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RESEARCH REPORT

Identification of cAMP-dependent phosphorylated proteins involved in the formation of environment-resistant resting cysts by the terrestrial ciliate *Colpoda cucullus*

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Abstract

In the terrestrial ciliate *Colpoda cucullus*, an elevation of the intracellular cAMP concentration was reported to be involved in environment-resistant resting cyst formation. In the present study, cAMP-dependently phosphorylated proteins of encystment-induced *C. cucullus* were isolated with Phos-tag agarose phosphate-affinity beads and subsequent SDS-PAGE. In a liquid chromatography/tandem mass spectrometry analysis of these phosphoproteins, 27-, 37- and 43-kDa proteins (p27, p37 and p43) were identified as Rieske iron-sulfur protein, histone H4 (hyperacetylated form), and actin, respectively.

Key Words: environment-resistant cyst; *Colpoda*; encystment; protein phosphorylation; cAMP

Introduction

The terrestrial protists, inhabitants of temporary puddles, form resting cysts when they detect approaching hostile environmental conditions. They excyst when conditions favorable to survive and to proliferate are regained. In general, the resting cysts of terrestrial protists are resistant to extreme environments. For example, dried cysts of the terrestrial ciliate *Colpoda cucullus* have been reported to survive up to about 120 °C (Taylor and Strickland, 1936). The resting cyst of this species is also resistant to environmental stresses such as desiccation, freezing and even extreme lower pH (1 M HCl) (Maeda *et al*., 2005; Sogame *et al*., 2011b). For better understandings of the mechanisms underlying the cellular morphogenesis and acquisition of tolerance during the encystment of *C. cucullus*, we studied this species from morphological and molecular perspectives, as follows.

The cyst formation (encystment) process involves remarkable cellular morphogenetic transformation as follows (Funatani *et al*., 2010): Some mitochondria are fragmented within 1 h after the onset of encystment induction and then digested by autophagy. Net-like globules called lepidosomes (Foissner *et al*., 2011) are formed inside the intracellular vacuoles, to be expelled within 1 - 2 h after the onset of encystment induction, and subsequently the ectocyst (an outermost layer of cyst wall) is formed. In this stage (2 - 3 h after the onset of encystment induction), small-sized chromatin granules extruded from the macronucleus are digested by autophagy, and the mitochondrial membrane potential begins to disappear (Sogame *et al*., 2014). Ectocyst formation is followed by the formation of several layers of endocyst between the ectocyst and plasma membrane for several days. Within 1 - 2 weeks, the structures characterizing vegetative cells such as the ciliary apparatus are disintegrated, and electron-lucent granules that may be reserve granules accumulate in the center of the mature resting cyst.

Encystment of *C. cucullus* can be induced by the overpopulation of vegetative cells in the presence of Ca^{2+} (Yamaoka *et al*. 2004; Matsuoka *et al*., 2009). Intracellular signaling pathways leading to the encystment of *C. cucullus* are triggered by an increase in the intracellular Ca^{2+} concentration (Sogame and Matsuoka, 2013), which is promoted by cell-to-cell mechanical stimulation in the presence of external Ca^{2+} (Matsuoka *et al.*, 2009). In these signaling pathways, the intracellular cAMP concentration is elevated (Matsuoka *et al*., 2009; Sogame *et al*., 2011a, 2011c), as are the

