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Ultrastructural Studies of Encystment in *Allomyces macrogynus*

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Ultrastructural organization of encysting zoospores of Allomyces macrogynus was examined using the methods of cryofixation and freeze substitution. During encystment, obvious changes were observed at the surface of the plasma membrane and in the structure of gamma particles. Many multivesicular bodies associated with the plasma membrane were observed at early stages of encystment. After induction of encystment, vesicles were found within the gamma particles. These vesicles appeared to leave gamma particles after forming multivesicular bodies. This study suggests that the cell wall formation during encystment is mediated by the fusion of multivesicular bodies with the plasma membrane.

Key Words: ***Allomyces macrogynus*, encystment, freeze substitution, gamma particle, zoospore.**

Fungal zoospores lack walls and become surrounded by new cell walls during encystment. As a natural protoplast, zoospores of many fungi are employed in investigations of the mechanism of wall formation. In *Allomyces macrogynus*, ultrastructure of the zoospores and encysting zoospores has been thoroughly examined using the method of conventional chemical fixation (Fuller and Olson, 1971; Barstow and Pommerville, 1980). It was suggested that the gamma particles are primarily responsible for the production of cytoplasmic vesicles and the formation of cell wall is mediated by the fusion of cytoplasmic vesicles with the plasma membrane (Barstow and Pommerville, 1980). But the role of the gamma particles in wall formation during encystment has been questioned and the organelles directly involved in wall formation has not been identified.

In ultrastructural studies of fungal hyphae, freeze substitution was introduced as a method to overcome the limitations of chemical fixation (Howard and Aist, 1979; Hoch and Howard, 1980; Hoch and Staples 1983; Heath et al., 1985). Freeze substitution has also proved to be an extremely useful technique for the preservation of zoospores and encysting zoospores with vesicular and vacuolar structures that often collapse with conventional chemical fixation (Cho and Fuller, 1989a, 1989b, 1989c). Yet, freeze substitution has not been applied to the developmental studies of the monoflagellate zoospores. The objectives of this study were to apply the methods of cryofixation and freeze substitution to ultrastructural studies of encysting zoospores of *Allomyces macrogynus* and to examine the vesicular structures involved in wall formation.

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Materials and Methods

Organism

The culture of *Allomyces macrogynus* (strain Burma 3-35) used in this study was generously provided by Dr. M. S. Fuller. Zoospore preparation and induction of synchronized encystment were adopted from the methods described by Barstow and Pommerville (1980).

Electron microscopy

Cryofixation and freeze substitution were done using the methods described in Cho and Fuller (1989a). For freezing, suspensions of zoospores and encysting zoospores spread on a gold slot grid were quickly plunged into propane cooled with liquid nitrogen. Encysting cells were frozen at 1~2 min, 3~5 min, 8~10 min, and 13~15 min after induction of encystment. After substitution, cells were infiltrated with Spurr's (1969) resin in ethanol and flat embedded (Koetzel, 1973). Sections were examined on a Hitachi H-7100 transmission electron microscope at 75 kV.

In this study, about 200 flat-embedded cells were selected with the light microscope and examined with the electron microscope. Among these cells, 12 cells were selected and thoroughly examined.

