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Possible Involvement of cAMP and Protein Phosphorylation in the Cell Signaling Pathway for Resting Cyst Formation of Ciliated Protozoan *Colpoda cucullus*

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Summary. It has been suggested that encystment of *Colpoda cucullus* is mediated by intracellular Ca^{2+}-activated signaling pathways involving an increase in the cAMP concentration. In the present study, cAMP enzyme immunoassay (EIA) and chemiluminescence detection for phosphorylated proteins using anti-phosphoserine antibody, anti-phosphothreonine antibody and biotinylated phosphate-binding tag molecules (Phos-tag) showed that the intracellular cAMP concentration in *Colpoda* cells was raised and the phosphorylation level of serine/threonine residues was elevated in many proteins prior to the cyst formation. Such encystment induction and protein phosphorylation were suppressed by the addition of an intracellular Ca^{2+} chelating reagent (BAPTA-AM) or the encystment inhibitor, chlorophyllin in all or most of these proteins, respectively. The phosphorylation level of some proteins was slightly elevated by the addition of IBMX, which tended to promote encystment induction, and tended to be slightly suppressed by the addition of H-89 (PKA inhibitor), which also suppressed encystment induction. These results suggest that Ca^{2+}-activated signaling pathway involving cAMP/PKA-dependent protein phosphorylation may be responsible for the encystment induction of *C. cucullus*.

Key words: *Colpoda*, cyst, encystment induction, cAMP, PKA, phosphorylation.

INTRODUCTION

It has been shown that resting cysts of protists are a cryptobiotic form (Gutiérrez *et al.* 2001), and that the process of the resting cyst formation (encystment) and excystment may be a gene-regulated morphogenetic process (Grisvard *et al.* 2008). During encystment, some cell structures are resorbed as a cyst wall forms. Earlier studies of signal transduction for cellular morphogenesis including encystment and excystment in protists focused on protists such as *Entamoeba*, *Hartmannella*, *Trypanosoma*, *leishmania*, etc. Investigators showed that it is involved in an increase in cAMP level (Raizada and Krishna Murti 1972, Tsim *et al.* 1996, Coppi *et al.* 2002, Frederick and Eichinger 2004) and changes in the protein phosphorylation level (Dell and Engel 1994, Ogueta *et al.* 1998, Franco *et al.* 2000,

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Abel *et al.* 2001, Meza *et al.* 2006). Additionally, an increase in internal level of Ca^{2+} which leads to activation of Ca^{2+}-binding proteins is reported to be responsible for protein phosphorylation in *Trypanosoma* (Ogueta *et al.* 1998) and signal transduction pathways for encystment in *Entamoeba* (Makioka *et al*. 2001). These seem to be conserved pathways as similar mechanisms have been reported to govern encystment in free-living ciliated protist *Colpoda*.

Encystment of the *Colpoda* occurs during the latter stages of growth cycle when cells are maintained on wheat grass medium supplemented with bacteria. Following inoculation, cells enter logarithmic growth and as the population increases, cells enter stationary phase of the growth cycle and begin to encyst (Akematsu and Matsuoka 2008). Induction of encystment has been ascribed to starvation, build up of harmful waste products or lack of oxygen – normal exobiotic factors known to induce this shift in growth rate as a result of overpopulation of cells or extracellular micromolar quantities of Ca^{2+} (Asami *et al.* 2010). This process is called spontaneous encystment. In fact, spontaneous encystment is promoted by an increase in external Ca^{2+} concentration (Yamaoka *et al*. 2004) and/or overpopulation of vegetative cells (Maeda *et al*. 2005). One idea might be that as the population increases, cells encounter each other and thereby intracellular Ca^{2+} concentration is raised as a result of an inflow of external Ca^{2+} (Matsuoka *et al*. 2009) or a release of Ca^{2+} stored in organelles. Interestingly, it has been shown that encystment in *Colpoda* is inhibited by factors released by bacteria (Yamasaki *et al*. 2004) which suggests that as more and more bacteria are consumed during growth, the titer of the inhibitor drops below a threshold suppressing encystment. Additionally, compounds contained in plant leaves, molecules derived from chlorophyll (Maeda *et al*. 2005) and artificial porphyrin analogues such as chlorophyllin-Cu (Tsutsumi *et al*. 2004) inhibit encystment. When the cells suspended in Ca^{2+}-free medium containing cAMP or IBMX (a non-selective inhibitor of phosphodiesterases) at low cell density (500–1,000 cells/ml), encystment is induced, but it is suppressed by the addition of a P-site inhibitor of adenylate cyclase (Matsuoka *et al*. 2009). Taken together, these results show that Ca^{2+} initiates encystment in *C. cucullus* through an adenylate cyclase-activated signaling pathway as suggested by Matsuoka *et al*. (2009).

