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Localization of Acid Phosphatase in *Saccharomyces cerevisiae*: a Clue to Cell Wall Formation

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Acid phosphatase is present in two layers of the cell envelope of *Saccharomyces cerevisiae*. These are separated by another layer, which is free of acid phosphatase. We have evidence that the cell wall is built up in two stages, which are independent. In the first stage, the cell wall is built up during the formation of the bud. Glucanase vesicles are involved in this process. In the second stage, a thick layer is deposited at the inside against the new cell wall. This results in the thick, rigid wall of the mature yeast cell. This latter layer is probably assembled on the outer surface of the plasmalemma.

Early in the cell cycle (13) a discrete morphological entity, the bud, appears on the yeast cell. The site at which this bud arises becomes a channel (the neck), which connects the mother and the daughter cell. For the formation of this channel, local destruction of the cell wall is necessary. Hydrolases, excreted by the yeast cell, are responsible for this local destruction (2, 10, 12, 22). They are thought to be transported to the cell periphery by the so-called glucanase vesicles (7, 8, 24).

Concomitant with local destruction, a new cell wall is built up. The material from which this wall is manufactured is inserted in the tip of the bud (11, 17, 19, 30, 31).

Besides glucan and chitin, the yeast cell wall contains a large amount of mannan (27). We have learned more in recent years about this mannan moiety (for a review, see reference 1). Ballou (1) suggested, as a working hypothesis, that the cell wall mannan is differentiated by location. It has a structural component that occurs interspersed with the glucan and that also covers the surface of the cell. The mannan occurring in enzymes such as invertase and acid phosphatase is possibly located in the periplasmic space. Others consider the enzymes as part of the cell wall matrix (28). The precise location of these mannoproteins in the cell wall remains to be established.

The structure of the mannan moiety of the mannoproteins is very similar to that of the cell wall mannan (29). We assume, for the time being, that there is no difference in the synthesis of the two mannan moieties either. The study of the biosynthesis of mannan has made good progress in the past few years (for a review, see reference 1). This is in contrast to the question of the site of mannan synthesis in the cell, which is still open. Mannan synthetase activity is mainly found in association with the endoplasmic reticulum (8, 19) and, to a lesser extent, with the glucanase vesicles (7, 8) and the plasmalemma (8, 19). The synthesized mannan has to be transported to the cell periphery, and Nečas (26) suggested two possible ways: (i) secretion of polysaccharides by exocytosis, and (ii) production of mannan in close association with the plasmalemma. In *Saccharomyces cerevisiae* protoplasts, no evidence for the involvement of vesicles in secretion has ever been found (26, 33). Meyer and Matile (23), also working with yeast protoplasts, were not able to demonstrate invertase secretion associated with vesicles, and they concluded that invertase is somehow transported through the membrane. Others, working with intact cells, have determined that exocytosis is the mechanism of secretion of mannoproteins (7, 8, 15, 21, 31).

We studied the yeast *S. cerevisiae*, using acid phosphatase as a representative of extracellular mannoproteins (3, 32, 33). The synthesis of this enzyme is repressed when the proper amount of phosphate is present in the growth medium (3, 32). The enzyme can easily be demonstrated biochemically (3, 32). It also can easily be localized at the ultrastructural level (33).

In this study, we localized this enzyme in intact repressed and derepressed cells to learn more about its location in the cell wall and about the way it is secreted by the yeast cell. Here we present evidence that the deposition of acid phosphatase outside the cell occurs both by exocytosis, in which vesicles are involved, and

by a process in which the enzyme is assembled on the plasmalemma.

MATERIALS AND METHODS

Organism and cultivation. Repressed and derepressed *S. cerevisiae* cells were grown and harvested as described previously (32).

Electron microscopy. Cells were prefixed for 10 min with 3% glutaraldehyde and 5% dimethyl sulfoxide in 50 mM sodium acetate, pH 5.0, rinsed, and, if required, treated with snail gut enzyme as described by Byers and Goetsch (6) for 15 to 60 min. This was followed by a period of incubation for acid phosphatase, fixation in trialdehyde, and postfixation in OsO_4 as described previously for protoplasts (33). The fixed cells were dehydrated in graded acetone to which, in the last step, a few drops of 2,2-dimethoxypropane (25) were added, and embedded in Araldite. Both poststained and unstained ultrathin sections were investigated in the electron microscope.