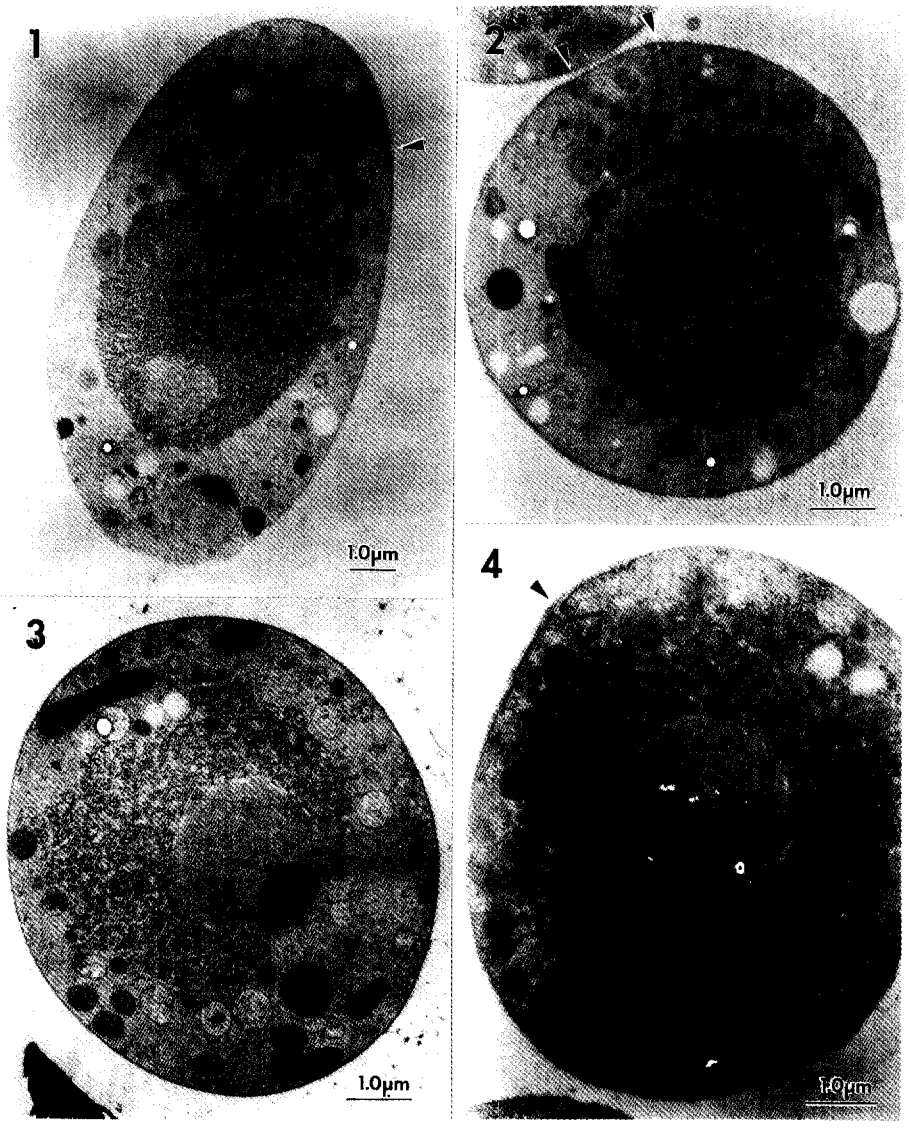

Fig. 1-4. General morphological changes during encystment in Allomyces macrogynus. Fig. 1. Longitudinal section of a zoospore. Arrowhead indicates a pit. Fig. 2. Section of a cell fixed 3~5 min after induction of encystment. Fig. 3. Section of a cell fixed 8~10 min after induction of encystment. Fig. 4. Section of a cell fixed 13~15 min after induction of encystment.

Results

The profile of the freeze substituted zoospores was smooth (Fig. 1). Flat vesicles and spherical vesicles were found near the plasma membrane (Figs. 1, 5) and pits were also associated with the plasma membrane (Figs. 6, 7). In zoospores, gamma particles were associated with fibrillar materials (Fig. 8), and not associated with vesicles.

In cells fixed 3~5 min after induction of encystment, nuclear caps were still intact and wall-like materials was observed at the surface of the plasma membrane (Fig. 13). In these cells, irregular shapes of multivesicular bodies associated with the plasma membrane were observed (Figs. 9-12). The shapes of the vesicles within these multivesicular bodies were also irregular. In all the induced cells examined in this study, small invaginations of the membrane that might be due to the vesicular fusion were not observed. In these cells, gamma particles had a crystalline lattice associated with matrix (Figs. 13-17). Small vesicles were observed between the matrix and crystalline lattice. Although the gamma particle's surrounding unit membrane (GS membrane, Myers and Cantino, 1974) was not well preserved in these cells, there seemed to be no space between the GS membrane and the matrix associated with the lattice.

