ISJ 3: 77-83, 2006 ISSN 1824-307X

Research Report

Cyst wall formation in the ciliated protozoan *Colpoda cucullus*: cyst wall is not originated from pellicle membranes

A Kida, T Matsuoka

Institute of Biological Science, Faculty of Science, Kochi University, Kochi 780-8520, Japan

Accepted 18 July 2006

Abstract

Ultrastructural changes during encystment (resting cyst formation) of *Colpoda cucullus* were observed with special reference to cyst wall formation. Within 1.5 h after encystment induction, fragmentation of some of the mitochondria occurred, followed by the appearance of a number of net-like globules in the cytoplasm, which were expelled to outside and then involved in cell-to-cell or cell-to-substratum adhesion. The cells were transformed into a spherical shape, and a number of ellipsoidal vacuoles in which ectocyst precursor (amorphous substance) was contained appeared near the cell surface (some of these opened to the outside). In this stage, the ectocyst (outermost layer) was completed. In 3 ~ 10 h toluidine blue-stained substance (TBS), which was probably the precursor for the first synthesized layer of endocyst (en-1), was released from a point near the cell surface and diffused over the cell surface (diffused into between the ectocyst and plasma membrane). Thereby, the ectocyst was lined by the en-1. Thereafter, several layers of endocyst were periodically formed for 1 ~ 2 weeks. Finally a number of reserve grains were accumulated, and cilia were resorbed.

Key words: *Colpoda*; encystment; cyst wall; ectocyst; endocyst

Introduction

When the protozoans face hazardous environments such as dessication, lack of food organisms and overpopulation, some of them are transformed into resting cysts which can survive hostile environments. The species of genus *Colpoda* (Foissner, 1993) are one of the protozoans that have been widely studied with regard to the resting cyst formation (encystment). Since the beginning of the last century (for review see Corliss and Esser, 1974), and especially for the last 50 years, ultrastructural research with special reference to the cyst wall formation has been reported (Kawakami and Yagiu, 1963a, b; Tibbs, 1968; Janisch, 1980; Ruthmann and Kuck, 1985; Martín-González *et al*., 1992, 1994; Frenkel, 1994; Delmonte Corrado, 1996; Chessa *et al*., 2002).

The knowledge about the structure of the cyst wall of colpodid ciliates and the process of wall formation reported in the previous studies could be summarized as follows:

(1) The cyst wall of the resting cyst is composed of a single outermost layer (ectocyst) and several inner layers (endocyst) [classified into ecto-, meso- and endocysts in some reports]; (2) In some studies, it has been suggested that the cyst wall may originate from pellicular membrane (Kawakami and Yagiu, 1963a; Ruthmann and Kuck, 1985); (3) In the precystic cells, ellipsoidal vesicles that are believed to be mucocysts

Corresponding author:
Tatsuomi Matsuoka
Institute of Biological Science, Faculty of Science, Kochi University, Kochi 780-8520, Japan
Email: tmatsuok@cc.kochi-u.ac.jp

(Frenkel, 1994) open to the outside, and the their content, which is presumably cyst wall precursor, is excreted (Martín-González *et al*., 1992, 1994; Frenkel, 1994). However, we have neither evidence for membrane-derived cyst wall formation nor any images of electron micrographs showing that the ecto- and/or endocyst are just being formed from materials excreted from presumed precursor-containing vacuoles. The present study revealed that ectocyst and endocyst of *C. cucullus* did not originate from pellicle membranes but instead were synthesized with different precursors excreted from the different types of vacuoles.

Materials and methods

Colpoda cucullus was cultured in a 0.1 % (w/v) infusion of dried cereal leaves inoculated with bacteria (*Enterobacter aerogenes*) at 23 ^{o}C in the dark. Bacteria were cultured on agar plates containing 1.5 % agar, 0.5 % polypeptone, 1 % meat extract and 0.5 % NaCl. The cultured vegetative cells were collected by centrifugation (1,000 xg, 1 min) and subsequently suspended in a standard saline solution containing 1 mM $CaCl_2$, 1 mM KCl and 5 mM Tris-HCl (pH 7.2). The cells were rinsed 2-3 times by repeating the sedimentation and suspension in standard saline solution, and finally suspended in the solution to induce encystment.

