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# Secretory Function of the Test Cell in Larval Tunic Formation in the Ascidian *Ciona intestinalis*: An Immunoelectron Microscopic Study

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**ABSTRACT**—The test cells, one of the two accessory cell groups of the ascidian oocyte, persist around the developing embryos and the hatched tadpole larvae, but their function during embryogenesis is not fully understood. We have produced monoclonal antibodies that specifically recognize the test cells in *Ciona intestinalis* (Takamura *et al.*, Zool. Sci. 13: 241–251, 1996). In the present paper, we demonstrate the ultrastructural localization of the antigen reactive to one of the test cell-specific antibody UA165 by immunogold labeling. The mature test cells in *Ciona intestinalis* contain two types of cytoplasmic inclusions, i.e., irregular vacuoles with electron-dense cores and spherical vesicles with a moderately electron-dense content. UA165-immunogold particles specifically labels the content of the spherical vesicles. Concomitantly with larval tunic formation at the early tailbud stage, the spherical vesicles begin to rupture and the UA165 antigen is exocytosed into the perivitelline space surrounding the embryo. The released antigen gradually deposits on the outermost layer (outer cuticular layer) of the larval tunic. Shortly after the beginning of exocytosis, the test cells scatter in the perivitelline space, move to the embryonic surface, and adhere to the outer surface of the larval tunic. These findings indicate that during the tailbud stage the test cells secrete a component that is necessary for the completion of the larval tunic.

#### INTRODUCTION

The ascidian eggs have two different types of accessory cells, follicle cells and test cells (reviewed by Kessel, 1983; Satoh, 1994). In *Ciona intestinalis*, both types of accessory cells differentiate from the primary follicle cells at the previtellogenetic stage of oogenesis (Sugino *et al.*, 1987). The test cells are embedded in the peripheral cytoplasm of the oocytes during vitellogenesis. In the mature eggs ovulated into the oviduct, the test cells are excluded from the egg surface, forming a continuous layer separated from the egg by a newly formed perivitelline space. In the tailbud embryos, the test cells scatter in the perivitelline space and adhere to the larval tunic, a protective covering of the tadpole larva. They remain there until the outer layer of the larval tunic is cast off at the time of metamorphosis.

Several functions of test cells during oogenesis have been suggested: nourishing the oocyte in *Molgula manhattensis* (Kessel and Kemp, 1962), providing the oocyte with yolk precursors in *Ciona intestinalis* (Mancuso, 1965) and *Ascidia malaca* (Dilly, 1969), producing pigment granules in *Styela* (Kessel, 1962; Kessel and Beams, 1965), and transmitting vanadium compounds to the oocyte in *Ascidia pygmaea* (Kalk, 1963; Hori and Michibata, 1981). The evidence for these suggestions, however, is not yet convincing (Kessel, 1983).

It has also been proposed that test cells play a role in the formation of the larval tunic during embryogenesis (Cavey, 1976; Satoh et al., 1982; Cloney and Cavey, 1982). In the embryos of Halocynthia roretzi, the test cells disperse in the perivitelline space and move to the embryonic surface when the larval tunic begins to form there. With actively extending numerous filopodia, the test cells creep around the outer surface of the embryo (Satoh et al., 1982). In some ascidian species, the test cells release a structure called "ornament" that becomes deposited on the outer surface of the larval tunic (Cavey, 1976; Cloney and Cavey, 1982 Cavey and Cloney, 1984; Cloney, 1990). We have produced monoclonal antibodies (UA165 and UA464) that specifically recognize the test cells in Ciona intestinalis (Takamura et al., 1996). In the immunofluorescence examination reproted in the preceding paper (Takamura et al., 1996), the antibodies stained not only cytoplasmic inclusions of the test cells during oogenesis and embryogenesis, but the larval tunic enclosing the hatched tadpole larvae. These findings suggest that the test cells synthesize components used for the formation of the larval tunic. In order to confirm the function of the test cells associated with the larval tunic formation, we examined the ultrastructural localization of UA165 antigen during oogenesis and embryogenesis by immunogold labeling technique.

## MATERIALS AND METHODS

#### Animals

*Ciona intestinalis* were collected from the subtidal zone near the Ushimado Marine Laboratory. Eggs removed from the oviduct were fertilized in plastic dishes with a suspension of sperm obtained from the spermduct of another individual. Embryos kept at 20°C reached the early tailbud stage about 7.5 hr after fertilization, and hatched about 15 hr after fertilization.