In the cells fixed 8~10 min after induction, boundary of the nuclear caps was not clear (Fig. 3). Wall-like ma-

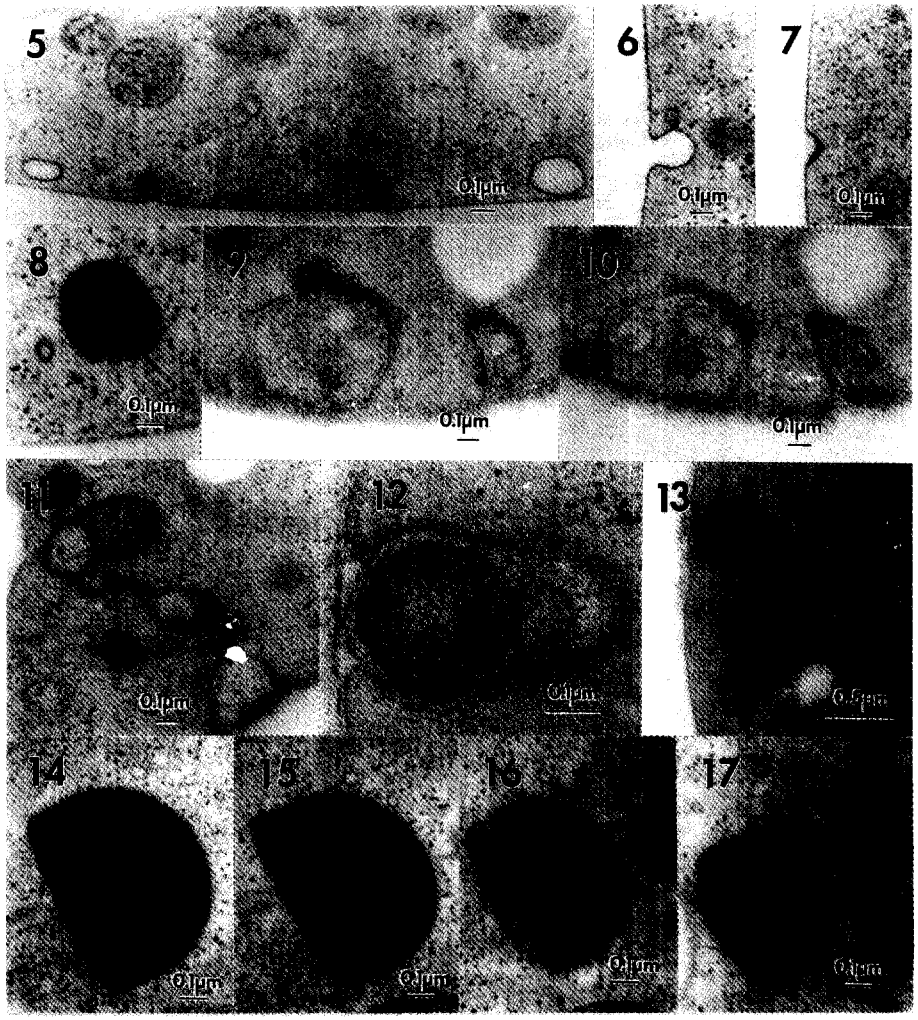

Fig. 5-8. Zoospore. Fig. 5. Various types of vesicular structures near the plasma membrane. Serial section of a cell in Fig. 1. Fig. 6. A pit associated with the plasma membrane. Fig. 7. Enlarged view of a pit in Fig. 1(arrowhead) Fig. 8. A Gamma particle containing crystalline materials. Serial section of a cell in Fig. 1. Fig. 9-17. Sections of cells fixed 3~5 min after induction. Fig. 9-12. Various types of multivesicular bodies fused with the plasma membrane. Serial sections of a cell in Fig. 2. Fig. 9, 10. Consecutive serial sections of a multivesicular body. Fig. 13-17. Gamma particles. Fig. 13. Wall materials are observed outside the plasma membrane. Fig. 14-17. Serial sections of a gamma particle.

terials deposited near the invaginated plasma membrane were observed (Fig. 18). The degree of penetration into the cytoplasm of these tubular invaginations was variable in different cells. Dense electron-opaque wall-like material was seen over the surface of the cell. Occasionally, multivesicular bodies that seemed to be connected with the gamma particles were observed (Fig. 19). Large vesicles were also observed in some gamma particles (Fig. 20). But, most of the gamma particles had shapes similar to those of 3~5 min cells (Fig. 21).