For vital staining of precystic cells with toluidine blue, 0.1 % toluidine blue dissolved in the standard saline solution was added to an equal volume of cell suspension, and kept for 5 ~ 10 min.

For prefixation of the precystic cells (0 ~ 1 h after encystment induction), one volume of the suspension of cells was mixed with 6 volumes of a glutaraldehyde (GA) fixative containing 6 % glutaraldehyde, 1 % OsO_4, 100 mM cacodylate buffer (pH 7.2) and 4 mM sucrose. After 10-min incubation, the prefixed samples were rinsed 5 times in 100 mM cacodylate buffer (pH 7.2) and then postfixed for 2 h in a fixative containing 1 % OsO_4, 100 mM cacodylate buffer (pH 7.2) and 2 mM sucrose. The precystic cells and mature cysts (1 h ~ 2 weeks after encystment induction) were prefixed with GA fixative without OsO_4 for 6 h and postfixed with a fixative containing 1 % OsO_4, 100 mM cacodylate buffer (pH 7.2) and 2 mM sucrose for 1 week. The postfixed samples were rinsed several times in distilled water, dehydrated through a graded ethanol series (30, 40, 50, 60, 70, 80, 90 and 100 % ethanol) for 15 min each and finally suspended in acetone. The dehydrated samples were embedded in Spurr's resin. Ultrathin sections were stained with 3 % uranyl acetate and then with lead citrate (10 min each). The sections were observed under a transmission electron microscope (JEOL, 1010T).

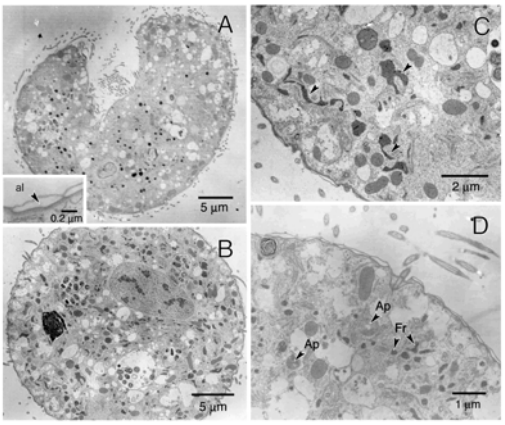

Fig. 1 Transmission electron micrographs of a vegetative cell of *C. cucullus* (Fig. 1A) and the precystic cell in the early phase of stage 1 (0 ~ 1 h after encystment induction) (Figs 1B-D). Fig. 1A (inset): a magnified picture of the cortical region, showing the alveolus (al). Fig. 1C: arrowheads, mitochondria that has just been torn and fragmented. Fig. 1D: Fr, mitochondria fragmented into small pieces; Ap, autophagosome-like structure.

Results

The morphological events during encystment are described by dividing them into stage 1 ~ 4. The parentheses show the typical terms after encystment induction in which the cytoplasmic events in each stage are observed in most cells.

Stage 1 (0 ~ 1.5 h after encystment induction). Fig. 1A is a longitudinal section of a vegetative cell, showing a number of small-sized vacuoles or vesicles in the cytoplasm and alveoli in the cortical region. Within 1 h after encystment induction, some of the mitochondria were fragmented (Figs 1B, C, D) and a number of autophagosome-like structures appeared (Fig. 1D). During the latter part of this stage, a number of net-like globules that had been described previously (Kawakami and Yagiu, 1963a) appeared in the cytoplasm (Figs 2A, B) and were subsequently released into extracellular space.

