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The Three-Dimensional Morphology of *Candida albicans* as Seen by High-Resolution Scanning Electron Microscopy

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The fine structure of *Candida albicans* has been repeatedly described by transmission electron microscopy, whereas studies by high-resolution scanning electron microscopy (HRSEM) are rare and devoted solely to the study of its external morphology. This report describes the results of an HRSEM study on *C. albicans* carried out by an osmium maceration protocol modified to better retain the structural characteristics of this yeast. Thus, we visualized various intracellular structures including invaginations of cell membrane (plasmalemmasomes), nuclear envelope, mitochondria, the vacuolar system, and two additional structures that might represent a form of endoplasmic reticulum and the Golgi apparatus. The present investigation, which for the first time shows the organelles of *C. albicans* at the 3D level, may lead to a better understanding of its cell physiology.

Keywords: high-resolution scanning electron microscopy, osmium maceration method, *Candida albicans*

Candida albicans is one of the most important human fungal pathogens because it causes severe mucosal and systemic infections in immunocompromised patients, including those receiving cancer chemotherapy, in diabetics and in premature infants (Odds, 1987; Cannon *et al.*, 1995). Furthermore, oral-pharyngeal candidiasis is so frequently associated with AIDS that it is regarded as a criterion for staging the progression of this disease (Coleman *et al.*, 1993). The internal structure of *C. albicans* has been described by transmission electron microscopy (TEM) (Tsukahara and Sato, 1964; Borges and De Nollin, 1974; Rajasingham and Cawson, 1984). In contrast, studies by SEM are limited (Barnes *et al.*, 1971; Tokunaga *et al.*, 1986; Kusamichi *et al.*, 1990; Portillo *et al.*, 1994) and are mostly devoted to the visualization of its surface morphology. This report describes the detailed fine structural morphology of *C. albicans* using an osmium maceration method (Riva *et al.*, 1999) modified to preserve the structure of yeast, combined with high-resolution scanning electron microscopy (HRSEM). In contrast to higher eukaryotic cells, yeast cells are particularly difficult to process for morphological studies, mainly due to the presence of a thick cell wall, which acts as a fixative diffusion barrier. Also, the presence of a rigid cell wall interferes with the sectioning of these cells, since the blade of the chopper shifts the cells instead of resecting them. To overcome these problems, fixation times have been lengthened, and cutting by chopper was replaced by shattering specimens that had been frozen in liquid nitrogen as in the original osmium maceration method by Tanaka and Mitsushima (1984). The fractured surface of *C. albicans* was subsequently exposed to the macerating solution, in order to remove most of the cytosolic soluble components. The present report shows unique images of the membranous cellular organelles of *C. albicans*. In fact, we observed plasmalemmasomes, the nuclear envelope, the mitochondria, the vacuolar system, and two more structures, which correspond to the endoplasmic reticulum (ER) and the Golgi apparatus of higher eukaryotes. The modified technique described here represents a new approach for examining the inner morphology of yeasts. Moreover, the outer morphology of *C. albicans* was investigated as well.

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Materials and Methods

Internal morphology

C. albicans cells were cultured in Sabouraud dextrose agar. Before fixation, a colony was transferred to Sabouraud dextrose broth at 37°C for 18 h to reach the stationary growth phase. Cells were then centrifuged and suspended in phosphate-buffered saline (PBS). After two more washes, pellets were fixed with 0.2% formaldehyde+0.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min. After washing in PBS (3×10 min), cells were postfixed in 1% OsO_4-1.25% $K_4Fe(CN)_6$ for 1 h in the dark. Following several rinses in PBS, cell pellets were warmed to 37°C and mixed in eppendorf tubes with a 5% agarose solution (Low Gelling Temperature Agarose, SIGMA, USA). Small conical blocks were plunged into 50% DMSO/water (v/v), at 4°C until they sank to the bottom. Afterward, the blocks were transferred into a container filled with liquid nitrogen and fractured by mechanical means. After three rinses in PBS, the fragments were treated with 0.1% OsO_4 in PBS for 72 h at 27°C. No second postfixation was performed. During maceration, no rotating agitator was used, in order to prevent complete removal of cytoplasmic organelles. After rinsing with PBS,