#### Monoclonal antibody

UA165, one of the monoclonal antibodies raised against homogenate of ovaries of adult *C. intestinalis*, was used. Details of production of the hybridoma clone that secretes UA165 antibody have been described in the preceding report (Takamura *et al.*, 1996). In immunofluorescence staining, UA165 antibody recognizes the cytoplasm of the test cells which are included in the vitellogenetic oocytes, and are enclosing the mature eggs and the embryos. UA165 antibody also stains the larval tunic covering the hatched tadpole larvae (Takamura *et al.*, 1996).

#### Transmission electron microscopy

Embryos, larvae, and small pieces of ovaries of *C. intestinalis* were fixed for 2 hr at room temperature with 2.5% glutaraldehyde dissolved in a sucrose containing buffer solution (0.4 M sucrose and 0.1 M Na-cacodylate, pH 7.4), washed with the sucrose-containing buffer solution, and then post-fixed for 1 hr at room temperature with 1% OsO<sub>4</sub> dissolved in a mixture of 1% K-ferrocyanide, 0.4 M NaCl and 0.1 M Na-cacodylate (pH 7.4) (Okada and Yamamoto, 1993). After fixation, the specimens were dehydrated through a graded series of ethanol and embedded in Spurr's resin (TAAB) following standard protocol. Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome and collected on copper grids. The sections were stained with 1% uranyl acetate dissolved in 50% ethanol and then 0.1% lead citrate, and examined with Hitachi H500H electron microscope operated at 75 kV.

#### Immunoelectron microscopy

For indirect immunogold labeling, samples fixed and dehydrated as described above were embedded in LR White resin (London Resin), and polymerized at 50°C for 24 hr. Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome and collected on nickel grids. Prior to immunolabeling, these grids were treated with calf serum diluted 1 : 10 in PBS for 15 min in order to block nonspecific binding of the antibody and washed in PBS. The grids were incubated with the hybridoma culture fluid containing UA165 antibody for 2 hr at room temperature. After rinsing in PBS, the grids were incubated with goat anti-mouse IgG + IgM conjugated to 10 nm colloidal gold (British BioCell) at a dilution of 1 : 20 in PBS for 1.5 hr at room temperature, thoroughly washed in PBS, and finally in distilled water. The grids were examined with a Hitachi H-500H electron microscope operated at 75 kV.

## RESULTS

In the oocyte where vitellogenesis has just begun (about 60  $\mu m$  in diameter), the test cells are embedded in

the peripheral cytoplasm of the oocyte (Fig. 1a). They are round or oval in shape with a relatively large round nucleus. The test cell cytoplasm is rich in the cisternae of rough surfaced endoplasmic reticulum (Fig. 1b). The Golgi complex is moderately well developed, associated with small vesicles with an electron-dense content. The test cells include many highly electron-dense granules with an irregularly wavy outline (about 1  $\mu$ m in diameter)(Fig. 1a, b). They correspond to the test granules described by Mancuso (1965). There is no structure labeled by UA165-immunogold particles in the test cell at this stage.

In the test cells embedded in the oocyte at a more advanced vitellogenetic stage (about 130  $\mu$ m in diameter), there appears an irregularly shaped vacuole containing slightly electron-dense homogeneous material. The vacuole is often found in the vicinity of the Golgi complex (Fig. 1c, d). This is the first structure that shows immunoreactivity to UA165 antibody; immunogold particles label the content of this vacuole (Fig. 1d). The profiles of rough surfaced endoplasmic reticulum have decreased in number and size in the test cell cytoplasm.

In the test cells embedded in the oocyte near the end of vitellogenetic stage (about 150 µm in diameter), many spherical vesicles appear in the cytoplasm (Fig. 2a, b). The vesicles measure about 1 µm in diameter, limited by a smooth membrane. They contain a slightly electron-dense homogeneous matrix and a little more electron-dense patches that are finely fibrous under a high power view (Fig. 2d). The electron-dense granules with irregular outlines (test granules) begin to vacuolate at this stage; a clear space appears around the electron-dense material within each granule. Thus, from this stage onwards, the test cells have two types of cytoplasmic inclusions: spherical vesicles newly formed at this stage and irregular vacuoles derived from the electron-dense granules (test granules) of earlier test cells. Immunoreactivity to UA165 antibody is restricted to the content of the spherical vesicles (Fig. 2b). In close examination, immunogold particles concentrate on the electron-dense patches within each spherical vesicle (Fig. 2d). The Golgi complex becomes inconspicuous and the cisternae of endoplasmic reticulum have disappeared from the test cell cytoplasm at this stage.