Stage 2 (1.5 ~ 3 h). The precystic cells became round, and cell movement was gradually slower and finally stopped. A number of spherical or ellipsoidal vacuoles, which were possibly similar organelles (mucocysts) previously reported in precystic cells of *Tillina magna* (Frenkel, 1994), were localized in the vicinity of the cell surface and opened to the extracellular space to excrete amorphous precursor material (Fig. 3B) for an outermost layer (ectocyst) (Figs 3A, B). The excreted materials were gradually deposited (Fig. 3C) and finally formed an electron-dense single layer (ectocyst) (Fig. 3D).

Stage 3 (3 ~ 10 h). By the time ectocyst completion occurred (Figs 4A, B), the cells had ceased swimming, but cells continued to rotate

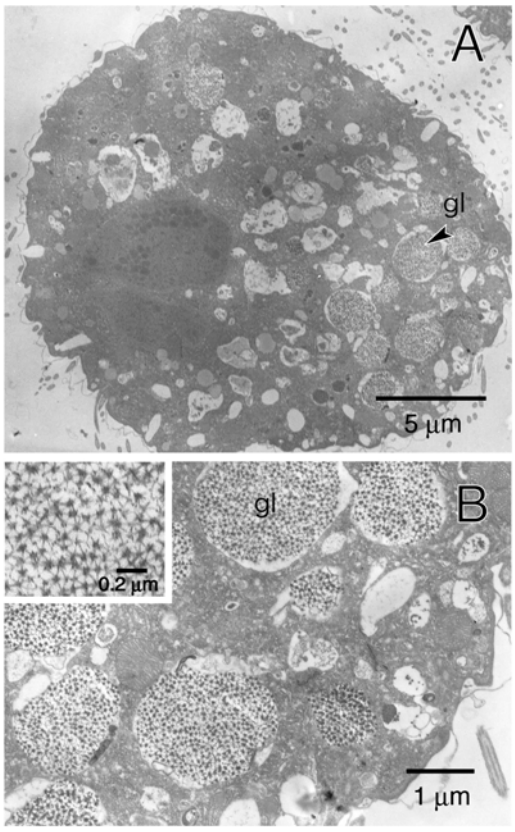

Fig. 2 Transmission electron micrographs of the precystic cells in the latter phase of stage 1 (1 ~ 1.5 h), showing net-like globules (gl) in the cytoplasm. Fig. 2A, a total view of the cell; Fig. 2B, a magnified picture and highly magnified one (inset) of net-like globules.

for a while (10~30 min) by ciliary movement inside the ectocyst envelope. During this stage, the number of electron-lucent vacuoles was reduced, while numerous endoplasmic reticula and mottled or electron-dense granules appeared (Figs 4A, B), which may be responsible for the next event, the synthesis of endocyst. A drastic event was visualized in toluidine blue-stained cells just after the precystic cells ceased to rotate inside the ectocyst envelope (Figs 4C, D). An extremely large vacuole (Fig. 4C) opened near the cell surface, and substance deeply stained with toluidine blue (TBS) diffused over the entire cell surface within a few minutes (Figs 4C, D). The TBS release occurred at 1.5 h in cases when the encystment was most quickly induced but proceeded much more slowly in the case of typical cells. The cells whose cortical region was deeply stained by the diffusion of TBS could hardly be crushed by a mechanical press. In the electron micrograph of this stage, fibrous materials constituting the first layer (en-1) of endocyst were observed between the ectocyst and plasma membrane (Fig. 4E), which probably correspond to TBS.

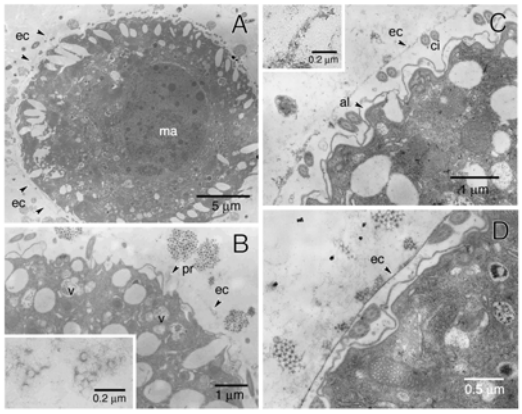

Fig. 3 Transmission electron micrographs of precystic cells in stage 2, showing ectocyst formation. Figs 3A-C: ec, ectocyst that has just been formed with amorphous precursor materials (Fig. 3B, pr) excreted from spherical or ellipsoidal vacuoles. ma, macronucleus; v, vacuoles containing amorphous materials; ci, cilia; al, alveolus. Fig. 3B (inset), a magnified picture of just excreted amorphous materials; Fig. 3C (inset), magnified image of ectocyst just being formed. Fig. 3D, an image showing a just completed ectocyst envelope (ec).