In cells fixed 13~15 min after induction, nuclear caps were partially dispersed (Fig. 4). Invaginations of the cell surface were still common (Figs. 22-25). In these cells, there was no increase in the amount of electron-opaque wall materials as compared to those of 8~10 min cells. In some gamma particles, separation of crystalline lattice and matrix was observed (Fig. 25). Dark objects that are believed to be the gamma particles were observed within multivesicular bodies (Figs. 26, 27).

Discussion

The cells at different stages of development showed different degrees of resistance to ice damage. Despite the smooth profile of the plasma membrane, the internal structures of all the zoospores examined in this study were associated with somewhat severe ice damages. Also, it was not possible to find a reasonably well-frozen cell among cells fixed 1~2 min after induction of encystment. Cells with complete cell walls were more resistant to ice

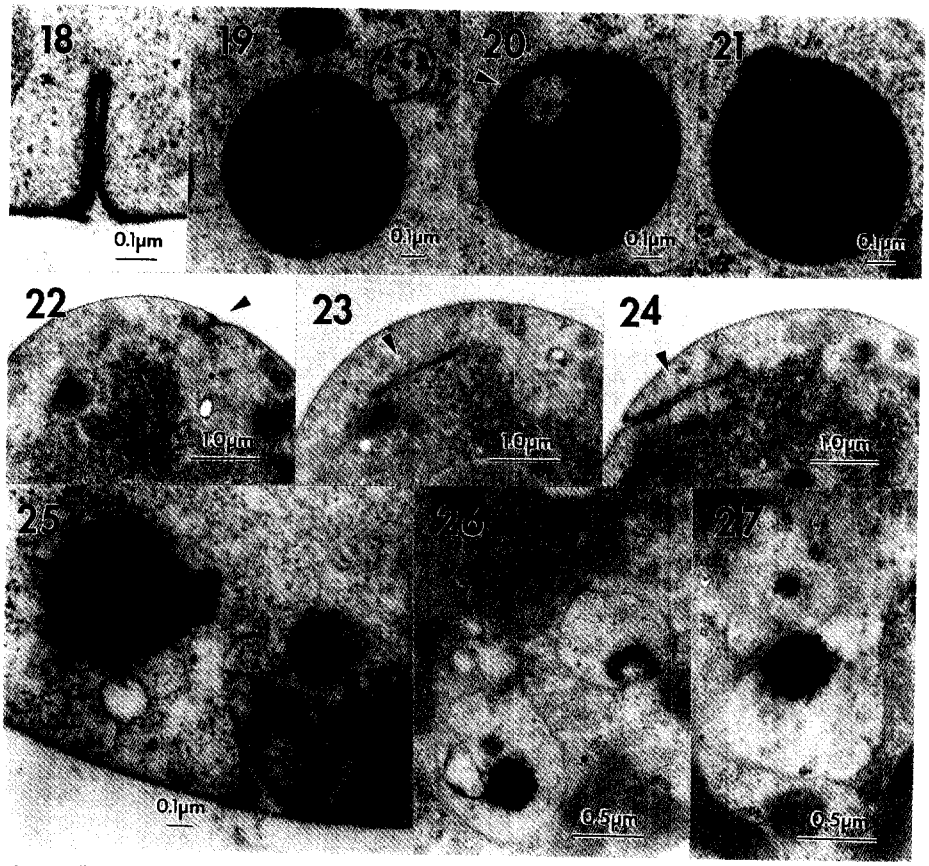

Fig. 18-27. Sections of cells fixed 8~10 min after induction. Fig. 18. Cell walls invaginated into the cytoplasm. Fig. 19. Multivesicular body that might be connected with the gamma particle. Fig. 20. Large vesicles within the gamma particle. Fig. 21. A section through the crystalline material in a gamma particle. Fig. 22-27. Sections of cells fixed 13~15 min after induction. Fig. 22-24. Serial sections of a cell in Fig. 4. Arrowheads indicate the tunnel made by the invaginated wall. Fig. 25. Separation of a crystal and matrix in a gamma particle. Arrow indicates the invaginated wall. 26-27. Electron-opaque objects that are believed to be gamma particles are observed within multivesicular bodies.

damage than the cells with incomplete cell walls. Although the degree of developmental synchrony was good, the developmental stage of each cell could be confirmed by the structure of nuclear cap.