The purpose of this study is to extend the study of encystment in *C. cucullus* by use of an enzyme immunoassay (EIA) to confirm that intracellular cAMP level is actually elevated prior to encystment, and identify proteins that are phosphorylated in response to activation of a Ca^{2+}/cAMP pathway.

MATERIALS AND METHODS

Organism

C. cucullus was cultured in 0.05% (w/v) cereal infusion inoculated with bacteria (*Klebsiella pneumoniae*) as food. The bacteria, which were kindly supplied by Prof. M. Fujishima of Yamaguchi University, were cultured on agar plates containing 1.5% agar, 0.5% polypepton, 1% meat extract and 0.5% NaCl. The vegetative *Colpoda* cells that had been cultured for 0.5~1 day were rinsed in 1 mM Tris-HCl (pH 7.2) on a membrane filter (10 μm pore size), and then suspended in test solutions at low density (1,000~2,000 cells/ml) or high density (30,000 cells/ml in encystment assays; 50,000~80,000 cells/ml in EIA and protein phosphorylation assays), concentrated by centrifugation at 1,500 g for 2 min.

Chemicals

3-isobutyl-1-methylxanthine (IBMX) and N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) dihydrochloride were purchased from Wako Pure Chemical Industries, ethylene bis (oxy-2, 1-phenylenenitrilo) tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) from Calbiochem, and chlorophyllin (coppered, sodium salt) from Sigma-Aldrich, Inc. BAPTA-AM and H-89 dihydrochloride were dissolved in dimethyl sulfoxide (DMSO) to give 10-mM stock solutions, respectively. In assays, these stock solutions were diluted 1,000 times to produce 10-μM solutions each containing 0.1% DMSO. IBMX and chlorophyllin-Cu were dissolved in pure water to give 50 mM and 1 mM stock solutions, respectively.

Assays for encystment induction (Figs 2a, 3d)

The number of encysted cells was estimated by counting swimming (vegetative) cells in a given volume of cell suspension using a thin pipette (Fig. 2a), or counted directly in a randomly chosen field of view containing 100–120 cells (Fig. 3d), and were expressed as a percentage of the total number of cells. Points (columns) and attached bars correspond to the means of 6 identical measurements and standard errors. Asterisks (*) in figures show significant differences among columns at $p < 0.05$ (Mann–Whitney test).

cAMP assay

Cyclic AMP concentration was determined using competitive enzyme immunoassay (EIA) kits (Cayman Chemical Company). For the EIA assay, cell suspensions were centrifuged (1,500 g, 2 min.) to discard the supernatant, and then 550 μl of 0.1 M HCl was added. Thereafter, the cells were disrupted using a sonicator (Bioruptor UCD-200, CosmoBio), and the supernatants were obtained by centrifugation (8,000 g, 2 min.). Determination of the cAMP concentration was performed according to the protocol of EIA kits. The supernatants were acetylated to improve signal detection.

SDS-PAGE and Western blotting analysis

For analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a cell pellet (centrifuged at 1,500 g for 2 min.) was mixed at a ratio of 1:1 (vol/vol) with a solution containing 2% SDS, 60 mM Tris-HCl (pH 6.8), 2% 2-mercaptoethanol and 20% glycerol, and subsequently boiled for 3 min. Total proteins (10 µl of the packed cells corresponding to 3,000 cells in each lane) were electrophoresed on a 10% polyacrylamide gel, followed by transfer to an Immobilon-P transfer membrane (Millipore) for 3 h at 50 V in a transfer buffer (pH 11.0) containing 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) and 10% methanol. For immunoblotting analysis, the membranes were then blocked for 2~3 h by incubation in a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.2) and 0.05% Tween-20 and supplemented with 5% skim milk. In order to detect the phosphoserine- or phosphothreonine-containing proteins, the blots were immunostained with 0.1 µg/ml mouse anti-phosphoserine monoclonal antibody (clone No. 4A4, Upstate) or 0.1 µg/ml anti-phosphothreonine monoclonal antibody (mixture of three clones: No. 1E11, No. 4D-11, and No. 14B3, Acris Antibodies GmbH) for 40 min. at 37°C, followed by incubation with 0.05 µg/ml peroxidase-labeled goat anti-mouse IgG (Kirkegaard & Perry Lab. Inc.) for 40 min. at 37°C. The antibodies were dissolved in a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 8.0) and 0.05% Tween-20 and supplemented with 0.1% BSA. The complex of biotin-pendant phosphate-binding tag molecule (Zn^{2+}-Phos-tag™ BTL-104; purchased from http:www.phos-tag.com) and HRP (horseradish peroxidase)-conjugated streptavidin (GE Healthcare Bio-Sciences) was prepared according to the method reported by Kinoshita *et al*. (2006). In both immunoblots and Phos-tag-labeled blots, the phosphorylated proteins were detected by the enhanced chemiluminescence (ECL) detection system (Amersham). After Western blotting analysis, blots were stained with 0.1% Coomassie brilliant blue R250 in a 40% (vol/vol) methanol, 1% glacial acetic acid solution, and destained in a 50% (vol/vol) methanol solution. For standard proteins, a molecular standards kit (Bio-Rad) was employed.