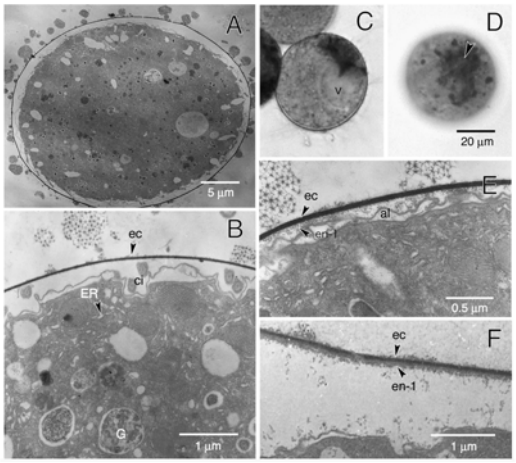

Fig. 4 Transmission electron micrographs (Figs 4A, B, E) of precystic cells and photomicrographs of toluidine blue-stained living cells (Figs 4C, D), showing the process of first layer (en-1) formation of endocyst. Fig. 4F [Stage 4]: a transmission electron micrograph of the cell at 24 h after encystment induction, showing a completed first layer of endocyst (en-1). Figs 4B, E, F: ER, endoplasmic reticulum; ci, cilia; G, mottled granules; ec, ectocyst; en-1, endocyst-1 (first synthesized endocyst layer); al, alveolus. Figs 4C, D: v, vacuole containing presumed precursor for endocyst layer (en-1); arrowhead, a point where toluidine blue-stained substance (TBS) was being released.

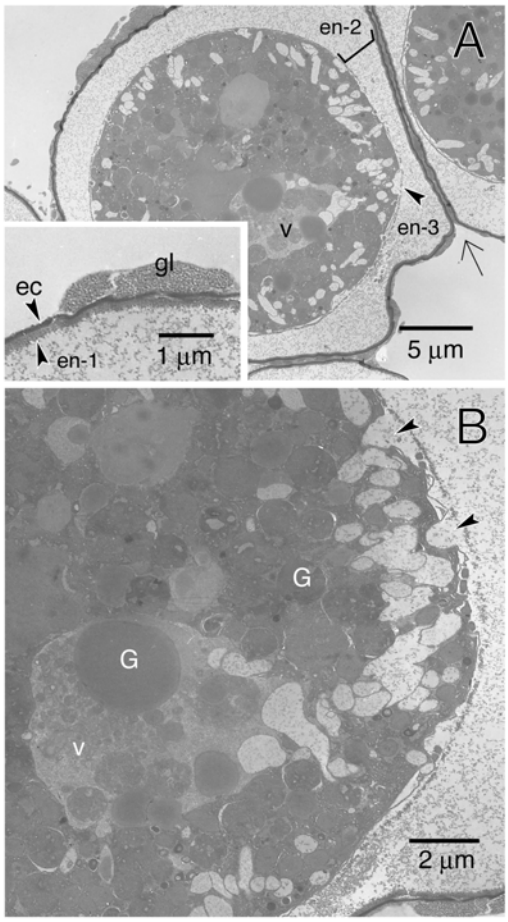

Fig. 5 Transmission electron micrographs of an immature cyst in the early phase of stage 4 (2 days after encystment induction), showing a third layer (en-3) of endocyst are being formed. Fig. 5A (inset), a magnified picture showing the ectocyst (ec) lined by endocyst-1 (en-1). en-2, second synthesized layer of endocyst; en-3, third synthesized layer of endocyst; v, vacuole containing presumable precursor for endocyst; gl, net-like globule attached to surface of ectocyst; G, electron-dense granule; arrow (Fig. 5A), a boundary of two cysts adhered to each other with crushed net-like globules; arrowheads in Fig. 5B, vacuoles just beginning to excrete the precursor for endocyst.