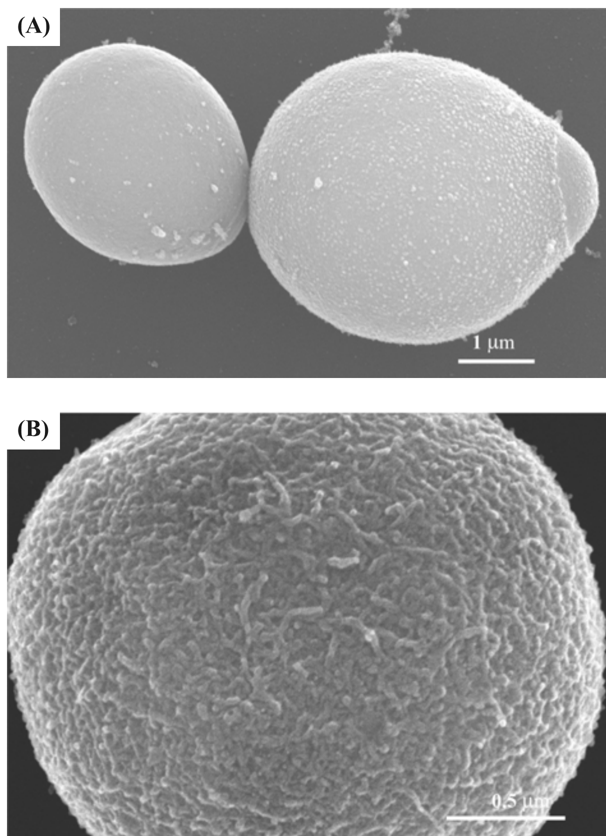

Fig. 1. External morphology of *C. albicans*. (A) Note the small protrusions of the outer surface. (B) At higher magnification, the external surface of some cells exhibited a reticulate structure.

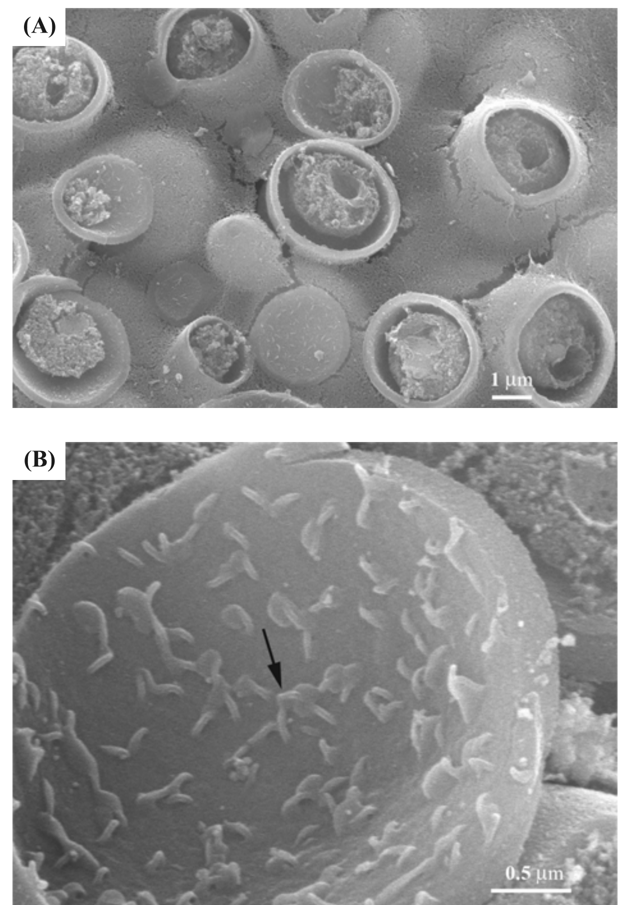

Fig. 2. (A) *C. albicans* cells embedded in agarose after cracking and osmium maceration at low magnification. The fractured surface of the pellet fragments reveals several broken cells. (B) *C. albicans* devoid of cytoplasmic structures shows the cytoplasmic surface of the plasmalemma. The arrow points to the randomly distributed finger-like invaginations (plasmalemmasomes).

the fragments were dehydrated through a series of acetones of increasing concentration, then they were critical point dried with CO_2 and coated with platinum (3 nm) in an Emitech K575 turbo sputtering apparatus. Specimens were observed in a field emission Hitachi (Tokyo, Japan) S4000 HRSEM operating at 20 kV.

External morphology

Stationary growth phase cells were fixed in 1% formaldehyde+1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min, rinsed, and allowed to sediment overnight on polylysine-coated coverslips. These were dehydrated through a series of graded acetones, critical-point dried with CO_2, and coated with platinum (3 nm).

Results

Surface morphology

All the *C. albicans* cells were ovate (Fig. 1A and B). At low magnification, the outermost layer of the cell wall exhibited an apparently wrinkled surface with small protrusions (Fig. 1A). However, at higher magnification, the external surface of some cells showed reticular structures (Fig. 1B), which might correspond to the fibrils (of a polysaccharidic nature), previously described by Kusamichi *et al.* (1990) and by Hazen and Hazen (1992), partially deformed by dehydration but well preserved compared to results obtained by other substructural techniques.