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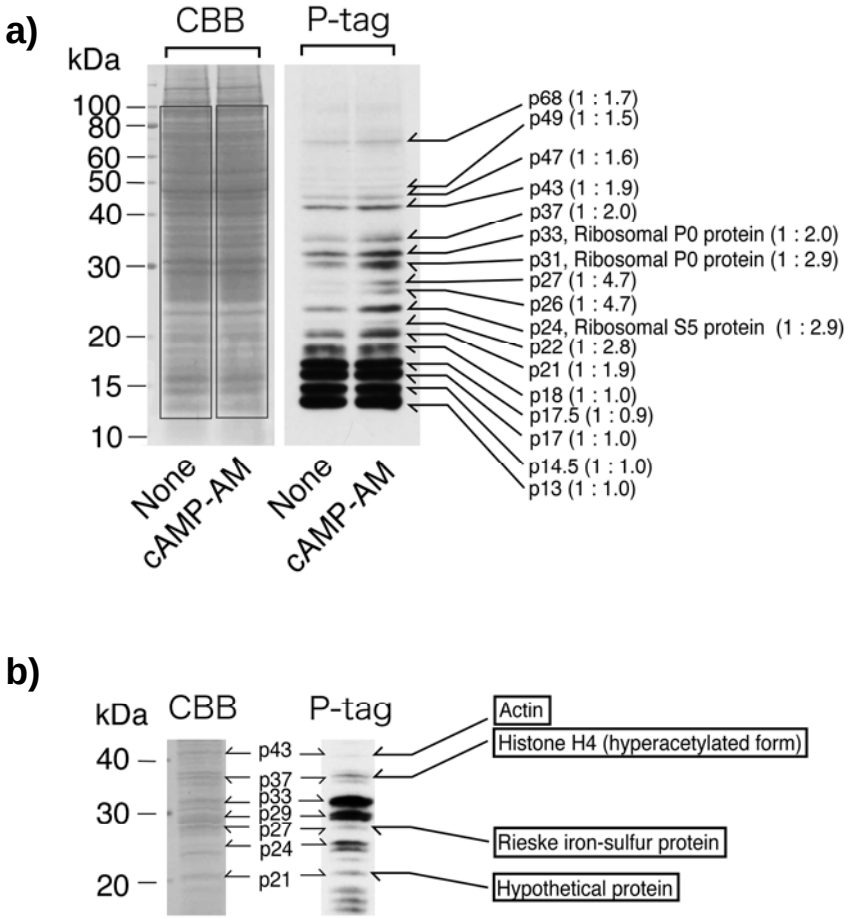

Fig. 1 cAMP-dependent protein phosphorylation detected by biotinylated Phos-tag/ECL assays (a),phosphoproteins isolated with Phos-tag agarose phosphate-affinity beads in encystment-induced *C. cucullus* (b). (a) 'CBB': Blots stained with CBB after the biotinylated Phos-tag/ECL detection. 'P-tag': Protein phosphorylation detected by biotinylated Phos-tag/ECL assays. Protein phosphorylation and encystment was induced by *C. cucullus* vegetative cells being suspended for 1 h at a low cell density (2,000 cells/ml) in 1 mM Tris-HCl (pH 7.2) containing 10 µM cAMP-AM (Fig. 1a, 'cAMP-AM'). As a control (a, 'None'), the cells were suspended in 1 mM Tris-HCl (pH 7.2) without cAMP-AM at a low cell density (2,000 cells/ml); (b) Isolation of phosphoproteins of encystment-induced *C. cucullus* (encystment induction by Ca^{2+}/overpopulation) with Phos-tag agarose phosphate-affinity beads and subsequent SDS-PAGE/Western blotting. CBB: Blots stained with CBB after the biotinylated Phos-tag/ECL detection ('P-tag'). 'P-tag': Biotinylated Phos-tag/ECL detection of phosphoproteins, isolated with Phos-tag agarose phosphate-affinity beads.

phosphorylation levels of several proteins (Sogame *et al*., 2012a), and followed by the alteration of protein expression (Sogame *et al*., 2012b, 2014). Among encystment-dependently phosphorylated proteins, some proteins (33-, 37-, 43-, 47- and 49-kDa proteins) have been reported to be cAMP-dependently phosphorylated (Sogame *et al.*, 2011c). In the present study, in addition to these proteins, several phosphoproteins were found to be also phosphorylated in cAMP-dependent manner. The purpose of the present study is to identify cAMP-dependently phosphorylated proteins in the early phase of encystment of *C. cucullus* by a liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis.