Stage 4 (10 h~ 2 weeks). In most cells, the formation of en-1 was completed within 1 day, and the ectocyst was lined by the en-1 (Fig. 4F). During this stage, several layers of endocyst were formed. As shown in Fig. 5, in the 2-day-old immature cyst, a third layer (en-3) of endocyst was observed to be just forming. In this case, an extremely large vacuole (Figs 5 A, B) associated with many electron-lucent small-sized vacuoles or ducts appeared near the cortical region, some of which opened to the space between the plasma membrane and the already synthesized

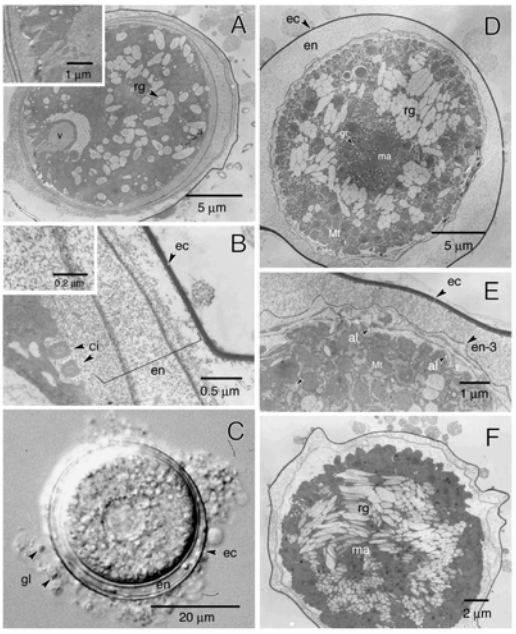

Fig. 6 A photomicrograph (Fig. 6C) and transmission electron micrographs (Figs 6A, B, D, E) of immature cysts at 1 week after encystment induction and in a 2-week-old mature cyst (Fig. 6F). Fig. 6A: 1-week-aged cyst. A vacuole (v) containing endocyst precursor opened to the space between the plasma membrane and the already synthesized innermost endocyst layer. Fig. 6B: a magnified picture of cyst wall. Inset, A highly magnified picture of endocyst showing that endocyst layers are composed of fine fibrous materials. Fig. 6C: a photomicrograph of 1-week-aged living cyst. Fig. 6D: a section of 1-week-aged cyst showing that cytoplasmic reconstruction rather progressed than the cyst shown in Fig. 6A. Fig. 6E: a magnified picture of the section shown in Fig. 6D. Fig. 6F: a section of 2-week-aged mature cyst. Figs 6A ~ F: rg, reserve grains; ec, ectocyst; en, endocyst; ci, cilia; gl, net-like globules; gr, small grains surrounding macronucleus; ma, macronucleus; Mt, mitochondria; en-3, third synthesized endocyst layer; al, alveolus; arrowhead in Fig. 6E, electron-lucent amorphous materials.

layer (en-2) of endocyst, and endocyst precursor was excreted (Fig. 5B, arrowheads). In addition, a number of electron-dense granules were seen inside and in the vicinity of the large vacuole (Figs 5B, G). The complex of the large vacuole and many small-sized vacuoles or ducts leading to the cell surface (Fig. 5 B) may correspond to the vacuole (Figs 4C, D) observed by light microscope. Fig. 6 show a photomicrograph of living cell (Fig. 6C) and electron micrographs (Figs 6A, B, D, E) of 7-day-old and 2-week-old cysts (Fig. 6F). Some of the cysts were still immature, and endocyst precursor was observed being released from a large vacuole (Fig. 6A)