In the freeze substituted zoospores, small pits were observed on the plasma membrane. These pits and the vesicles near the plasma membrane might suggest the dynamic movement of plasma membrane for water expulsion (Cho and Fuller, 1989b).

A previous study claimed that the fusion of vesicles with the plasma membrane was observed within 2~10 min after the induction of encystment (Barstow and Pommerville, 1980). Although we have examined 6 cells within 3~10 min after the induction of encystment, we have not observed the small invaginations that might be due to the vesicular fusion. Instead, we observed multivesicular bodies associated with the plasma membrane in the cells fixed 3~5 min after induction.

It is believed that the wall formation in the hyphal tip is mediated by the fusion of vesicles with the plasma membrane. But, the mechanisms of wall formation in the hyphal tip should be different from that in the encysting zoospores. Although tip growth requires the expansion of both wall and membrane, encystment occurs with little expansion of membrane and cell volume. If the wall formation during encystment is mediated by the fusion of vesicles with the plasma membrane, most of the vesicular membranes incorporated into the plasma membrane should be endocytosed. In electron micrographs, it is not easy to distinguish endocytosis from exocytosis. But, exocytosis should occur first prior to endocytosis if endocytosis occurs during encystment. In this study, we did not observe any membranous structures suggesting either endocytosis or exocytosis except the association of multivesicular bodies with the plasma membrane. Thus we propose that the multivesicular bodies are fused with the plasma membrane and endocytosis of the plasma membrane does not occur during encystment.

The ratio of the surface area to volume is much lower in multivesicular bodies than in small vesicles. Thus, the

fusion of multivesicular bodies with the plasma membrane will help to decrease the expansion of plasma membrane during encystment than the fusion of small vesicles. Walls deeply invaginated into the cytoplasm can also be regarded as means to maintain the effective surface area of the plasma membrane with no endocytosis of membrane. The shapes of the multivesicular bodies associated with the plasma membrane are similar to the extracellular vesicular structures observed in the encysting zoospores of *P. palmivora* (Cho and Fuller, 1989c). It is interesting that both structures were observed in the freeze substituted cells.

We have failed to collect any direct evidence that gamma particles are involved in cell wall formation. The vesicles formed within the gamma particles (G vesicles) are unusual structures in that they are formed within the membrane-bound compartment. By the same reasoning, the fate of G vesicles may not be similar to the fate of Golgi-derived vesicles. If we assume that the G vesicles have any roles during encystment, the contents of the G vesicles should be discharged out of the gamma particles. Fusion of G vesicles with the GS membrane may disrupt the selective transport of the contents of the G vesicles. One of the plausible possibility is that the GS membrane is extended into the cytoplasm and the vesicles surrounded by GS membrane are discharged as a multivesicular body. We have observed the multivesicular bodies that appeared to be associated with gamma particles. However, these associations could be artifacts caused by ice damage. Even if we assume that the multivesicular bodies are derived from gamma particles, vesicles within multivesicular bodies are still within the boundary of GS membrane. It is highly unlikely for vesicles and vesicular contents to pass through the GS membrane. We suggest that the vesicles within the gamma particles are discharged outside of the cytoplasm after the fusion of multivesicular bodies with the plasma membrane. After the fusion of multivesicular bodies with the plasma membrane, these vesicles may be activated in extracellular environment and begin to produce wall materials.

In the late stage of encystment, decaying gamma particles were observed within multivesicular bodies. The separation of gamma matrix from its GS membrane was observed only at this late stage. Vesicles formed within the gamma particles may begin to fuse with the GS membrane at this stage.

Acknowledgment

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