RESULTS

cAMP and phosphorylation assays during cell growth

When the vegetative cells of *C. cucullus* were transferred into fresh culture medium, they grew exponentially for 1 day (Fig. 1a, closed circles), and subsequently began to encyst (Fig. 1a, dashed line), a process termed "spontaneous encystment." The concentration of cAMP was elevated just before the cells began to encyst (Fig. 1a, open squares). Fig. 1b shows blots stained with Coomassie brilliant blue (CBB) after phosphorylation analysis (Fig. 1c-2), indicating the bands (arrows) corresponding to phosphorylated proteins. Immunoblot analyses using anti-phosphoserine (Fig. 1c-1) and anti-threonine (Fig. 1c-2) specific antibodies revealed that *in vivo* phosphorylation levels were elevated in several proteins prior to cyst formation.

cAMP and phosphorylation assays in Ca^{2+}/overpopulation-stimulated cells

The rate of encystment induction depends upon an elevation in intracellular Ca^{2+} concentration caused by an inflow of external Ca^{2+} which is promoted by cell-to-cell mechanical contact due to overpopulation of cells (Matsuoka *et al.* 2009 and works in preparation). In the present study, two different parameters ('addition of 0.1 mM Ca^{2+}' and 'overpopulation') were given so that intracellular Ca^{2+} necessary to cause a maximal response could be supplied. For control, the parameters ('Ca^{2+}-free' and 'low cell density') were given so that intracellular Ca^{2+} concentration is not raised as possible.

When vegetative cells at high density were transferred into 1 mM Tris-HCl buffer (pH 7.2) containing 0.1 mM $CaCl_2$, the cells began to cohere, and were observed to be surrounded by the outermost cyst wall termed an ectocyst in 2~6 h. At this stage, the cilia were being resorbed but plainly identifiable below the ectocyst wall. The cells suspended in Ca^{2+}-free (without the addition of Ca^{2+}) 1-mM Tris-HCl buffer (pH 7.2) at a low density of cells encysted slowly.

In 0.5~1 h after onset of encystment induction by overpopulation and an increase in Ca^{2+} in external medium, the intracellular cAMP concentration (Fig. 2b) and *in vivo* phosphorylation levels (detected by anti-phosphoserine antibody) of many proteins (Fig. 2c) were elevated. Fig. 2d shows that *in vivo* phosphorylation detected by Phos-tag (Fig. 2d, P-tag), anti-phosphoserine antibody (Fig. 2d, P-Ser) and anti-phosphothreonine antibody (Fig. 2d, P-Thr) at 1 h after encystment induction was stimulated by overpopulation and Ca^{2+}. Phosphorylated proteins detected by anti-phosphoserine or anti-phosphothreonine antibody were also detected by Phos-tag except for the 39 kDa, 53 kDa and 80 kDa proteins, although some of the signals including 54 kDa protein were quite faint (Fig. 2d).

Effects of BAPTA, IBMX, H-89 and chlorophyllin on *in vivo* protein phosphorylation and encystment induction

Fig. 3 shows the effects of an intracellular Ca^{2+} chelating reagent (BAPTA) (sample No. 7), a PKA inhibitor (H-89) (sample No. 6), a phosphodiesterase inhibitor (IBMX) (sample No. 5) and chlorophyllin-Cu (sample No. 8) on Ca^{2+}/overpopulation-induced *in vivo* phosphorylation of proteins (Figs 3a, b), and on the rate