Materials and Methods

Cell culture and encystment induction

Colpoda cucullus (Nag-1 strain) collected from the soil surface in Kochi Prefecture, Japan was cultured in a 0.05 % (w/v) infusion of dried wheat leaves, which was periodically inoculated with a non-pathogenic strain (6081) of *Klebsiella pneumoniae*. The bacteria were cultured on agar plates containing 1.5 % (w/v) agar (Wako Pure Chemical Industries, Osaka, Japan), 0.5 % (w/v) polypeptone (Nihon Pharmaceutical Co., Tokyo), 1 % (w/v) beef extract (Becton Dickinson, Lincoln Park, NY), and 0.5 % (w/v) NaCl. The vegetative cells of *C. cucullus* cultured for 1 - 2 days were collected by

centrifugation (1,500×*g* for 2 min) and resuspended in 1 mM Tris-HCl (pH 7.2). In order to induce protein phosphorylation and encystment, the cells collected again by centrifugation (1,500×*g* for 2 min) were suspended at a low cell density (2,000 cells/ml) in 1 mM Tris-HCl (pH 7.2) containing 10 µM adenosine 3', 5'-cyclic monophosphate acetoxymethyl ester (cAMP-AM) (encystment induction by cAMP), or suspended at a high cell density (50,000 cells/ml) in 1 mM Tris-HCl (pH 7.2) containing 1 mM $CaCl_2$ (encystment induction by Ca^{2+}/overpopulation). As a control of encystment induction by cAMP, the *Colpoda* cells were suspended in 1 mM Tris-HCl (pH 7.2) containing 0.1 % dimethyl sulfoxide (DMSO) at a low cell density (2,000 cells/ml).

Chemicals

Adenosine 3', 5'-cyclic monophosphate acetoxymethyl ester (cAMP-AM) was purchased from Sigma-Aldrich (St. Louis, MO). cAMP-AM was dissolved in DMSO to give 10 mM stock solution. Prior to the assays, the stock solution was diluted 1,000 times to produce 10 µM cAMP-AM solution containing 0.1% DMSO. Phenylmethylsulfonyl fluoride (PMSF) was purchased from Boehringer-Mannheim (Gaithersburg, MD), aprotinin from Sigma-Aldrich, leupeptin and pepstatin from Peptide Institute Inc. (Osaka, Japan), sodium fluoride (NaF) from Wako Pure Chemical Industries (Osaka, japan), and sodium orthovanadate from Sigma-Aldrich. PMSF and pepstatin were dissolved in DMSO to give 1 M and1 mg/ml stock solutions, respectively. For use, these stock solutions were diluted 1,000 times to produce solutions with final concentrations of 1 mM and 1 µg/ml, respectively, and containing 0.1 % DMSO. Leupeptin, aprotinin and sodium orthovanadate (Na_3VO_4) were dissolved in pure water to produce 1 mg/ml, 1 mg/ml and 1 M stock solutions, respectively. Prior to the assays, they were diluted 1,000 times to produce final concentrations of 1 µg/ml, 1 µg/ml and 1 mM solutions, respectively. NaF was dissolved in pure water to give a 200 mM stock solution, and diluted 200 times to produce a solution with a final concentration of 1 mM.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

We performed the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) essentially according to Laemmli's method (Laemmli, 1970). The *Colpoda* cells were solubilized in the SDS-sample buffer containing 30 mM Tris-HCl (pH 6.8), 1 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 10% glycerol, and then boiled for 3 min. The total proteins (approx. 50 mg) corresponding to 5,000 cells in each lane were electrophoresed on a 10 % (Fig. 1a) or 12.5 % (Fig. 1b) gel at 150 V.

Western blotting and biotinylated Phos-tag /ECL assay

Electrophoresed proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA) for 3 h at 350 mA in a transfer buffer (pH 11.0) containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) and 10 % methanol, transferred for 60 min at 100 mA using a semi-dry blotting system (Hoefer TE70, Amersham, Tokyo) with three different types of blotting solutions containing 20 % methanol each (solution A, 300 mM Tris; solution B, 25 mM Tris; solution C, 25 mM Tris-borate buffer [pH 9.5]). For the Phos-tag (phosphate-binding tag molecule) detection of phosphorylated proteins, we prepared a complex consisting of biotin-pendant phosphate-binding tag molecule (Phos-tag) (Zn^{2+}-Phos-tag™ BTL-104; available at http:www.phos-tag.com) and horseradish peroxidase (HRP)-conjugated streptavidin (GE Healthcare Bio-Sciences, Buckinghamshire, UK).Phosphorylated proteins were detected according to the method reported by Kinoshita *et al.* (2006). In this assay, the phosphoproteins on the transfer membranes were detected by an enhanced chemiluminescence (ECL) detection system (GE Healthcare) (exposed to Hyperfilm and developed at 20 °C). Thereafter, the blots were stained for 1 min with 0.1 % CBB R250, 40 % (v/v) methanol, 1% glacial acetic acid solution, and then destained in 50 % (v/v) methanol.