In the mature eggs ovulated from the ovary and deposited in the oviduct (about 200  $\mu$ m in diameter), the test cells are excluded from the egg surface, forming a continuous layer just inside the vitelline coat (Fig. 2c). An empty space (perivitelline space) separates the test cell layer from the oocyte surface. The nucleus of the test cell has decreased in volume and the nucleoplasm becomes condensed. The irregular vacuoles have extensively swollen to 2–3  $\mu$ m in diameter. Within each vacuole limited by an irregularly indented membrane, the electron-dense material is fragmented and scattered in the expanded clear space (Fig. 2c). The spherical vesicles remain unchanged in appearance. Since the spherical vesicles and the irregular vacuoles fill up the test cell cytoplasm, the cytoplasmic matrix is confined to



Fig. 1. Test cells included in the oocyte at the early (a and b) and the late (c and d) vitellogenetic stages. Specimens are embedded in Spurr's resin in a and b, and in LR White resin and immunolabeled in c and d. (a) The peripheral part of an oocyte (Oo) containing test cells with a relatively large nucleus (Nt). (b) A part of a test cell showing cisternae of rough-surfaced endoplasmic reticulum, a Golgi complex (G), and irregularly shaped electron-dense granules (Va). (c) A test cell showing a vacuole (arrow) in the vicinity of the Golgi complex (G). The vacuole is labeled by UA165-immunogold particles. (d) Magnified view of a Golgi-associated vacuole (arrow) labeled by UA165-immunogold particles. FC, follicle cell; VC, vitelline coat. Bar; 2 µm for a and c, and 0.5 µm for b and d.



Fig. 2. Test cells embedded in the oocyte cytoplasm at the late vitellogenetic stage (a and b), and excluded from the mature egg stored in the oviduct (c and d). Specimens are embedded in Spurr's resin in a, and in LR White resin and immunolabeled in b-d. (a) A test cell containing newly formed spherical vesicles with moderate electron-density (Ve), and irregularly shaped electron-dense granules (Va) which begin to vacuolate. (b) A part of a test cell containing many spherical vesicles (Ve) labeled by UA165-immunogold particles. Irregular vacuoles (Va) deriving from electron-dense granules of earlier test cells are not labeled by immunogold particles. (c) Test cells forming a cell layer just inside the vitelline coat (VC). A perivitelline space (PS) separates the cell layer from the egg surface (Eg). The test cells contain spherical vesicles (Ve) and largely swelled irregular vacuoles (Va). (d) Magnified view of spherical vesicles (Ve). Immunogold particles are mainly distributed on the fibrous patches (arrow) within the spherical vesicles. An irregular vacuole (Va) is not immunolabeled. FC, follicle cell; Nt, nucleus of test cell; Oo, oocyte cytoplasm; VC, vitelline coat. Bar; 2 μm for a and c, and 0.5 μm for b and d.



Fig. 3. Test cells and the embryonic surface at the early tailbud stage [7.5 hr (a,b) and 8.5 hr (c,d) after fertilization]. Specimens are embedded in Spurr's resin in a and c, and in LR White resin and immunolabeled in b and d. (a) Test cells (TC) beginning exocytosis. Arrows indicate spherical vesicles (Ve) open to the perivitelline space (PS). (b) The peripheral part of a test cell showing spherical vesicles (Ve) labeled by UA165-immunogold particles, one of which (arrow) is releasing the UA165-immunolabeled content into the perivitellin space (PS). Irregular vacuoles (Va) are not immunolabeled. (c) Test cells (TC) dispersing in the perivitellin space (PS). Filopodia (arrow head) arise from the surface of the test cells. (d) Magnified view of the surface of an embryonic epidermal cell (EC). A layer of moderately electron-dense material (arrow) is deposited on the plasmamembrane (Pm) of the epidermal cell. Immunogold particles are scattered in the perivitellin space (PS). FC, follicle cell; VC, vitelline coat. Bar; 3 μm for a and c, and 0.5 μm for b and d.



Fig. 4. The surface of embryos at the middle tailbud stage [10 hr after fertilization (a,b)] and just hatched tadpole larvae [15 hr after fertilization (c,d)]. Specimens are embedded in Spurr's resin in a and c, and in LR White resin and immunolabeled in b and d. (a) Test cells (TC) beginning to attach with filopodia (arrow head) to the larval tunic (C1) which covers the embryonic epidermal cells (EC). Test cells contain irregular vacuoles (Va) and only a few spherical vesicles. (b) Magnified view of the surface of a test cell (TC) and an epidermal cells (EC). The plasmamembrane (Pm) of the epidermal cell is covered by a larval tunic, which consists of an outer cuticular layer (C1) and outer compartment (OC). In the perivitelline space (PS), immunogold particles are distributed near the test cell plasmamembrane as well as near the outer cuticular layer (C1). (c) The larval surface enclosed by test cells (TC) adhering to the larval tunic (C1). (d) Magnified view of the part where the plasmamembrane (Pm) of a test cell (TC) is closely apposed to the larval tunic, which consists of an outer cuticular layer (C1), outer compartment (OC), inner cuticular layer (C2), and inner compartment (IC). UA165-immunogold particles label the fuzzy coat covering both the outer cuticular layer and the test cell plasmamembrane. FC, follicle cell; Va, irregular vacuole; VC, vitelline coat. Bar; 3 μm for a and c, and 0.5 μm for b and d.