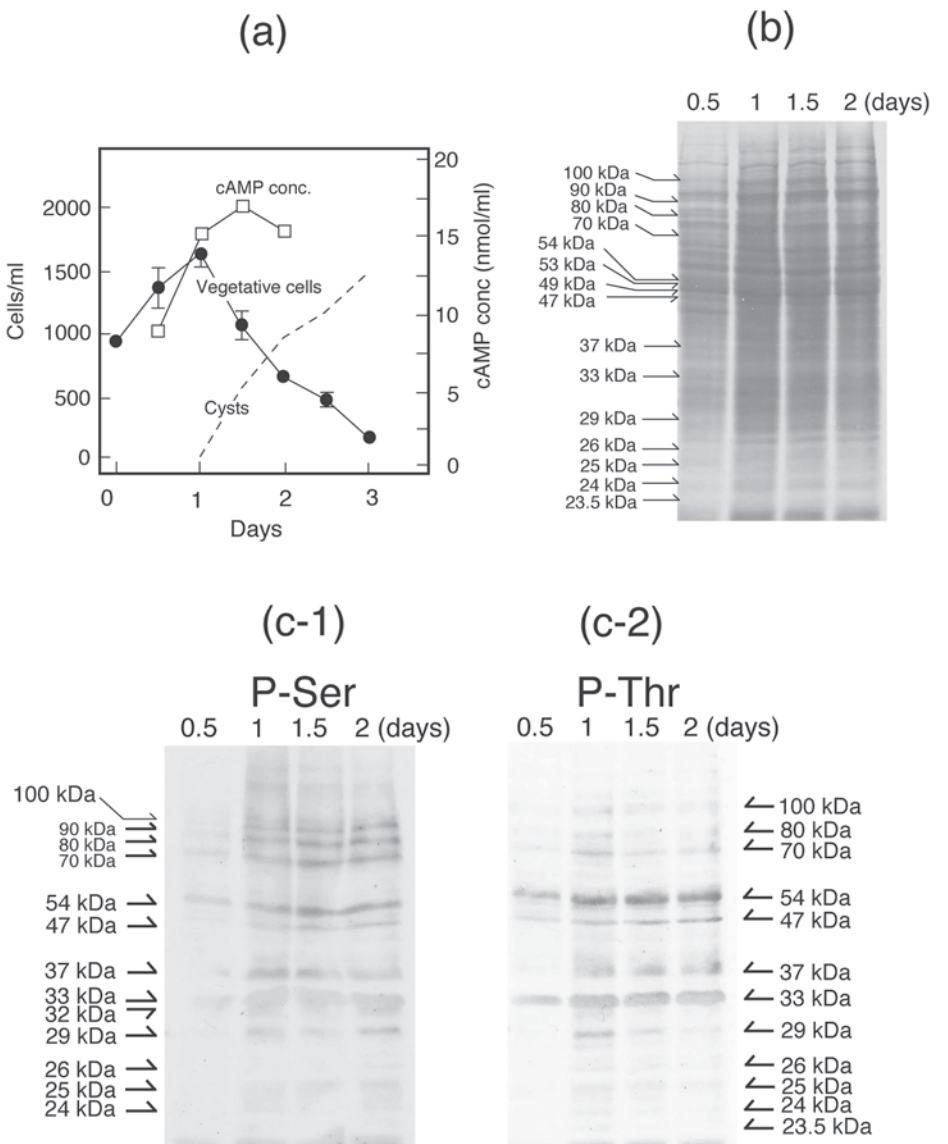

Fig. 1. Elevation of cAMP concentration and promotion of protein phosphorylation during cell growth in *C. cucullus*. **a** – cell growth curve (closed circles) of *Colpoda* in 0.05% cereal infusion and intracellular cAMP concentration (nmol/ml) (open squares). Closed circles and attached bars correspond to the means of 5 measurements and standard errors. Open squares are the result of one trial of measurement. The number of encysted cells (dashed line) was estimated from the number of vegetative cells. The culture was started at a cell density of 1,000 cells /ml of vegetative cells; **b** – blots stained with Coomassie brilliant blue (CBB) after the phosphorylation analysis performed in Fig. 1c-2; **c** – *in vivo* protein phosphorylation analyzed in immunoblots using anti-phosphoserine antibody [P-Ser]) (Fig. 1c-1) or anti-threonine antibody [P-Thr]) (Fig. 1c-2) at 0.5, 1, 1.5, 2 days after a start of culture. In both immunoblots, the same batch sample was analyzed.

of encystment induction (Fig. 3d); The numbers (1–8) labeled top or bottom of figures indicate the cell samples suspended in various test solutions whose components are shown in Fig. 3e. These assays focused on relatively distinct bands (29 kDa, 33 kDa, 37 kDa, 47 kDa, 49 kDa, 54 kDa, 70 kDa, 80 kDa, 90 kDa and 100 kDa proteins). Blots stained with CBB after phosphorylation analysis by anti-phosphoserine antibody (Fig. 3b, left) are shown in Fig. 3c, indicating the bands (arrows) corresponding to phosphorylated proteins.