ImageJ analysis

Theoptical density of each lane (indicated by the framework) of CBB-stained gels (Fig. 1a, left) was determined by an ImageJ analysis. Each Phos-tag detected band (Fig. 1a, right) was also analyzed by ImageJ, and the ratios of theoptical density of each corresponding band are indicated in the parentheses.

Isolation of phosphoproteins with Phos-tag agarose phosphate-affinity beads

For the isolation of phosphoproteins using Phos-tag agarose phosphate-affinity beads, the *Colpoda* cells were solubilized for 2 h on ice in 50 mM Tris-HCl (pH 7.4) containing 2.5 % (w/v) sodium deoxycholate, 2 % (v/v) Nonidet P-40 (NP-40), 1 mM N, N, N', N'-ethylenediaminetetraacetic acid (EDTA), 0.15 M NaCl, protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ ml leupeptin and 1 µg/ml pepstatin) and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF). The isolation of phosphoproteins using Phos-tag agarose phosphate-affinity beads and SDS-PAGE was performed according to the methods reported by Kinoshita-Kikuta *et al.* (2009).

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Prior to the LC-MS/MS analysis, the phosphoproteins trapped on Phos-tag agarose phosphate-affinity beads were separated by SDS-PAGE and then electroblotted to an Immobilon-P transfer membrane (Fig. 1b, left lane). The blots used in the Phos-tag detection assays were incubated in 1 N aqueous NH_3 for 15 min three times to remove the biotinylated Phos-tag complex (Nakanishi *et al.*, 2007). Protein bands (Fig. 1b, left lane) visualized by CBB staining were cut out and then subjected to reduction with 40 mM DTT (dithiothreitol) for 1 h at 37 °C and alkylation with 100 mM iodoacetic acid for 20 min at room temperature to generate the carboxymethylation of cysteine residues. To block the non-specific binding of protease, membrane pieces were treated in 100 mM

Table 1 Novel *Colpoda cucullus* proteins isolated with phosphate-affinity beads and tentatively identified by LC-MS/MS whose phosphorylation level is enhanced by encystment induction

Protein name	Bands obtained by SDS-PAGE	Sequences of exactly matched peptides	Partially matched peptides [2]	Accession No. (organisms)	Sequence coverage (%)
Actin	p43	AGFAGDDAPR, MPGIMVGMDQK, DSYVGDEAQSK, LTEAPLNPK, ELTALAPSTMK		gi 157093087 (*Karlodinium micrum*)	14 (52AA/376AA)
Histone H4 [1]	p37	ISGLIYEETR, TLYGFGG		gi 294888716 (*Perkinsus marinus*)	17 (17AA/103AA)
Rieske iron-sulfur protein	p27	LVEDK, PGNFGDHIDFK	HLVEDKPTFFVTSSR, VNIDNWFDENR, LYAMGVIGR, EENELPSNTLLDK, EVILSDAGNT	gi 146164447 (*Tetrahymena thermophila*)	26 (69AA/269AA)
Hypothetical protein	p21	LYDPNTFYEHGD-NPAFK	GTASEEELK NWDDFLQRDCK PVGSHGITK	gi 146142959 (*Tetrahymena thermophila*)	22 (46AA/210AA)

[1]Hyperacetylated form
[2]*De novo* sequences. The residues matched with those in sequences predicted by PEAKS online 5.3 are underlined

acetic acid containing 0.5 % polyvinylpyrrolidone (PVP40) for 30 min at 37 °C. On-membrane digestion of proteins was performed in 10 ml of 30 mM Tris-HCl (pH 8.5) containing 10 % acetonitrile and 1 pmol trypsin (Sigma-Aldrich) for 18 h at 37 °C. Peptides produced by tryptic digestion were separated by a 0% - 40% linear gradient with acetonitrile and analyzed with an ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC Qtof) system (Xevo®, Waters, Milford, MA). The raw data were processed with ProteinLynx Global Server 2.4 software (Waters). Proteins were identified by searching against the Alveolata protein sequences registered in NCBI Entrez protein records using PEAKS online 5.3 (Bioinformatics Solutions Inc., Waterloo, ON, Canada).