Internal morphology

As shown in Figs. 2∼6, the osmium maceration method allowed the visualization of *C. albicans* internal structures. High-resolution SEM images displayed the 3D morphology of the large vacuole, the secretory granules, the nuclear envelope, and the fractured cell wall with great clarity (Figs. 3 and 4). In every cell, a diffuse cytoplasmic granularity somewhat obscured the cell structures. The plasmalemma usually was adjacent to the cell wall, only occasionally being detached from it (Fig. 4A). In those cells whose organelles and cytoplasm had been completely removed, the inner surface of the plasmalemma and the invaginations became evident. The latter displayed a random distribution throughout the cytoplasm membrane (Fig. 2B), and at higher magnification, they appeared as small vesicular structures in continuity with the cytoplasmic membrane (Fig. 5, arrows). The vacuolar apparatus occupied the central part of the cell, whereas the va-

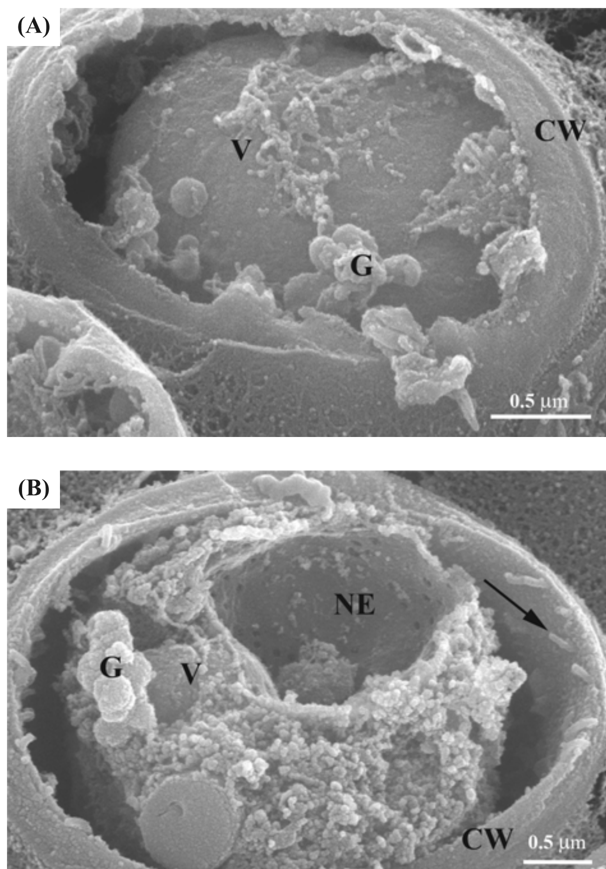

Fig. 3. *C. albicans* cells. (A) A large vacuole (V) and secretory granules (G) are evident. (B) Note the thickness of the cell wall (CW). The cytoplasmic side of the plasma membrane shows the plasmalemmasomes (arrow). The cytoplasm is filled with small particles. A portion of the internal nuclear envelope (NE) displays pores after removal of the chromatin.