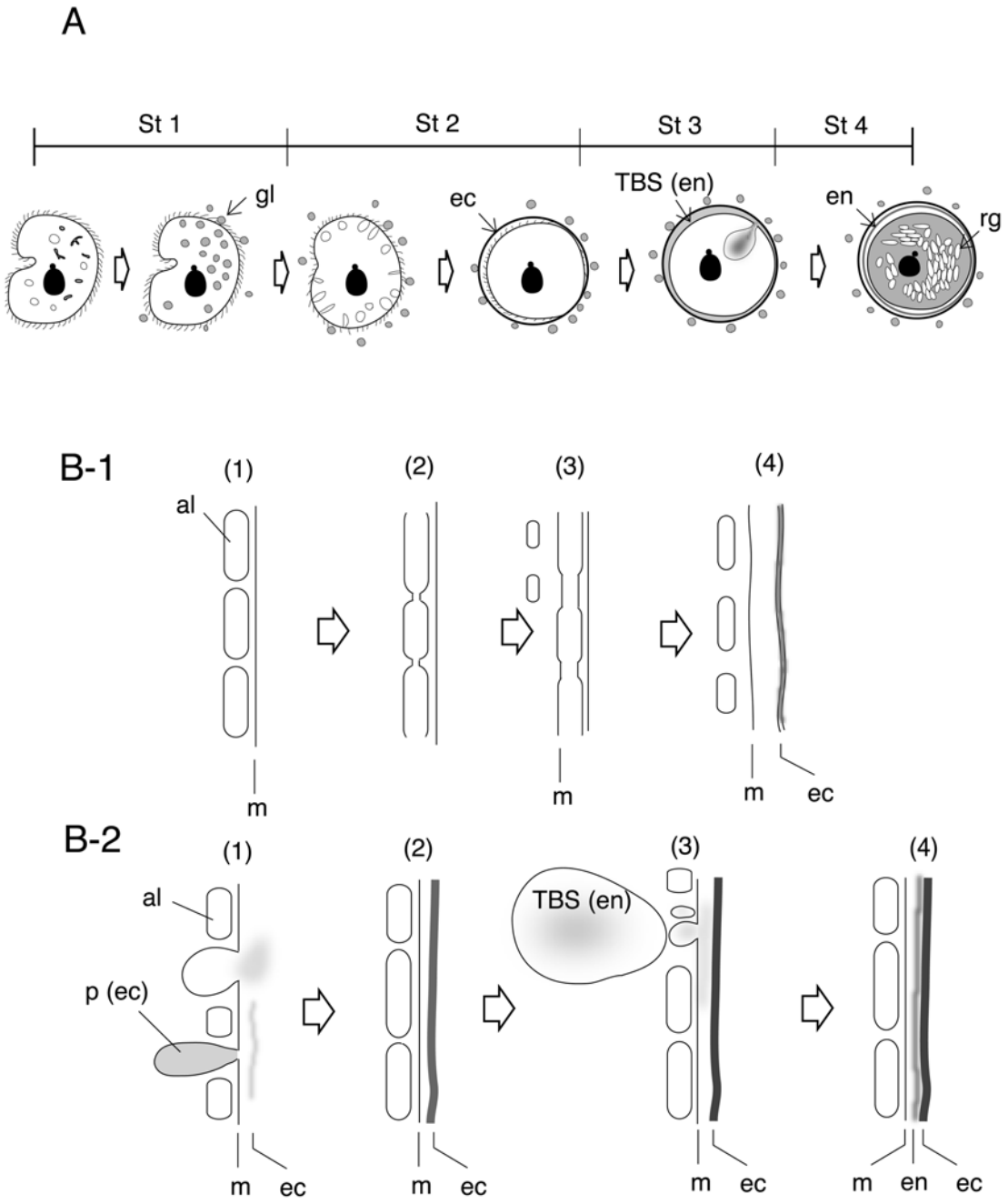

Fig. 7 Schematic diagrams showing the entire encystment process (Fig. 7A) and two different ideas for cyst wall formation (Fig. 7B). Fig. 7B-1: a hypothesis for ectocyst formation in which it originates pellicle membranes. Fig. 7B-2: a schematic diagram showing ecto- and endocyst formation based on the present results. ec, ectocyst; en, endocyst; gl, net-like globules; TBS (en), toluidine blue-stained substance (endocyst precursor); rg, reserve grains; al, alveolus, m, plasma membrane; p (ec), precursor for ectocyst.

