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# Cell Walls of Normal and Lysozyme-Damaged Blastoconidia of *Candida albicans*: Localization of Surface Factor 4 Antigen and Vicinal-Glycol Staining

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**The fungicidal effect of lysozyme on *Candida albicans* involves ultrastructural modifications previously described (G. Marquis, S. Montplaisir, S. Garzon, H. Strykowski, and P. Auger, Lab. Invest. 46:627–636, 1982). To further define the action of lysozyme on the yeast cell wall, we used the following: (i) the periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) method to highlight vicinal-glycol-reactive sites of complex carbohydrates; (ii) a monospecific antiserum and a protein A-gold complex to study the expression of surface factor 4, a major *Candida* antigen; and (iii) the periodic acid-silver methenamine method to stain cell wall glycoproteins. All *Candida* cells were found to express surface factor 4 antigen. In normal blastoconidia, surface factor 4 was located in a glycoprotein-rich cell wall layer, underneath radially oriented bundles of filaments which form the outermost wall layer. In lysozyme-treated blastoconidia, this glycoprotein-rich layer was lost and the regular brushlike organization of the outer fibrillogranular layer was disrupted. PA-TCH-SP staining and localization of surface factor 4 antigen demonstrated an altered arrangement of bundles of filaments in the outer wall layers of blastoconidia which were morphologically intact but had abnormal cell wall appearance. Next, there was a reduction in thickness of the outer layer and the expression of surface factor 4 antigen was limited to the cytoplasmic membrane area. Later on, the cell wall was almost uniformly highlighted by PA-TCH-SP staining. These data evinced a highly plastic architecture of the cell wall in *C. albicans*.**

Hen egg-white lysozyme is known to be fungicidal to the blastoconidia of *Candida albicans* under defined in vitro conditions (6, 7). We previously published a detailed study on the ultrastructural modifications which occur in *C. albicans* cells following lysozyme exposure (7). As described (7), the early events seen in lysozyme-treated blastoconidia were changes in the layering of the cell wall. Later events were a progressive disappearance of cytoplasmic organelles, with or without unremitting accumulation of complex carbohydrate material between the wall and plasmalemma, and the focal retraction of the cytoplasmic membrane from the adjacent cell wall; next, the cytoplasmic content, pulled inward by the accumulating material, was progressively destroyed through the concurrent plasmolysis. To further define the action of lysozyme on the yeast cell wall, we investigated the distribution of a major *Candida* antigen known as surface factor 4 and of vicinal-glycol-containing complex carbohydrates in normal and lysozyme-treated blastoconidia. The antigenic formulas of medically important yeasts have been defined by the pattern of reactivity in agglutination with rabbit antisera directed at thermostable immunodeterminants, i.e., factor antigens (17). Specific monofactorial sera that were prepared by cross-adsorption and then purified are commercially available for rapid immunoidentification of *Candida* species (15), but very few ultrastructural observations on their binding sites in the *Candida* cell wall have been published (5, 10). Surface factor 4 antigen is known to be present in *Candida* species such as *C. albicans*, *C. tropicalis*, *C. guilliermondii*, and *C. stellatoidea* but absent in *C. krusei*, *C. pseudotropicalis*, and *C. parapsilosis* (15). After vicinal-glycol-reactive sites were stained, the cell walls of normal *C. albicans* blastoconidia were reported to appear as a sandwich structure, with an outer set of layers of high electron density, an intermediate set of layers of lower electron density, and an inner layer of high electron density (3, 11, 12).

## MATERIALS AND METHODS

**Organism and culture conditions.** *C. albicans* 4454M, a serotype A isolate (Institut Pasteur, Paris, France), was maintained on Sabouraud dextrose agar. For use in the present investigation, exponential-phase organisms were obtained from a tryptose-phosphate broth culture (Difco Laboratories, Detroit, Mich.), grown in a controlled-environment incubator shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 37°C and 200 rpm. Yeast cells were collected by centrifugation and resuspended in a specially