RESULTS

Pretreatment of fixed yeast cells for optimal acid phosphatase localization. Prefixed, derepressed cells, incubated for acid phosphatase, showed good localization of the enzyme in the cell wall, whereas the cytoplasm was devoid of lead precipitate (Fig. 1A). Poor penetration of substrate or capturing agent or both into the cell is considered a major problem in yeast ultrastructure preservation (6, 18) and yeast histochemistry (4, 9). Several successful methods to overcome this problem have been proposed (4, 6, 9). The method of Byers and Goetsch (6) offered the greatest potential for our purpose.

Indeed, only after snail enzyme pretreatment of the prefixed cells were we able to demonstrate acid phosphatase activity in the cytoplasm of derepressed cells (Fig. 1B). There was no difference with respect to acid phosphatase location in the cell wall (compare the cell walls shown in Fig. 1A and 1B). So the cell wall itself presents no barrier to rapid penetration. A brief treatment of about 15 min with snail enzyme is sufficient for cytoplasmic localization and causes little cell wall destruction.

Acid phosphatase localization. (i) Cytoplasmic structures. Acid phosphatase was found in derepressed cells in the central vacuole, in the Golgi structure, and in vesicles (Fig. 1B and 2–5). We especially observed these vesicles in the apical part and in the neck region of the bud (Fig. 2–5). They show considerable morphological resemblance, with respect to location in the cell and dimensions, to those observed by Moor (24), Sentandreu and Northcote (28), Byers and Goetsch (6), and Bowers et al. (5). They are probably identical to the glucanase vesicles (7, 8).

(ii) Cell wall. Very striking is the occurrence of acid phosphatase outside the cell (Fig. 1A and B and 6A), located in two layers (*1* and *3*), separated by an acid phosphatase-free layer (*2*), giving the cell wall a three-layered appearance. This three-layered structure can also be observed in wall fragments of lysed cells after

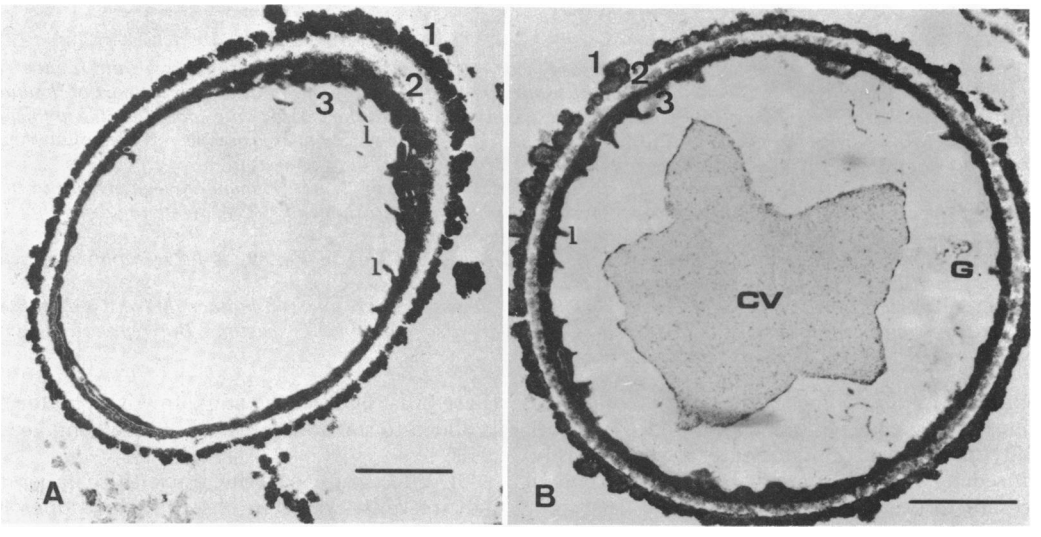

FIG. 1. *(A) Unstained section of a derepressed yeast cell incubated for acid phosphatase. Note the absence of lead precipitate in the cytoplasm. (B) Unstained section of a derepressed yeast cell incubated for acid phosphatase after a pretreatment with snail gut enzyme. Note the difference in contrast of layer (2) between the snail gut enzyme-treated and the untreated yeast cells. Acid phosphatase is present in the central vacuole (CV) and in the Golgi structure (G). I, Invagination of the plasmalemma. Bar represents 1 μm.*