forming the innermost layer of endocyst. During this stage, the compact and narrow layers and broad layers of endocyst were observed to be alternatively formed (Figs 6A, B). Both of the layers seem to be composed of identical fibrous materials (Fig. 6B). The broad layers of endocyst might not be produced by the fixation and dehydration processes for electron microscopy, because a broad space (layer) was seen in a living cyst (Fig. 6C). After the endocyst layers were completed, the reserve grains gradually accumulated in the central region (Fig. 6D), and

the cytoplasm became dappled by the appearance of amorphous electron-lucent structures (Figs 6D, E), which seem to develop into the reserve grains. In addition, the macronucleus was surrounded by a number of small grains (Fig. 6D). Finally, electron-lucent amorphous structures were replaced by a large amount of regularly stacked ellipsoidal reserve grains (Fig. 6F), and, as a result, the central region of cytoplasmic space was occupied mainly by the reserve grains and mitochondria located in the peripheral region. In the final stage (Figs 6D-F), cilia were resorbed.

Discussion

The outline of the encystment process with special reference to cyst wall formation is schematically summarized in Fig. 7. The first drastic events in the stage 1 are the fragmentation of mitochondria and appearance of autophagosome-like structures (Figs 1B, C, D, 7A). The fragmented mitochondria are probably digested inside the autophagosomes. The net-like globules appeared and excreted in the latter phase of this stage (Figs 2, 7A) are probably involved in a cell-to-cell adhesion (see Fig. 5A, arrow) or adhesion of the cells to the substratum.

If the ectocyst originated from pellicle membranes, as suggested in the previous reports (Kawakami and Yagiu, 1963a; Ruthmann and Kuck, 1985; Watoh *et al*., 2005), alveoli might be fused with one another to produce new plasma membrane and outermost double membranes developing into the ectocyst which are deposited with a precursor substance (Fig. 7B-1). Although electron microscopic images implying the fusion of alveoli are observed occasionally (Watoh *et al*., 2005), no images showing that the outer double membranes produced by fusion of alveoli further developed into ectocyst or endocyst have been observed in the previous study (Watoh *et al*., 2005). On the other hand, in the present study, we succeeded in obtaining successive images showing that the ectocyst was just being formed with amorphous substance excreted from vacuoles (Figs 3A-D, ref. Fig. 7B-2). In this process, neither the fusion of alveoli nor the appearance of new alveoli was detected. As a result, we believe that the images of fused alveoli observed in the previous report (Watoh *et al*., 2005) may have been artifacts produced by the fixation and/or dehydration processes.

The ectocyst formation is followed by a diffusion of TBS which is probably endocyst precursor (Figs 4C, D). In the previous report (Watoh *et al*., 2005), the TBS is suggested to diffuse between the first-formed layer of endocyst (en-1) and the plasma membrane based on the photomicroscopic observation. This idea should be modified, because the present electron microscopy revealed that during this stage, fibrous endocyst materials probably corresponding to TBS were observed in the space between the ectocyst and the plasma membrane (Fig. 4E, ref. Fig. 7B-2).

The formation of endocyst-1 (en-1) is followed by the formation of several layers of endocyst, the precursor of which is probably supplied by a periodic excretion into the space between the plasma membrane and the innermost endocyst layer (Figs 5, 6A). In this case, the newly formed endocyst shows the replica-like configuration of the cell surface, i.e. the smooth endocyst layers beneath the smooth cell surface (Figs 6A, B) and undulate layers for furrowed cell surface (Figs 6D, E). The alternative formation of the narrow and broad layers of endocyst may be responsible for the periodic shrinkage of the cell body. That is, broad endocyst layers may be formed when the endocyst precursor is excreted into the broad space between the plasma membrane and innermost endocyst layer which is produced by the cell shrinkage. Every layer of endocyst is composed of fine fibrous materials (Fig. 6B), indicating that it is derived from a common precursor material, which is expected to be deeply stained with toluidine blue.