FIG. 1. PA-TCH-SP staining of normal (a) and lysozyme-treated (b through f) blastoconidia of *C. albicans*. The normal cell wall is seen to consist of a brushlike fibrillogranular layer (i.e., bundles of filaments oriented radially), an amorphous highly dense layer, an intermediate layer in which discrete silver grains are deposited, and an innermost dense layer which is appressed on the plasmalemma. The amorphous highly dense layer is never seen in lysozyme-treated blastoconidia. Moreover, the outer fibrillogranular cell wall layer is progressively reduced in thickness (b to d) and a random arrangement of bundles of filaments is demonstrated. Later on, the cell wall is almost uniformly highlighted by the PA-TCH-SP reaction and aggregates of glycogenlike particles are seen near the plasmalemma (e and f). Duration of exposure to lysozyme (in hours): 2 (b), 3 (c), 4 (d), and 6 (e and f). Bars, 0.5 μm for panels a to e and 0.2 μm for panel f.

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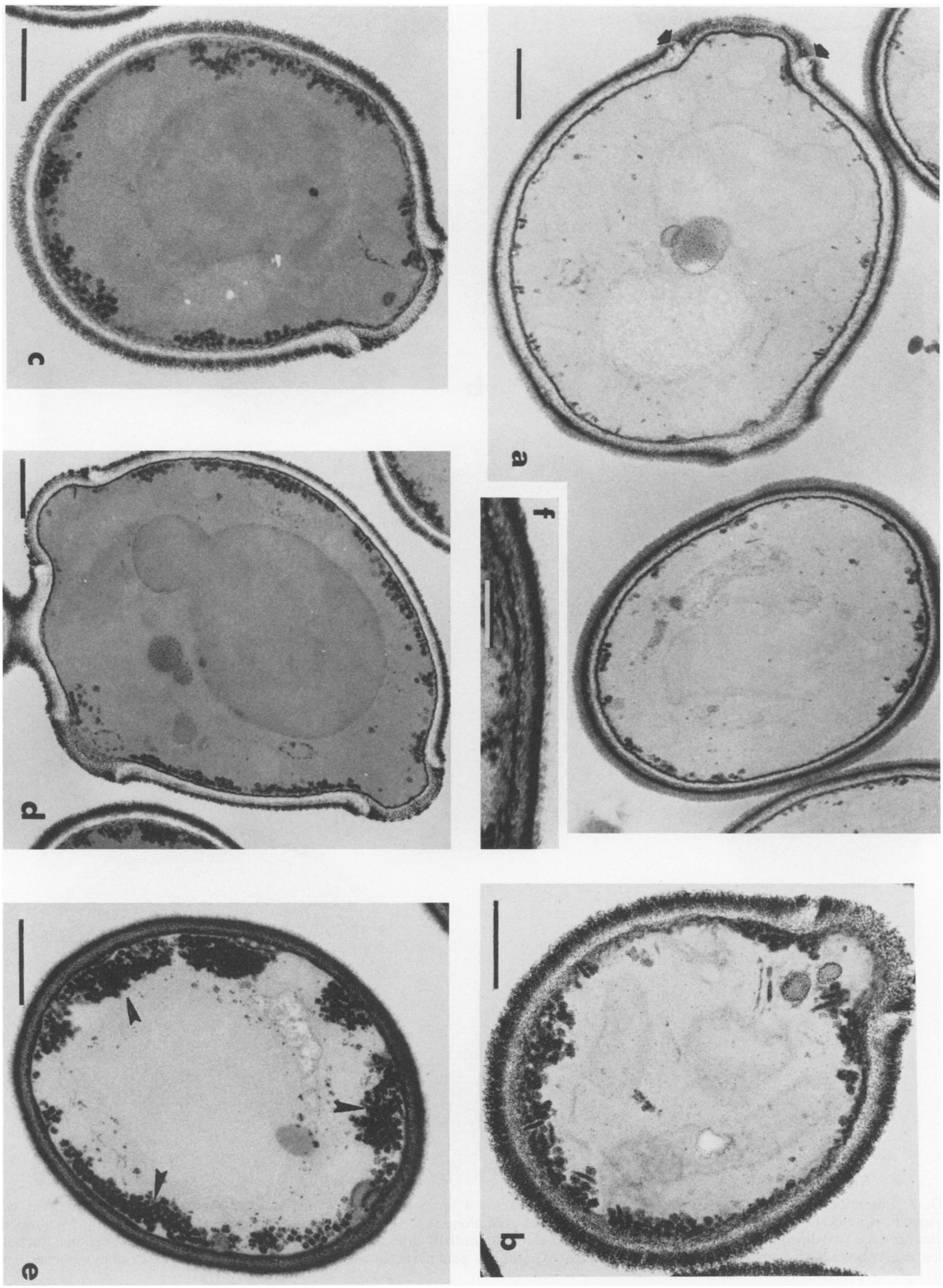
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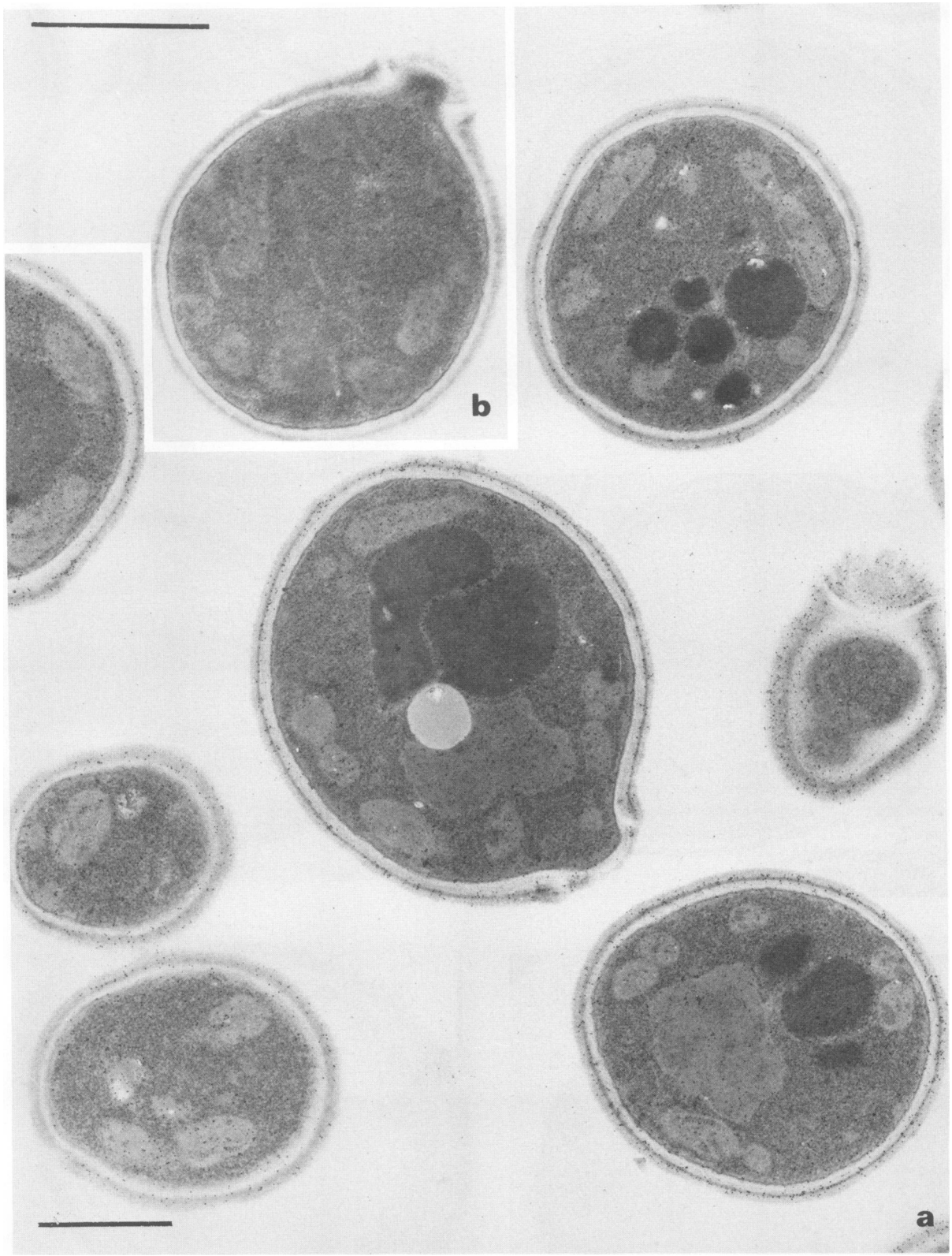