a narrow space in the periphery of the test cell. After fertilization and during early stages of embryogenesis up to the early tailbud stage, test cells show no significant changes as to the arrangement and the internal ultrastructures. In immunogold labeling, only the content of the spherical vesicles is reactive to UA165 antibody during this period.

At the early tailbud stage (about 7.5 hr after fertilization), exocytosis of spherical vesicles begins to be observable in test cells. Some spherical vesicles open to the perivitelline space through a fusion of the limiting membrane with the adjacent plasmamembrane (Fig. 3a, b). Immunogold labeling shows that substance reactive to UA165 antibody is released from such spherical vesicles into the perivitelline space (Fig. 3b). No significant change is detectable in appearance of the irregular vacuoles.

At a little advanced early tailbud stage (about 8.5 hr after fertilization), test cells become free from the continuous cell layer and disperse into the perivitelline space (Fig. 3c). Test cells often protrude filopodia toward the embryonic surface. Exocytosis of the spherical vesicles continues; the vesicles gradually decrease in number in the test cells (Fig. 3c) and immunogold particles designating the presence of the secreted UA165 antigen increase in density in the perivitelline space (Fig. 3d). At the same time with the dispersion of test cells, the first sign of larval tunic formation becomes discernible as a thin deposit of moderately electron-dense extracellular substance over the plasmamembrane of embryonic epidermal cells (Fig. 3d). The nascent larval tunic is not reactive to UA165 antibody at this stage.

At the middle tailbud stage (about 10 hr after fertilization), the larval tunic becomes evident over the entire surface of the embryonic epidermal cells (Fig. 4a). It consists of an electron-dense membranous layer coated with an electron-dense fuzzy substance on its outer surface [outer cuticular layer (Dilly, 1969)], and a space filled with a flocculent matrix between the outer cuticular layer and the epidermal cell plasmamembrane [outer compartment (Dilly, 1969)] (Fig. 4b). Test cells scattering in the perivitellin space begin to adhere to the outer surface of the outer cuticular layer with many cytoplasmic processes. The outer surface of the test cell plasmamembrane is also covered with an electrondense fuzzy coat (Fig. 4b). In the perivitelline space, immunogold particles mainly distribute around the surface of the test cells as well as along the outer surface of the outer cuticular layer (Fig. 4b).

In the tadpole larvae shortly after hatching (about 15 hr after fertilization), almost the entire surface of the larval body is covered with test cells adhering to the larval tunic (Fig. 4c). In the portion where the test cell is closely apposed to the larval tunic, there is always a clear gap of 50–100 nm between the test cell plasmamembrane and the outer cuticular layer of the larval tunic (Fig. 4d). Another membranous layer [inner cuticular layer (Dilly, 1969)], which is less electron-dense than the outer cuticular layer at this stage, appears inside the outer cuticular layer (Fig. 4d). Immunogold particles label the fuzzy coat covering both the outer surface

of the outer cuticular layer of the larval tunic, and the surface of the test cell plasmamembrane facing the larval tunic (Fig. 4d).

#### DISCUSSION

In this paper we present evidence that the test cells in *Ciona intestinalis* play a secretory role in the larval tunic formation. A component synthesized in the test cells during oogenesis is stored in one of two types of cytoplasmic inclusions (spherical vesicle) until exocytosed into the perivitelline space concomitantly with the beginning of the larval tunic formation at the early tailbud stage. The secreted substance gradually deposits on the surface of the outer cuticular layer of the larval tunic, and on the surface of the test cell plasmamembrane closely facing to the larval tunic.