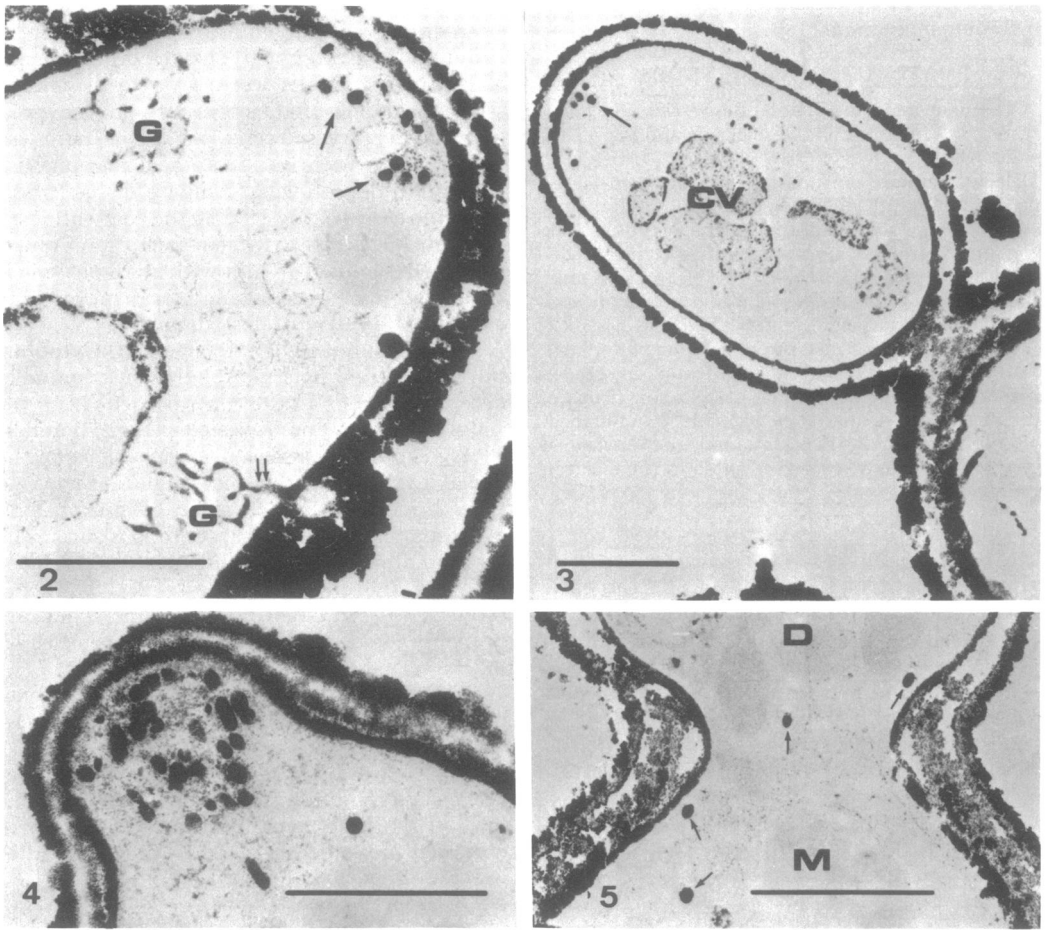

FIG. 2. *Detail of the tip of a bud of a derepressed yeast cell pretreated with snail gut enzyme and incubated for acid phosphatase. Glucanase vesicles, in which enzyme is present, are found at the apical part of the bud only (arrows). A Golgi structure (G) appears to have a connection with the periplasmic space (double arrows), a phenomenon we seldom observed. Tilting of the sections in the electron microscope revealed that these structures have no open connection but are situated at different levels in the section.*

FIG. 3. *Unstained section of a yeast cell treated as described in Fig. 2. Acid phosphatase is present in two layers in the cell wall, in glucanase vesicles (arrow), and in the central vacuole (CV). Note the difference in cell wall thickness between the mother and the daughter cell.*

FIG. 4. *Unstained section of a just-initiated bud of a yeast cell as in Fig. 2. A large number of acid phosphatase-filled vesicles can be seen. Bar represents 1 μm.*

FIG. 5. *Detail of a yeast cell as in Fig. 2, showing the channel that connects a mother (M) and a daughter (D) cell. Glucanase vesicles filled with enzyme (arrows) are also found in these regions. Bar represents 1 μm.*

acid phosphatase incubation. This suggests that the inner layer (*3*) is bound to the cell wall and that the lead precipitate is not entirely caused by a soluble acid phosphatase that is present in the periplasmic space.

The newly formed cell wall already has a three-layered appearance (Fig. 2–4 and 7). The inner layer (*3*) of the bud is thinner than that of a mature cell (Fig. 7). This means that, after the formation of the new cell wall in the bud, there must be a second stage in which material is added to the new cell wall in maturing yeast cells.