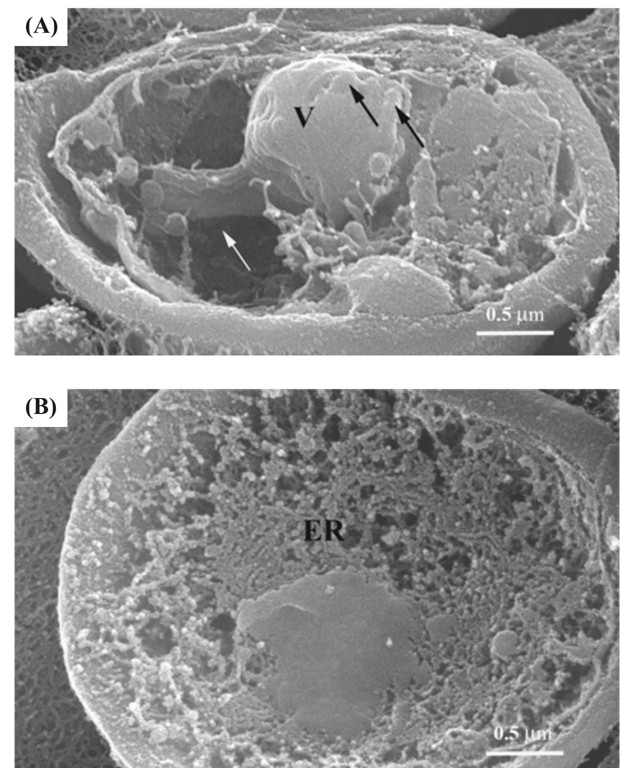

Fig. 4. *C. albicans* inner morphology. (A) The vacuolar apparatus (V) occupies the central part of the cell and is in continuity with the plasmalemma (white arrow). Note the presence of numerous bulges on the limiting membrane of the vacuole (black arrows) and the appearance of small spherical bodies (vesicles) in proximity to the plasmalemma or near to the vacuolar membrane. The space in between the cell wall and the plasmalemma can be appreciated. (B) A well developed endoplasmic reticulum (ER) occupies most of the cell. Spherical bodies are located among the elements of the ER.

cuole limiting membrane established intimate contacts with the plasmalemma through flattened protrusions (Fig. 4A). A large portion of the vacuole outer surface was irregular and sometime exhibited bulges. A vast membranous network, made up of branched and twisted tubules, occupied a large part of the cellular space, clearly showing interconnections with the vacuole (Fig. 4B). Mitochondria were located immediately beneath the plasma membrane (Fig. 5). Mitochondrial cristae were well defined and displayed both lamellar and tubular morphology. In Fig. 6, some tubular structures with nodular swelling (probably corresponding to a primitive Golgi apparatus) can be observed.

Discussion

By cryofracture and the osmium maceration method modified to fit the structural characteristics of *C. albicans*, we showed for the first time the three-dimensional inner morphology of this yeast. This was obtained despite the particulate cytoplasm, which, according to TEM studies, could be related both to the great number of ribosomes (Borges and

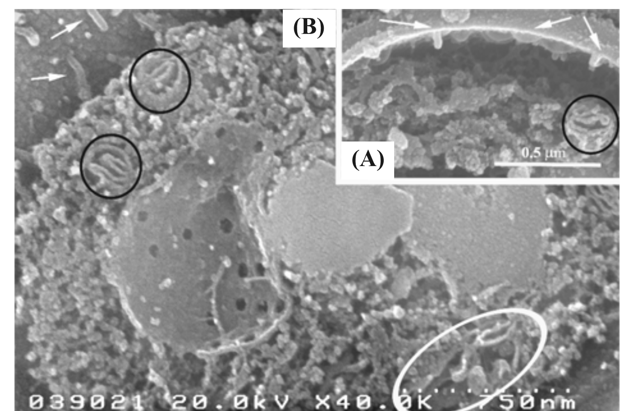

Fig. 5. *C. albicans* organelles. (A) Details of the peripheral cytoplasm containing a few tubules of the ER and a mitocondrion with lamellar cristae. (B) Sectioned mitochondria exhibiting both lamellar (black outline) and tubular (white outline) cristae are seen close to plasmalemma. The nuclear envelope with pores is evident. Arrows: plasmalemmasomes.

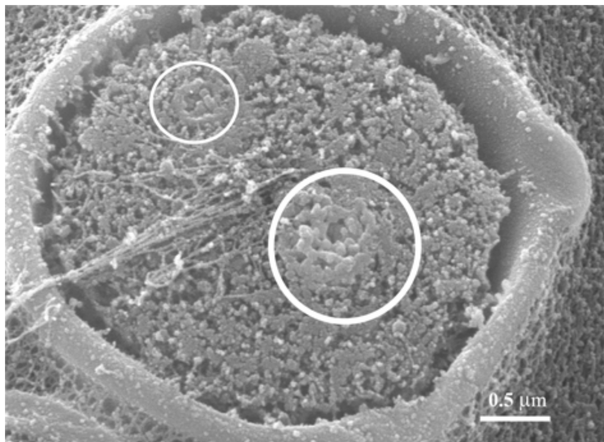

Fig. 6. The encircled tubular structures likely represent portions of the *C. albicans* Golgi apparatus.