We have confirmed the presence of two types of cytoplasmic inclusions that Mancuso (1965) described in the test cells during oogenesis of C. intestinalis. The one type termed test granules by Mancuso (1965) appears as electron-dense granules at the early vitellogenetic stage and changes into large irregular vacuoles at the end of the vitellogenetic stage. Several authors have speculated on the nature of such granules (vacuoles) that they are vanadiumcontaining structures (Kalk, 1963; Hori and Michibata, 1981), pigment granules (Kessel, 1962; Kessel and Beams, 1965), or precursors of yolk granules (Mancuso, 1965). In the test cells of some ascidian species such as Corella and Styela, large vacuoles contain particulate or fibrous electron-dense materials named ornaments by Cavey (1976). The ornaments are released from the test cells at the late tailbud stage and deposited on the outer surface of the larval tunic of the hatched tadpole larvae (Cavey, 1976; Cloney and Cavey, 1982; Cavey and Cloney, 1984; Cloney, 1990). In the present materials, no significant changes were detectable in the large irregular vacuoles during embryogenesis. The other type of the cytoplasmic inclusions is a spherical vesicle with moderately electron-dense content. Mancuso (1965) has reported that such cytoplasmic vesicles arise at the late vitellogenetic stage. However, little attention has been paid to these vesicles until now. In the present study we demonstrate that the vesicles contain a component reactive to UA165 antibody (UA165 antigen). UA165 antigen was exocytosed into the perivitelline space during the tailbud stage, and the secreted antigen attached to the outer cuticular layer of the larval tunic. During oogenesis, UA165 antigen was first detectable in the vacuoles associated with the Golgi complex. The rough-surfaced endoplasmic reticulum that was well developed in the test cells at the early vitellogenetic stage disappeared in accordance with the formation of UA165 antigen-containing spherical vesicles near the end of the vitellogenetic stage. These findings suggest that the precursor of UA165 antigen is synthesized in the test cells during vitellogenetic stage, acquires antigenicity to UA165 antibody through processing in the Golgi complex, and the antigen accumulates in the spherical vesicles.



Fig. 5. Immunoelectron micrograph of a part of an ornament-containing vacuole in the test cell of *Styela partita*. The ornaments are strands of fine particles similar in appearance to those reported in other ascidian species (Cavey, 1976; Cloney and Cavey, 1982). Labeling by UA165-immunogold particles indicates that UA165 antibody does not recognize the ornaments, but the amorphous matrix (arrow) of the ornament-containing vacuole. Bar, 0.5 μm.

We briefly review the studies on the test cell function related to the larval tunic formation. Early workers believed that test cells secreted the larval tunic . However, recent ultrastructural studies have repeatedly showed the occurrence of secretory activities in the embryonic epidermal cells at the time of larval tunic formation (Dilly, 1969; Terakado, 1970; Reverberi, 1978; Gianguzza and Dolcemascolo, 1980; Cavey and Cloney, 1984). It has been demonstrated in dechorionation experiments that the embryos from which the test cells are removed at an early developmental stage can produce all of the major structural elements of the larval tunic (outer and inner cuticular layers, and the ground substance) (Mancuso, 1974; Reverberi, 1978; Cavey and Cloney, 1984; Cloney, 1990). These results indicate that the epidermal cells rather than the test cells play a principal role in larval tunic morphogenesis. On the other hand, Cloney and Cavey (1982) have presented evidence that the test cells are also functional in the completion of the larval tunic. In Corella inflata and Ascidia callosa, the larval tunic is hydrophobic and very sticky if embryos are dechorionated at the neurula stage; larvae tend to clump or adhere to the culture dishes (Cloney and Cavey, 1982). Cloney (1990) has

proposed that secretions of the test cells render the larval tunic hydrophilic. Production of ornaments is another function of the test cells as to the completion of the larval tunic. The larval tunic that was formed by *Corella* embryos dechorionated at the late tailbud stage was not sticky in spite of the absence of ornaments (Cloney and Cavey, 1982). Hence the ornaments *per se* can not be the factor that makes the larval tunic hydrophilic. In *Ciona* and *Ascidia*, the test cells do not produce ornaments, but test cells themselves firmly adhere to the larval tunic (Cloney, 1990).

We can suppose two possible functions of UA165 antigen. The antigen may correspond to the factor that is presumed by Cloney and Cavey (1982) to be secreted from test cells of Ascidia and Corella during the tailbud stage and prevent the larval tunic from being hydrophobic. The other possibility is that UA165 antigen may be a factor necessary for test cells to adhere to the surface of the larval tunic. In immunofluorescence histochemistry, UA165 antibody also stained the cytoplasm of the test cells in Styela partita. Immunogold labeling, however, showed that the component reactive to UA165 antibody in the test cells of Styela partita was not the ornaments but the amorphous matrix within the ornament-containing vacuoles (Fig. 5). These data indicate that UA165 antigen in Ciona intestinalis are not homologous to the substance composing ornaments in Styela partita. A molecular analysis is necessary to understand the exact function of UA165 antigen in the larval tunic formation.

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