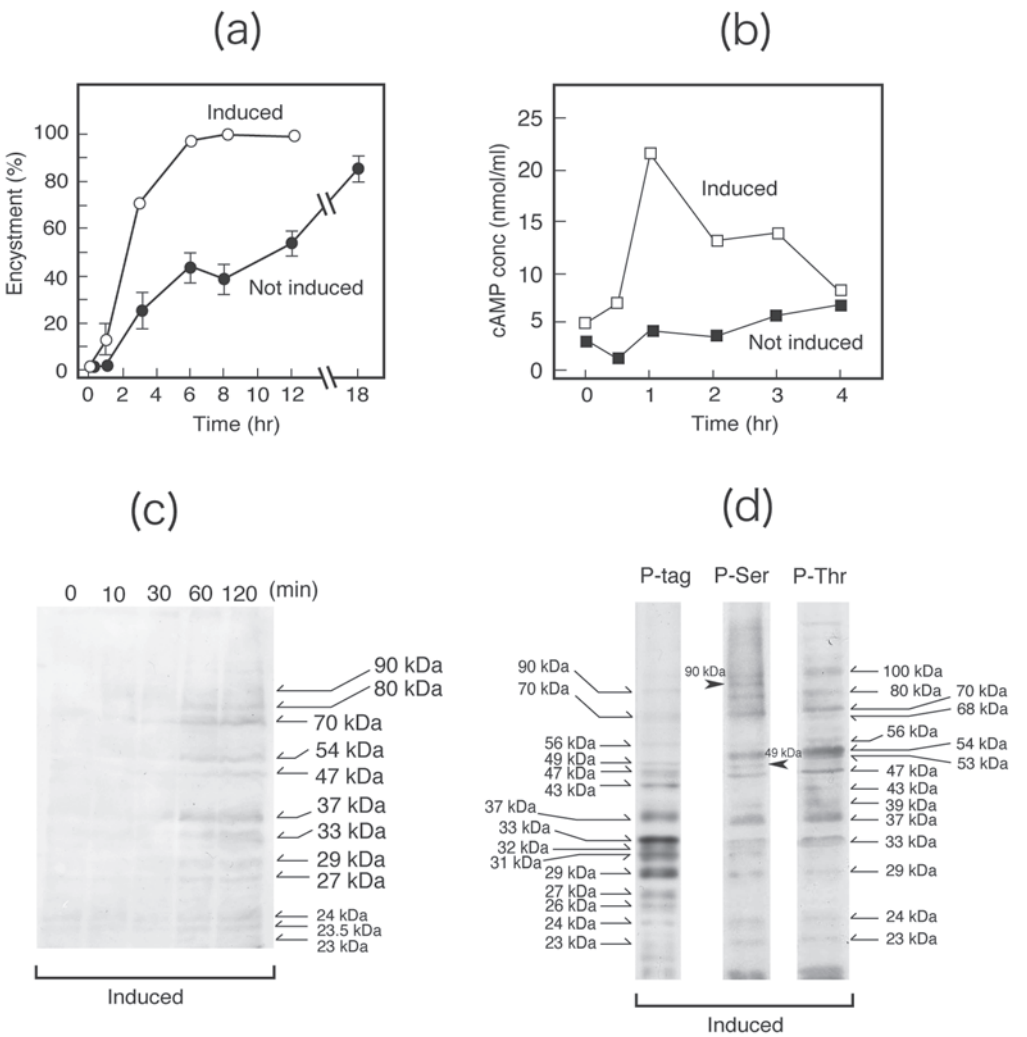

Fig. 2. Promotion of encystment induction (Fig. 2a), elevation of cAMP concentration (Fig. 2b) and protein phosphorylation (Figs 2c, d) of *C. cucullus*, which was stimulated to encyst by being suspended in 0.1 mM Ca^{2+}-containing Tris-HCl (pH 7.2) at a high cell density ('Induced'). **a** – open circles ('Induced'). Encystment rate (%) of cells stimulated to encyst. Closed circles ('Not induced'). Encystment rate (%) of cells suspended in Ca^{2+}-free Tris-HCl (without addition of $CaCl_2$) at a low cell density; **b** – a temporal elevation of intracellular cAMP concentration after stimulated to encyst (one trial of measurement). Open squares ('Induced'). Intracellular concentration of cAMP of cells stimulated to encyst. Closed squares ('Not induced'). Intracellular cAMP concentration of cells suspended in Ca^{2+}-free medium at a low cell density; **c** – *in vivo* protein phosphorylation detected by anti-phosphoserine antibody. The samples were prepared at 0, 10, 30, 60 and 120 min. after the cells were stimulated to encyst; **d** – detection of *in vivo* protein phosphorylation by a Phos-tag (Fig. 2d, P-tag), anti-phosphoserine antibody (Fig. 2d, P-Ser) and anti-phosphothreonine antibody (Fig. 2d, P-Thr) at 1 h after the vegetative cells were stimulated to encyst.

Detection by anti-phosphoserine antibody (Figs 3a, b, left)

The phosphorylation assay performed with anti-phosphoserine antibody is shown in Figs 3a, b (left, P-Ser). Elevation of the phosphorylation level caused by overpopulation and an increase in external Ca^{2+} concentration was detected in the 29 kDa, 33 kDa, 37 kDa, 47 kDa, 49 kDa and 54 kDa proteins, with a slight tendency of elevation in the 70 kDa, 80 kDa, 90 kDa and 100 kDa proteins (compare sample No. 1 with No. 3, or sample No. 2 with No. 4). In the presence of BAPTA-AM in the external medium, the Ca^{2+}/overpopulation-enhanced phosphorylation level tended to be reduced