Results and Discussion

When the cells were suspended at a low cell density in 1 mM Tris-HCl (pH 7.2) containing 10 µM cAMP-AM, the phosphorylation level was evidently elevated (greater than about twofold) in 21-, 22-, 24-, 26-, 27-, 31-, 33-, 37-, and 43-kDa proteins (p21 - p43) (Fig. 1a, 'P-tag'), indicating that these proteins may be phosphorylated through cAMP-dependent kinase (protein kinase A; PKA). The ratios indicated in the parentheses in Fig. 1a ('P-tag') are the ratio of the optical density between each band in each lane ('None' and 'cAMP-AM'), which reflects the degree of protein phosphorylation. In this case, the total protein content contained in each lane was equivalent, because the ImageJ analysis of the CBB-stained Western blots (Fig. 1a, 'CBB') showed the equivalent optical density between the two lanes. Among the cAMP-dependently phosphorylated proteins, p24 has already been identified to be ribosomal S5 protein, and p31 and p33 to be ribosomal P0 protein (Sogame *et al*., 2012a).

The phosphorylated proteins contained in encystment-induced cells (1 h after the onset of encystment induction) which were isolated with Phos-tag agarose phosphate-affinity beads, separated by SDS-PAGE, and the Western blots (Fig. 1b, 'CBB') were analyzed by biotinylated Phos-tag/ECL (Fig. 1b, 'P-tag') prior to CBB staining. CBB-stained protein bands (p21, p27, p37, and p43)

on the transfer membrane whose phosphorylation level was evidently elevated in cAMP-dependent manner, were analyzed by LC-MS/MS, followed by a database search. In the present assays, the amino acid sequences of protease-digested fragments of p21, p27, p37 and p43 completely and/or partially coincided with the sequences of the *Tetrahymena thermophila* hypothetical protein, Rieske iron-sulfur protein (RISP) of *T. thermophila*, histone H4 (hyperacetylated form; http://datasheets.scbt.com/sc-34264.pdf) of *Perkinsus marinus*, and actin of *Karlodinium micrum*, respectively (Table 1).The hypothetical protein (p21) failed to be identified, because no proteins highly homologous to the *T. thermophila* hypothetical protein were found.

RISP has been suggested to have functions other than a core polypeptide of Complex III of a mitochondrial electron transport chain. That is, RISP may form part of the mitochondrial permeability transition pore complex, and the phosphorylation and dephosphorylation of this protein may be involved in the regulation of the mitochondrial permeability transition (He and Lemasters, 2005) which reduces mitochondrial membrane potential. In encystment-induced *C. cucullus*, the mitochondrial membrane potential begins to disappear 2 - 3 h after the onset of encystment induction (Sogame *et al*., 2014). The encystment-dependent phosphorylation of a *Colpoda* RISP (p27) may be involved in the disappearance of the mitochondrial membrane potential through the regulation of the mitochondrial permeability transition.

The tails of the histone proteins including histone H4 can be epigenetically modified by acetylation, methylation, or phosphorylation. It is known that histone phosphorylation is involved in diverse nuclear events such as DNA damage repair, transcription regulation, and apoptosis-induced chromatin condensation (Rossetto *et al*., 2012). In the early stage of *Colpoda* encystment (2 - 3 h after the onset of encystment induction), chromatin condensation occurs in a macronucleus, and subsequently many condensed chromatin granules are extruded from the macronucleus to be digested by autophagy (Funatani *et al*., 2010). The cAMP-dependent phosphorylation of *Colpoda* histone H4 (p37) in encystment-induced *C. cucullus* may contribute to the chromatin condensation of the macronucleus.

The phosphorylated form of G-actin has no polymerizing activity (Sonobe *et al.* 1986; Furuhashi *et al*., 1998), suggesting that the cAMP-dependent phosphorylation of *Colpoda* actin (p43) in encystment-induced *C. cucullus* may be involved in the disintegration of actin filaments of the vegetative cell structure.

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