On micrographs of the thick third layer of mature cells, we often observe white lines in the dark regions corresponding to lead precipitate. The distance between these white lines is fairly constant (about 0.1 μm), and they run parallel to the cell surface. They are caused by folds in the plasmalemma due to ingrowing of

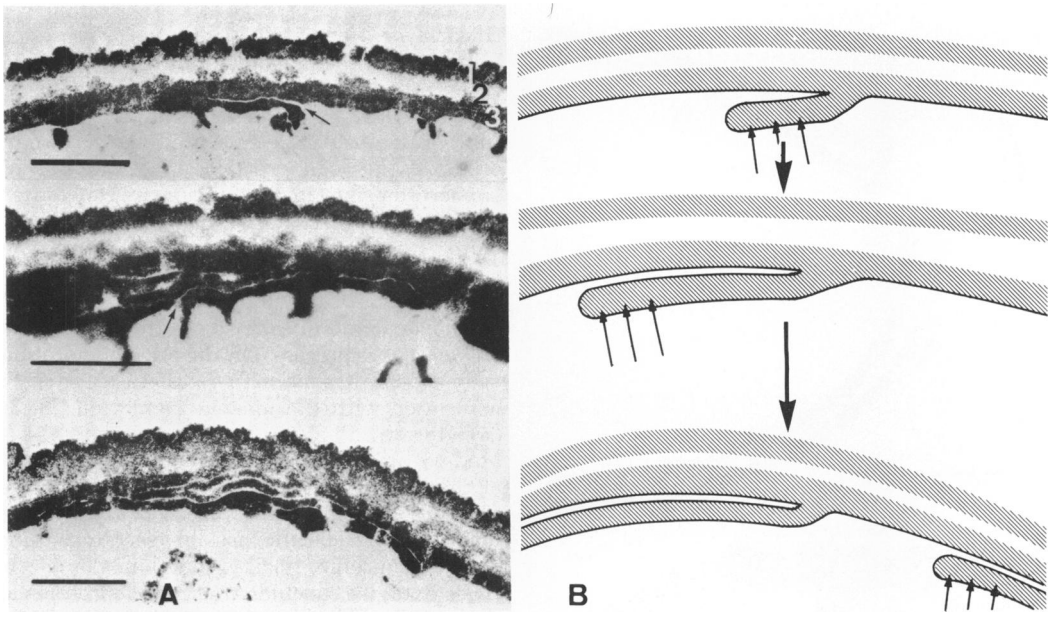

FIG. 6. *(A) Details of a yeast cell stained for acid phosphatase activity. (1) Outer layer, acid phosphatase positive; (2) mid-layer, acid phosphatase negative; and (3) inner layer, acid phosphatase positive. The white lines in the dark areas of layer 3 originate from the folded plasmalemma (arrows). Bars represent 0.5 μm. (B) Scheme of the second stage of cell wall formation as described in the text. The supposed preselected site of the plasmalemma, which grows into the cell, is indicated by the small arrows. The shaded areas represent the acid phosphatase-positive layers and the black line represents the plasmalemma.*

this third layer into the cytoplasm (arrows in Fig. 6A). Figure 6B shows how we think this process of ingrowing works.

Control experiments of the acid phosphatase reaction. As controls, we used yeast cells repressed in their acid phosphatase synthesis and derepressed cells in which the acid phosphatase was inhibited by NaF.

In repressed cells, an aspecific lead precipitate was found in the central vacuole only. This precipitate can also be seen after inhibition of acid phosphatase by NaF. This means that it is probably due to polyphosphate present in the central vacuole (16).

In derepressed cells, incubated in the presence of NaF, no lead precipitate was found. This means that (i) the precipitate in the central vacuole of derepressed cells after acid phosphatase incubation is due solely to acid phosphatase action, and (ii) the activity of the constitutive enzyme, if present, is too low to be detected with this histochemical technique.

The acid phosphatase present in the outer layer (*1*) is not due to adsorption of secreted acid phosphatase from the medium or of acid phosphatase present in the snail gut juice. This can be deduced from the following observations: (i) this outer layer is not observed in snail enzyme-treated, repressed cells, (ii) it cannot be found in repressed yeast cells kept in a medium to which yeast acid phosphatase is added, and (iii) it is present on derepressed cells when snail enzyme treatment is omitted.