De Nollin, 1974) and to the abundance of glycogen granules which, as reported by Rajasingham and Cawson (1980), seems to be the primary form of storage of this polysaccharide. The organelles visualized here included the nuclear envelope, the mitochondria, the vacuolar system, two structures that represent the endoplasmic reticulum and the Golgi apparatus of higher eukaryotes, and the invaginations of the plasmalemma. These membrane refoldings were named plasmalemmasomes by Edwards (1962). Marchant and Robards (1968), noted that, beside these structures, there were vesicular structures interposed between the capsule and the plasmalemma, which subsequently were called lomasomes (Moore and Mclear, 1961). Such a distinction was confirmed by Rajasingham and Cawson (1984), although the functional significance of these structures remained obscure (Mulholland *et al.*, 1994; Hazen *et al.*, 1995). Findings obtained here did not reveal the presence of structures that could be related to lomasomes. Our impression is that the vesicular structures, seen with TEM by the above mentioned authors, might simply represent plasmalemmasomes in cross section. Vacuolar apparatus complexity could be appreciated through the visualization of its intermembranous connections to the plasmalemma. The functions and the morphology of the vacuole have been well defined (Gow and Gooday, 1982; Klionsky *et al.*, 1990; Baba *et al.*, 1997). Vacuoles in yeasts contain various types of hydrolases and have been considered to be a lytic compartment analogous to lysosomes in animal cells (Matile and Wiemken, 1967; Wiemken *et al.*, 1979). A major function of the vacuole is the degradation and recycling of cell components. The numerous bulges observed on the surface of the vacuole could represent transport vesicles that, after blending with the membrane, spill their content inside the vacuole to be degraded. The yeast cells possess conveyors for retrograde transport of resident proteins and also endocytotic conveyors conducting extracellular ligands into the vacuole via the prevacuolar compartment (Vorisek, 2000). These exocytotic or endocytotic conveyors could correspond to the small vesicles seen in proximity to the plasmalemma or adjacent to the vacuolar membrane. Another interpretation regarding these small vesicles is that they correspond to lysosomal structures, as reported by Montes *et al*. (1965). The intermembranous connections between the plasmalemmal protrusions and the vacuolar membrane may be partially responsible for transporting material required for cell development, especially during budding (Palmer *et al.*, 2003). The vast tubular network clearly showing interconnections with the vacuole could be a part of the ER. Little is known about the structure of ER in *C. albicans*. Nevertheless, immunofluorescence studies (Rose *et al*., 1989; Preuss *et al.*, 1991) proved that the peripheral ER in yeast is a tubular network, similar in structure to the ER of higher eukaryotes. Such an organization is fully confirmed by findings reported here that also show the close apposition of the ER tubules to the vacuole membrane. We believe that the elongated tubular structure found into the cytoplasm of *C. albicans* may correspond to the Golgi apparatus. By TEM, the Golgi apparatus of yeast has been described as a circumscribed tubular network consisting of arrays of two or three parallel layers (Rambourg *et al*., 1995). This is in contrast with the mammalian cells in which the Golgi apparatus is composed of stacks of closely superimposed and parallel flattened saccules, usually adjoined to form a single organelle (Rambourg and Clermont, 1990). In *C. albicans*, we observed that this tubular membrane appears strongly folded on itself and that it shows dilations along its length. Dilations could correspond to secretory granules that are about to undergo exocytosis. Indeed, it has been postulated in mammalian cells that the secretory granules are formed by the rupture of tubular networks (Rambourg *et al.*, 1993), which would be equivalent to the transtubular or trans Golgi network (Griffiths and Simons, 1986). Portions of nuclear envelope with pore complexes were clearly visible after removal of the chromatin. The nuclear envelope and the vacuole often were adjacent. This is in agreement with a number of yeast studies (Koning *et al.*, 1993; Pan *et al.*, 2000), which reported that the yeast nucleus and the vacuole are physically linked by protein-protein interactions. While much is known about functions, shape, and size of yeast mitochondria (Egner *et al.*, 2002), little is known about their internal structure. It is difficult to elucidate the presence of mitochondria with tubular and lamellar cristae through a simple morphological analysis. In fact, this is the first time that in yeast cells different morphologies were observed in mitochondrial cristae. The *C. albicans* treated for the study of the external morphology always exhibited a uniformly rough surface. Moreover, even the cells processed by the osmium maceration method showed the same morphology. Possibly, the procedures used here were unable to preserve the ultrastructure of the *C. albicans* cell wall resulting in the loss of the outermost fibrillar layer or in changes from its native state. As reported by Kusamichi *et al*. (1990), the polysaccharide chains of the fibrillar structure are especially sensitive to alcohol dehydration. In fact, the latter causes the precipitation of the sugar component of the mannoproteins, which make up the fibrillar network, resulting in a drastic deformation and disappearance of the fibrillar layer (Tokunaga *et al.*, 1986). We first showed the internal 3D structure of *C. albicans* by the application of the osmium maceration method combined with cryofracture. These permit optimal observation of yeast cells and, in par-

ticular, allow detailed observation of their intracellular organelles, which traditionally have been the purview of transmission electron microscopy.

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