorylation requires an inflow of Ca^{2+} [5]. The protein phosphorylation follows processes of structural and functional reorganization such as the degradation of vegetative cell structures, cyst wall formation or the arrest of mitochondrial activity, which each begin more than 1 h after the onset of encystment induction [7].

The vegetative cells of *C. cucullus* cultured in a cereal (wheat leaves) infusion containing bacteria have been shown to grow rapidly for 1–1.5 days; thereafter, the cells begin to encyst (spontaneous encystment) [8]. Our preliminary observation showed that it was difficult to induce rapidly growing cells to encyst, while post-growing cells were apt to encyst. If protein phosphorylation is indeed involved in encystment induction and/or its process, the phosphorylation level is also expected to be influenced by culture age. In the present study, therefore, the protein phosphorylation level and encystment rate were compared between rapidly growing cells and post-growing cells, and it was shown that they were difficult to elevate in rapidly growing cells. This finding may be attributed to the lower membrane permeability to Ca^{2+} in the rapidly growing cells. Therefore, the present study also aimed to compare,

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by means of fura 2 ratiometry assays [9], the relative intracellular Ca^{2+} concentration between rapidly growing cells and post-growing cells.

Materials and Methods

Cell Culture and Encystment Assays

Colpoda cucullus was cultured in a 0.05 % (w/v) infusion of dried wheat leaves which was sometimes inoculated with bacteria (*Klebsiella pneumoniae*). The bacteria were cultured on agar plates containing 1.5 % agar, 0.5 % polypepton, 1 % meat extract and 0.5 % NaCl. The cultured cells of *C. cucullus* were washed in 1 mM Tris–HCl (pH 7.2) by centrifugation ($1,500\times g$ for 2 min). In order to induce encystment, the cells concentrated by centrifugation ($1,500\times g$ for 2 min) were suspended in an encystment-inducing medium [1 mM Tris–HCl (pH 7.2), 0.1 mM CaCl_2] at a high cell density (50,000 cells/ml). As a control, the cells were suspended in 1 mM Tris–HCl (pH 7.2) at a low cell density (2,000 cells/ml).

For determination of the growth curve (Fig. 1a), the number of vegetative cells in a given volume of cell suspension was counted using a thin glass pipette, and the number of resting cysts was estimated by directly counting the number adhering to a given bottom area of the petri dish. In Fig. 1c, the number of encysted cells was counted in a randomly chosen field of view containing 100–200 cells, and was expressed as a percentage of the total number of cells.

Measurement of Free Ca^{2+} Concentrations

The concentration of free Ca^{2+} ($[\text{Ca}]_f$) was calculated as

$$[\text{Ca}]_f = K_d[\text{Ca}]_t / ([\text{EGTA}] - [\text{Ca}]_t)$$

where K_d is the dissociation constant (151 nM in pH 7.2; [10] of EGTA for Ca^{2+} , $[\text{Ca}]_t$ is the concentration of total Ca^{2+} , and $[\text{EGTA}]$ is the EGTA concentration. For fura 2 ratiometry, the membrane-permeable fura 2-AM (1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane- N,N,N',N' -tetraacetic acid, pentaacetoxymethyl ester) (Dojindo Laboratories) was dissolved in dimethyl sulfoxide (DMSO) to give a 5 mM stock solution. The stock solution was diluted 1,000 times to produce 5 μM solution containing 0.1 % DMSO. For fura 2-AM loading, the cells were suspended for 30 min in a solution containing 1 mM Tris–HCl (pH 7.2), 10 μM EGTA and 5 μM fura 2-AM, and then washed twice ($1,500\times g$ for 2 min) in a solution containing 1 mM Tris–HCl (pH 7.2) and 0.1 mM CaCl_2 , or in 1 mM Tris–HCl (pH 7.2). The relative intracellular Ca^{2+} concentrations were

represented as the ratios (F_{340}/F_{380}) of the fluorescence intensities of the cell suspension (2,000 or 50,000 cells/ml) excited by 340- and 380-nm lights. The fluorescence intensities were measured with a Simadzu RF-510 spectrofluorophotometer. Throughout measurement, the cell suspension was stirred to avoid sedimentation of cells.

SDS-PAGE and Western Blotting Analysis

For analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were mixed at a ratio of 1:1 (v/v) with a double-strength sample buffer [2 % SDS, 60 mM Tris–HCl (pH 6.8), 10 % 2-mercaptoethanol and 20 % glycerol], and then boiled for 3 min. A sample containing proteins corresponding to 5,000 cells in each lane was electrophoresed on a 12.5 % gel at 150 V. After SDS-PAGE, the proteins were transferred to an Immobilon-P transfer membrane (Millipore) for 1 h at 100 mA using a semi-dry blotting system (Amersham; Hoefer TE70) with three kinds of blotting solutions [300 mM Tris containing 20 % methanol, 25 mM Tris containing 20 % methanol, 25 mM Tris–borate buffer (pH 9.5) containing 20 % methanol]. For the detection of phosphorylated proteins, a complex of biotin-pendant phosphate-binding tag molecules (Zn^{2+} -Phos-tag^{TM} BTL-104; purchased from <http://www.phos-tag.com>) and horseradish peroxidase-conjugated streptavidin (GE Healthcare Bio-Sciences) was prepared. The phosphoproteins on the membranes were detected according to the method reported by Kinoshita et al. [11]. Blots were stained with a solution containing 0.1 % Coomassie brilliant blue R250 (CBB), 40 % (v/v) methanol and 1 % glacial acetic acid, and then destained in a 50 % (v/v) methanol solution.