DISCUSSION

The ellipsoid form of the yeast cell is a result of combined spherical and polar growth of the bud. Insertion of new cell wall material at the tip of the bud (11, 17, 19, 30, 31) provides for this polar growth. We have frequently observed glucanase vesicles at the tip of the bud which contain acid phosphatase. We have also shown that the newly formed yeast cell wall contains acid phosphatase in the outer layer and that this is a specifically bound enzyme. There is much evidence that mannan covers the cell surface of the yeast cell (1). We conclude that acid phosphatase is associated with this mannan layer where it is deposited during cell wall formation. From these clues, we deduce that at least one of the cell wall components is transported to its site of incorporation by means of the glucanase vesicles.

It is now generally accepted that hydrostatic

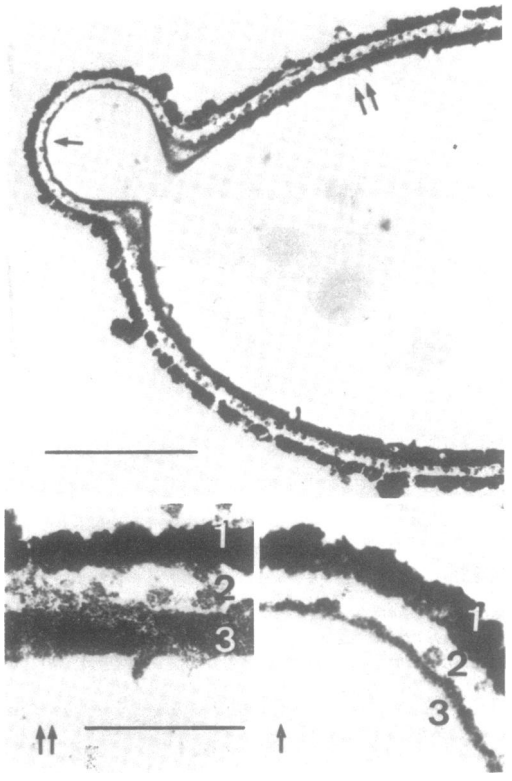

FIG. 7. *Budding yeast cell stained for acid phosphatase. Bar represents 2 μm. (Insets) Difference in thickness between the wall of the mature cell (double arrows) and the wall of the bud (single arrow) is seen to be due solely to differences in thickness of layer 3. Bar represents 0.5 μm.*

pressure is the principle driving force of spherical extension in the yeast cell (26, 27). Elastic properties of the bud cell wall, the presence of which is suggested on the micrograph of Fig. 8, make this possible. Some time later in development, the cell wall becomes more rigid. After its formation in the bud, the cell wall has to be completed with respect to its structure during maturation, and hence its mechanical properties change.

We have shown that the inner layer of the cell wall of mature yeast is thicker than that of the bud (Fig. 7). There is no difference between the dimensions of layers (*1*) and (*2*) in buds and mature cells. So, there is a second stage of cell wall building in yeast cells, which seems to be responsible for (i) thickening of the cell wall during aging, and (ii) fixation of the ultimate form of the cell by increasing the rigidity of the wall.

We can get a clue to the question of how this layer is formed from micrographs of the thick, third layer. Formation occurs via a process of ingrowing of the plasmalemma adjacent to this layer into the cytoplasm, parallel to the cell wall (Fig. 6A and B). The plasmalemma eventually remains visible as white lines in the dark areas of the lead precipitate. We find some support for this interpretation in conventionally stained sections of yeast cells (Fig. 9). Here, we are able to see sets of membranes running parallel to the cell surface, which are associated with the inner part of the cell wall. We never found glucanase or other vesicles involved in this process. On the other hand, it has been shown that mannan synthetase activity is associated with the plasma membrane (8, 34, 35). Hereward has found fibril synthesis associated with plasmalemma grooves on protoplasts of the yeast *Schizosaccharomyces pombe* (14). She suggests that these areas of the plasmalemma have a specific, possibly secretory, function in relation to fibril production. Combining these data, we conclude that mannoproteins for this third layer must be assembled on the outer surface of the plasmalemma which, together with some predilection of site, causes the localized ingrowing of the plasmalemma.

In this report, we have offered material for a working hypothesis on cell wall building in yeasts, based on the histochemical and morphological analysis of a large number of individual cells. We think that the cell wall is built up in two sequential modes. Mode I, in which glucanase vesicles are involved, is used to lay down the new cell wall during bud formation only. This process is, therefore, cell cycle dependent.