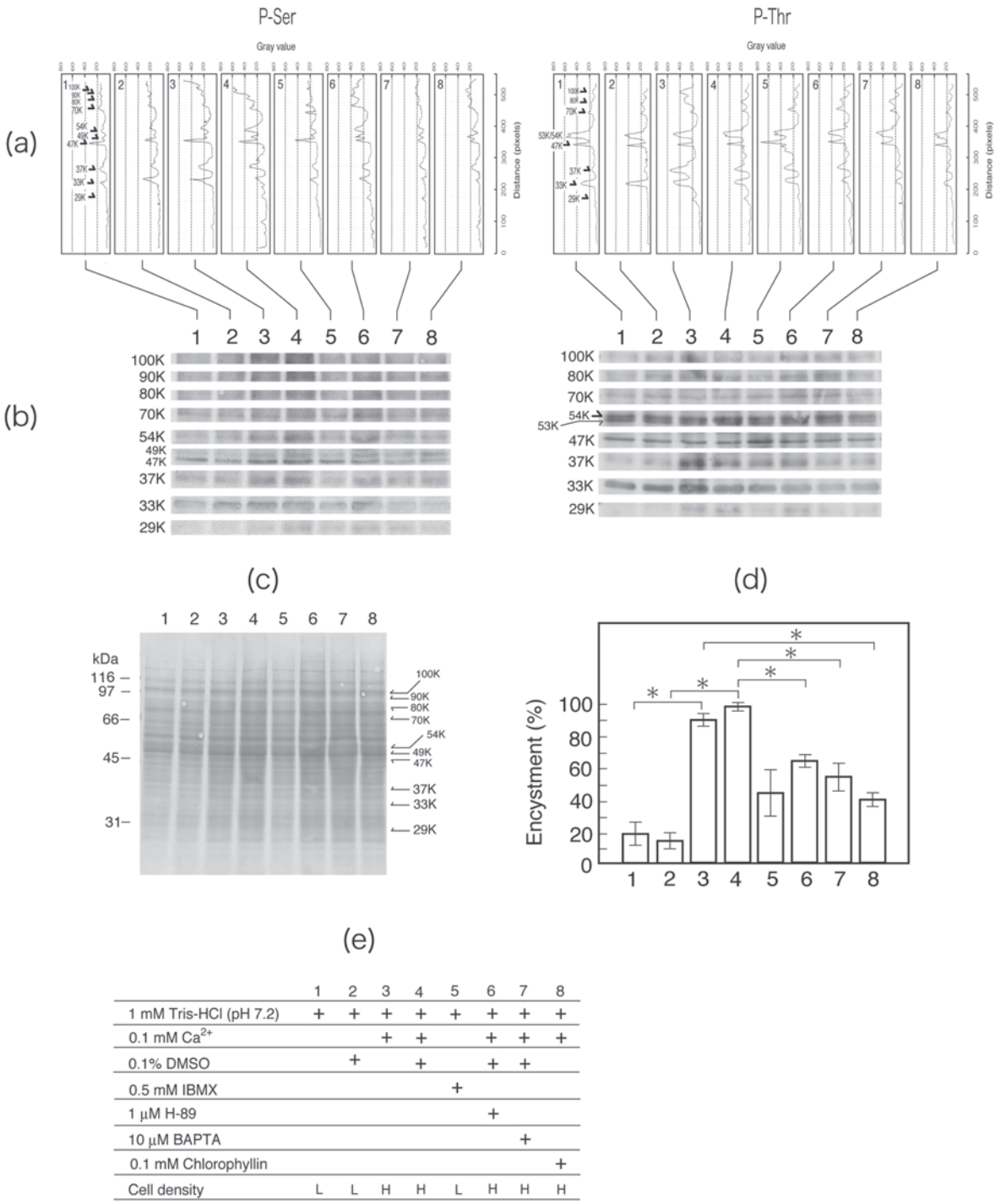

Fig. 3. Effects of BAPTA, IBMX, H-89 and chlorophyllin-Cu on *in vivo* protein phosphorylation (Figs 3a, b) and encystment induction (Fig. 3d) of *C. cucullus*, which was stimulated to encyst by being suspended in 0.1 mM Ca^{2+}-containing Tris-HCl (pH 7.2) at a high cell density. **a** – NIH Image analyses of each lane of immunoblots shown in Fig. 3b; **b** – (left and right). Immunoblots of phosphorylated proteins analyzed using anti-phosphoserine antibody [P-Ser]) (Fig. 3b, left) or anti-threonine antibody [P-Thr]) (Fig. 3b, right) at 1 h after the vegetative cells were stimulated to encyst; **c** – blots stained with Coomassie brilliant blue (CBB) which were used in the immunoblotting analysis with anti-threonine antibody (Fig. 3b, right); **d** – rates of encystment (%) measured 7 h after the onset of encystment induction; **e** – components contained in 8 test solutions (No. 1–8) and the density of the cells suspended in each solution. The cells were suspended in each solution either at a low cell density (L) or at a high cell density (H). The numbers (1–8) labeled on the top of each lane of immunoblots (Fig. 3b) and CBB-stained blots (Fig. 3c), and on the bottom of each column (Fig. 3d) correspond to those labeled on the top of table (Fig. 3e).

in these proteins (compare sample No. 4 with No. 7). In the presence of IBMX, slightly elevated phosphorylation was detected in the 47 kDa and 54 kDa proteins (compare sample No. 1 with No. 5). In the presence of H-89, it was reduced slightly in the 37 kDa, 47 kDa and 54 kDa proteins (compare sample No. 4 with No. 6). In the presence of chlorophyllin-Cu (encystment inhibitor), it was reduced in most (29 kDa, 33 kDa, 37 kDa, 47 kDa and 54 kDa) of the proteins (compare sample No. 3 with No. 8).