Results and Discussion

As shown in Fig. 1, the vegetative cells of *C. cucullus* transferred into fresh culture medium grew rapidly for 1 day (Fig. 1a, open circles) and then began to encyst, with most cells encysting in 2 days (termed ‘spontaneous encystment’; Fig. 1a, closed circles). The start of resting cyst formation depended upon the culture condition; for example, encystment was delayed (beginning at 1.5–2 days) in some cultures.

When the vegetative cells of *C. cucullus* cultured for 0.5 or 1 day were stimulated to encyst, the protein phosphorylation level was enhanced in 1-day cultured cells (Fig. 1b). In this case, the enhancement of the Phos-tag signal should be attributed to a real enhancement of the protein phosphorylation level (not to a difference in the amounts of proteins between the lanes), because the densities were almost equivalent among the lanes of CBB-stained blots (Fig. 1b).

Fig. 1 Cell growth (a), and the effects of culture age (0.5 and 1 day) on in vivo protein phosphorylation (b), the rate of encystment induction (c), and intracellular Ca^{2+} concentration (F_{340}/F_{380} ratio) (d) in *C. cucullus* designed to stimulate ('Induced') or not stimulate ('Not Induced') cells to encyst. **a** Growth curve of the cells cultured in a 0.05 % wheat leaf infusion. One thousand vegetative cells/ml were inoculated in fresh culture medium. Open and closed circles indicate the density (cells/ml) of vegetative cells and resting cysts (cells enveloped with ectocyst), respectively. **b** Protein phosphorylation was analyzed after the vegetative cells of *Colpoda* were stimulated for 1 h to encyst ('Induced') or placed in low-density, no- CaCl_2 (non-inducement) conditions for 1 h ('Not Induced'). Left two lanes ('CBB'), CBB-stained blots after Phos-tag assay was performed. Right two lanes ('P-tag'), proteins detected by biotinylated Phos-tag/

ECL. **c** The rate of encystment was determined at 7 h after the vegetative cells of *Colpoda* were placed in encystment-inducing conditions ('Induced'), or low-density, no- CaCl_2 (non-inducement) conditions ('Not Induced'). **d** The F_{340}/F_{380} ratio of the cell suspension measured at 1 h after the onset of encystment induction. As a control, the ratio of the cell suspension placed in low-density, no- CaCl_2 conditions ('Not Induced') was measured at 0 and 1 h after the cell suspension was transferred into a cuvette and subsequently stirred every 10 min. Columns (points) and attached bars correspond to the means of 5 (a and d) or 6 (c) identical measurements and standard errors. Asterisks (*) and double asterisks (**) in the figures represent significant differences among columns at $p < 0.05$ and < 0.01 , respectively (Mann–Whitney test)

In the 0.5-day cultured cells (rapidly growing cells), the encystment was difficult to induce (Fig. 1c, '0.5 day/Induced'), but it could be prominently induced in the 1- and 1.5-day cultured cells (post-growing cells) (Fig. 1c). As mentioned above, the protein phosphorylation and encystment induction were difficult to promote in the rapidly growing cells. This is possibly attributable to a lower increase in intracellular Ca^{2+} concentration in the rapidly growing cells. However, the intracellular Ca^{2+} concentration (F_{340}/F_{380} ratio) of the 0.5-day cultured cells was raised as much as that of the 1.5-day cultured cells (Fig. 1d) when the cells were stimulated for 1 h to encyst. Thus, the lower responses in the rapidly growing cells may not be attributed to a lower increase in intracellular Ca^{2+} concentration, but rather to the occurrence of less starvation. Actually, the incorporation into the cell interior of D-glucose or the accumulation of its metabolic products has been shown to suppress encystment induction [12].

The intracellular Ca^{2+} concentration was raised, even when the cells were kept in a non-inducible condition (Fig. 1d). This may result from mechanical stimulation of the cells, because during measurement, the cell suspension was stirred to avoid sedimentation of the cells.

Encystment was induced spontaneously in 1- and 1.5-day (Fig. 1c) cultured cells, even under low-density, no- CaCl_2 (non-inducement) conditions. Spontaneous cyst formation also gradually occurred in the post-growing phase (Fig. 1a, closed circles). 'Starvation' is known to be a common factor in encystment induction among a number of protozoans [13]. Starvation-mediated encystment may be involved in the consumption of intracellular encystment-suppressing nutrient molecules and their metabolic products [12]. Spontaneous encystment in 1- or 1.5-day cultured cells (Fig. 1c) may result from a drop in the intracellular encystment-suppressing nutrients and their metabolic products.

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