In the present study, we defined a “mature cyst” of *C. cucullus* as a cyst in which every cytoplasmic event (formation of ecto-and endocysts, resorption of cilia, accumulation of reserve grains, aggregation of mitochondria in the peripheral region of cytoplasm) during the resting cyst formation has occurred and further prominent change is not observed (see Fig. 6F). It has been reported that in other species of *Colpoda*, the silver impregnation method indicates that some pairs of kinetosomes disappear during resting cyst formation (Martín-González *et al*., 1991). Unfortunately, the present microscopy failed to conclude whether the kinetosomal structures completely disappear in the mature cysts, because the cytoplasm of mature cysts is compacted and highly electron-dense.

Acknowledgement

We thank Ms Watoh T for her technical assistance in electron microscopy.

References

Chessa MG, Largana I, Trielli F, Rosati G, Politi H, Angelini C, Delmonte Corrado MU. Changes in the ultrastructure and glycoproteins of the cyst wall of *Colpoda cucullus* during resting encystment. Eur. J. Protist. 38: 373-381, 2002.

Corliss JO, Esser SC. Comments on the role of the cyst in the life cycle and survival of free-living protozoa. Trans. Amer. Micros. Soc. 93: 578-593, 1974.

Delmonte Corrado MU, Chessa MG, Pelli P. Ultrastructural survey of mucocysts throughout the life cycle of *Colpoda cucullus* (Ciliophora, Colpodea).

Acta Protozool. 35: 125-129, 1996.
Foissner W. Colpodea (Ciliophora). Gustav Fischer Verlag, Stuttgart, 1993.
Frenkel MA. The cyst wall formation in *Tillina magna* (Ciliophora, Colpodidae). Arch. Protistenkd. 144: 17-29, 1994.
Janisch R. A freeze-etch study of the ultrastructure of *Colpoda cucullus* protective cysts. Acta Protozool. 19: 239-246, 1980.
Kawakami H, Yagiu R. An electron microscopical study of the change of fine structures in the ciliate, *Colpoda cucullus*, during its life cycle. II. From the preencystment stage to the early stage of the formation of the first layer of resting cyst membrane. Zool. Mag. 72: 146-151, 1963a.
Kawakami H, Yagiu R. The electron microscopical study of the change of fine structure in the ciliate, *Colpoda cucullus*, during its life cycle. III. From the stage of completion of the first layer of resting cyst membrane to the completion of the resting cyst. Zool. Mag. 72: 224-229, 1963b.
Martín-González A, Benítez L, Gutiérrez JC. Cortical and nuclear events during cell division and resting cyst formation in *Colpoda inflata*. J. Protozool. 38: 338-344, 1991.
Martín-González A, Benítez L, Gutiérrez JC. Ultrastructural analysis of resting cysts and encystment in *Colpoda inflata*. 2. Encystment process and a review of ciliate resting cyst classification. Cytobios 72: 93-106, 1992.
Martín-González A, Palacios G, Gutiérrez JC. Cyst wall precursors of *Colpoda inflata*: a comparative ultrastructural study and a review of ciliate cyst wall precursors. Cytobios 77: 215-223, 1994.
Ruthmann A, Kuck A. Formation of the cyst wall of the ciliate *Colpoda steinii*. J. Protozool. 32: 677-682, 1985.
Tibbs J. Fine structure of *Colpoda steinii* during encystment and excystment. J. Protozool. 15: 725-732, 1968.
Watoh T, Sekida S, Yamamoto K, Kida A, Matsuoka T. Morphological study on the encystment of the ciliated protozoan *Colpoda cucullus*. J. Protozool. Res. 15: 20-28, 2005.