Detection by anti-phosphothreonine antibody (Figs 3a, b, right)

The phosphorylation assay performed with the anti-phosphothreonine antibody is shown in Figs 3a, b (right, P-Thr). Elevation of the phosphorylation level caused by overpopulation and an increase in external Ca^{2+} concentration was detected in the 29 kDa, 33 kDa and 37 kDa proteins, and tended to increase in the 80 kDa and 100 kDa proteins (compare sample No. 1 with No. 3). In this case, however, in the presence of DMSO, the phosphorylation level was not elevated in the 33 kDa and slightly elevated in 37 kDa proteins (compare sample No. 2 with No. 4). In the presence of BAPTA-AM, Ca^{2+}/overpopulation-mediated phosphorylation was suppressed in the 29 kDa, 33 kDa and 37 kDa proteins (compare sample No. 4 with No. 7). In the presence of IBMX, elevated phosphorylation was detected in the 37 kDa and 47 kDa proteins (compare sample No. 1 with No. 5). In the presence of H-89, the phosphorylation was slightly suppressed in the 37 kDa protein (compare sample No. 4 with No. 6). In the presence of chlorophyllin-Cu, the phosphorylation was suppressed in the 29 kDa, 33 kDa and 37 kDa proteins (compare sample No. 3 with No. 8). These results are summarized in Table 1. In Table 1, the antibodies (anti-phosphoserine or anti-phosphothreonine antibody) by which the protein phosphorylation was detected are shown in parentheses.