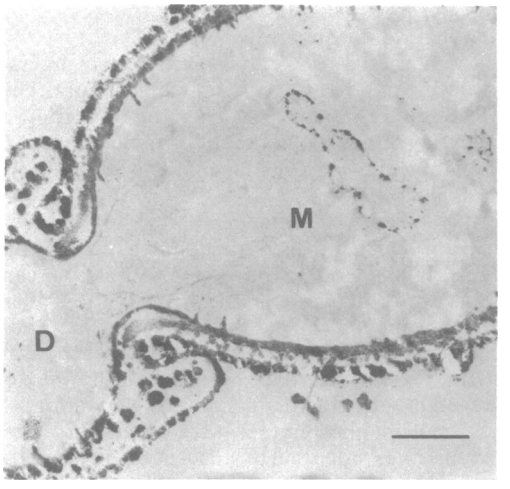

FIG. 8. *Snail gut enzyme-treated yeast cell. The wall of the daughter cell (D) is digested at the tip of the bud and is snapped back due to its high elasticity. M, Mother cell. Bar represents 1 μm.*

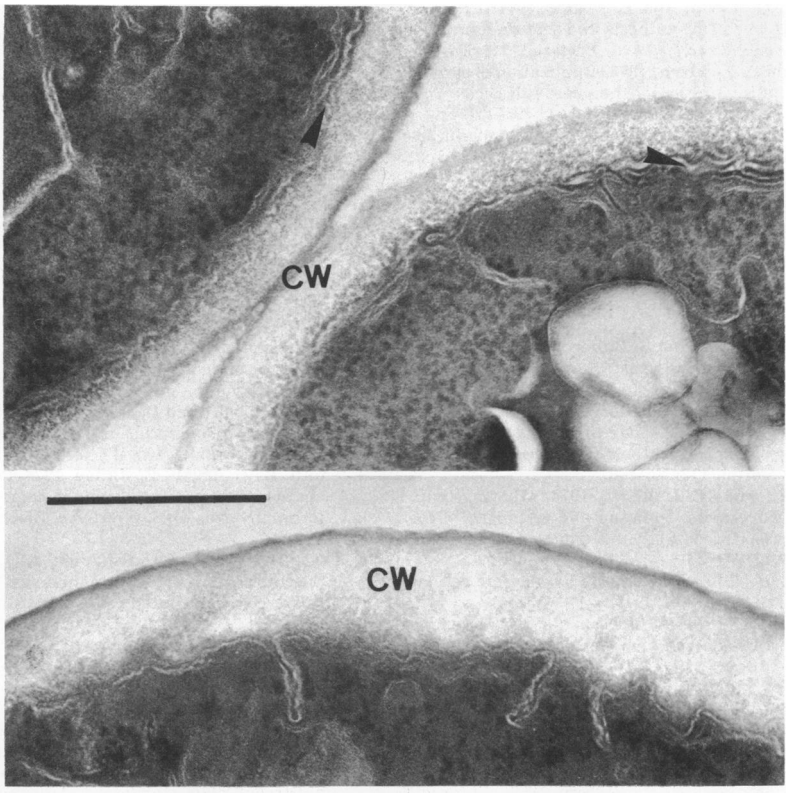

FIG. 9. *Details of a yeast cell fixed in trialdehyde fixative and postfixed in OsO_4. The thin sections are poststained with lead citrate and uranyl acetate. In the inner part of the cell wall, a set of membranous structures running parallel to the cell surface can be seen (arrows). CW, Cell wall. Bar represents 0.5 μm.*

Mode II, in which no vesicles are involved, is used to complete the wall in aging yeast cells. We have no reason to assume that this process acts during a discrete time interval in the cell cycle, for we have found it in both older buds and maturing cells. The mannan is probably manufactured on the plasmalemma.

We are not able to exclude structural alterations in the other two layers of the wall that arise concomitantly with the process described as mode II. Such structural alterations might also be responsible for a change in cell wall properties.

From our work on protoplasts (32, 33), we derived additional evidence that the cell wall is built up in two independent stages, in which different mechanisms operate.

Yeast protoplasts kept in liquid medium are unable to regenerate a cell wall (26, 27). Cell division is also repressed in protoplasts (for a review, see reference 26). This means that mode I, which we think occurs only during bud formation, is also repressed. Indeed, in derepressed, acid phosphatase-secreting protoplasts (33), we were unable to find any glucanase vesicles or any acid phosphatase on the cell surface. In protoplasts, this enzyme is probably secreted into the medium via mode II (3, 32, 33).

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