Assays of encystment induction

In the presence of BAPTA-AM, H-89 or chlorophyllin-Cu, encystment induction was significantly suppressed ($p < 0.05$, Mann–Whitney test) (Fig. 3d). In the presence of IBMX, the degree (%) of encystment tended to be raised, but not significantly ($p > 0.05$, Mann–Whitney test) (Fig. 3d, compare sample No. 1 with No. 5).

DISCUSSION

Based on the pharmacological assays, it has been speculated that intracellular signaling pathways for encystment induction in *C. cucullus* may be activated by an increase in intracellular Ca^{2+} concentration which is caused by a Ca^{2+} inflow stimulated by cell-to-cell contact; the cAMP concentration may be elevated in the downstream of this pathway (Matsuoka *et al*. 2009). The present study confirmed that the intracellular cAMP concentration of *C. cucullus* was actually elevated (Fig. 1a, Fig. 2b) prior to encystment, and revealed that concomitantly the phosphorylation level was elevated in many proteins (Fig. 1c, Figs 2c, d). Among them, the Ca^{2+}/overpopulation-enhanced phosphorylation in 29 kDa, 33 kDa, 37 kDa, 47 kDa, 49 kDa and 54 kDa proteins (Figs 3a, b, Table 1) and encystment induction (Fig. 3d) were suppressed in the presence of BAPTA-AM. These results suggest that an increase in intracellular Ca^{2+} concentration promoted by cell-to-cell co-stimulation (Matsuoka *et al*. 2009) may be responsible for enhancement of the phosphorylation in these proteins. The fact that among the proteins in which phosphorylation level was enhanced by encyst-

Table 1. Promotion and suppression of *in vivo* protein phosphorylation summarized based on the results of Fig. 3.

Promoted by Ca^{2+}/ overpopulation	Suppressed by BAPTA	Promoted by IBMX	Suppressed by H-89	Suppressed by chlorophyllin
54 kDa (Ser)	54 kDa (Ser)	54 kDa (Ser)	54 kDa (Ser)	54 kDa (Ser)
49 kDa (Ser)	49 kDa (Ser)			
47 kDa (Ser)	47 kDa (Ser)	47 kDa (Ser, Thr)	47 kDa (Ser)	47 kDa (Ser)
37 kDa (Ser, Thr)	37 kDa (Ser, Thr)	37 kDa (Thr)	37 kDa (Ser, Thr)	37 kDa (Ser, Thr)
33 kDa (Ser, Thr)	33 kDa (Ser, Thr)			33 kDa (Ser, Thr)
29 kDa (Ser, Thr)	29 kDa (Ser, Thr)			29 kDa (Ser, Thr)

ment induction, phosphorylation in the 37 kDa, 47 kDa and 54 kDa proteins was promoted by IBMX, a non-selective inhibitor of phosphodiesterases (Mehats *et al*. 2002) and tended to be slightly suppressed by H-89, which is a PKA inhibitor (Chijiwa *et al*. 1990) (Figs 3a, b, Table 1), implies that these proteins may be phosphorylated by PKA.

In the presence of chlorophyllin-Cu, an inhibitor of encystment induction of *C. cucullus* (Tsutsumi *et al.* 2004), enhancement of phosphorylation was suppressed in most of the proteins (Figs 3a, b, Table 1), indicating that chlorophyllin-Cu may affect an event upstream of protein phosphorylation.

The pattern of protein phosphorylation prior to spontaneous encystment occurring after exponential cell growth during culture (Fig. 1c) seems to be almost identical with that of protein phosphorylation in the cells which were stimulated to encyst by overpopulation in the presence of 0.1 mM Ca^{2+} (Figs 2c, d). This suggests that a common signaling pathway may be utilized in the spontaneous encystment and Ca^{2+}/overpopulation-promoted encystment.

An elevation in the phosphorylation level of most proteins was detected commonly by anti-phosphoserine and anti-phosphothreonine antibodies (Figs 2d, 3), possibly because the anti-phosphothreonine antibody may mis-react with phosphorylated serine residues of the proteins, or *vice versa*, though it is likely that some proteins contain two different phosphorylation sites for serine and threonine residues. The bands corresponding to the 39 kDa, 53 kDa and 80 kDa proteins (Figs 2d, 3b) detected by the anti-phosphoserine or anti-phosphothreonine antibody were not detected by the Phos-tag assay (Fig. 2d, P-tag). The signal of 54 kDa protein which was detected distinctly by the antibodies was quite faint or difficult to be detected by Phos-tag. Presumably, phosphate groups bound to these proteins may fail to be captured by the Phos-tag, or anti-phosphoserine or anti-phosphothreonine antibody may be bound to these proteins nonspecifically.

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