FIG. 2. Immunoelectron microscopy detection of surface factor 4 antigen. Osmium-fixed normal blastoconidia were treated with monospecific antibody and protein A-gold (a) or with protein A-gold alone (b). All *C. albicans* cells express surface factor 4. Immunogold particles are seen to delineate a cell wall layer immediately underneath radially oriented bundles of filaments. In tangential sections, the outer fibrillogranular layer is also labeled. In the negative control experiment, gold particles were rarely found over the yeast cell cytoplasm. Bars, 1 μm.

devised medium, named MBG, containing 0.15% malt extract, 0.05% beef extract, and 2.5% glycerol.

**Treatment with lysozyme.** MBG flasks seeded with $10^6$ blastoconidia per ml were placed in the shaking incubator for 4 to 6 h (37°C, 200 rpm). Yeast cells (approximately $10^7$/ml) were collected by centrifugation and resuspended to their initial volume, using either fresh MBG or MBG supplemented with 100 μg of hen egg-white lysozyme per ml. Reincubation was carried out under the same conditions. A dye exclusion technique (1) was used to follow the progressive decline in viability that took place in cultures to which lysozyme had been added. Treated cultures, containing 50 to 70% viable cells, were processed with untreated cultures (more than 97% viable cells) for electron microscopic examination. Treated cultures were sampled after 2 to 6 h of exposure to lysozyme.

**Preparation of specimens.** Yeast cells were fixed in sequence with 3% glutaraldehyde (3 h) and 1% osmium tetroxide (3 h) in 0.1 M 1,4-piperazinediethane-sulfonic acid buffer, pH 7.4, at room temperature, with buffer rinses after each fixative. Cell pellets were infiltrated with 2% agar, dehydrated in a series of graded alcohols, taken into propylene oxide, and embedded in Araldite 502. Ultrathin sections (80 to 100 nm) were cut with a diamond knife on a LKB ultratome and picked up on naked 400-mesh gold or nickel grids.

**Histochemical and immunocytochemical methods.** Sections on gold grids were stained by the periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) method of Thiéry (16) to highlight vicinal-reactive sites of complex carbohydrates. Monospecific rabbit antibody to *Candida* factor 4 antigen (Candida Check; Iatron Laboratories, Chiyoda, Tokyo, Japan) was applied at a dilution of 1:50 in sequence with a protein A-gold complex for the ultrastructural localization of this major surface antigen in normal and lysozyme-treated blastoconidia. The protein A-gold complex (Au = 8 nm), prepared by the method of Bendayan (2), was stardardized to an optical density of 0.1 at 525 nm. Sections on nickel grids stained by immunogold for the localization of surface factor 4 were highlighted with uranyl acetate and lead citrate prior to examination with a Philips EM 300 microscope. The periodic acid-silver methenamine procedure, performed as described by Rambourg (13), was used to assess the distribution of glycoproteins within the normal *Candida* cell wall.

## RESULTS

**Demonstration of vicinal-reactive sites.** As revealed by the PA-TCH-SP staining procedure, the cell walls of normal *Candida* blastoconidia were seen to consist of an outer brushlike fibrillogranular layer, an amorphous highly dense layer, an intermediate layer in which discrete silver grains are deposited, and an innermost dense layer which intimately follows convolutions of the cytoplasmic membrane in the interior of the cell (Fig. 1a). The fibrillogranular and amorphous dense cell wall layers were interrupted in the bud scar region (arrows). After exposure to lysozyme for 2 to 4 h (Fig. 1b to d), the following changes were noted: (i) disruption of the regular brushlike organization of the outer fibrillogranular layer; (ii) disappearance of the amorphous highly dense layer; and (iii) progressive reduction in the thickness of the outer fibrillogranular layer. Later events are presented in Fig. 1e and f. The cell wall of this blastoconidium, exposed to lysozyme for 6 h, appeared almost uniformly highlighted by the deposition of silver proteinate. In addition, cytoplasmic organelles were no longer visible and large aggregates of PA-TCH-SP-reactive glycogenlike particles (arrowheads) were seen in the vicinity of the cytoplasmic membrane.

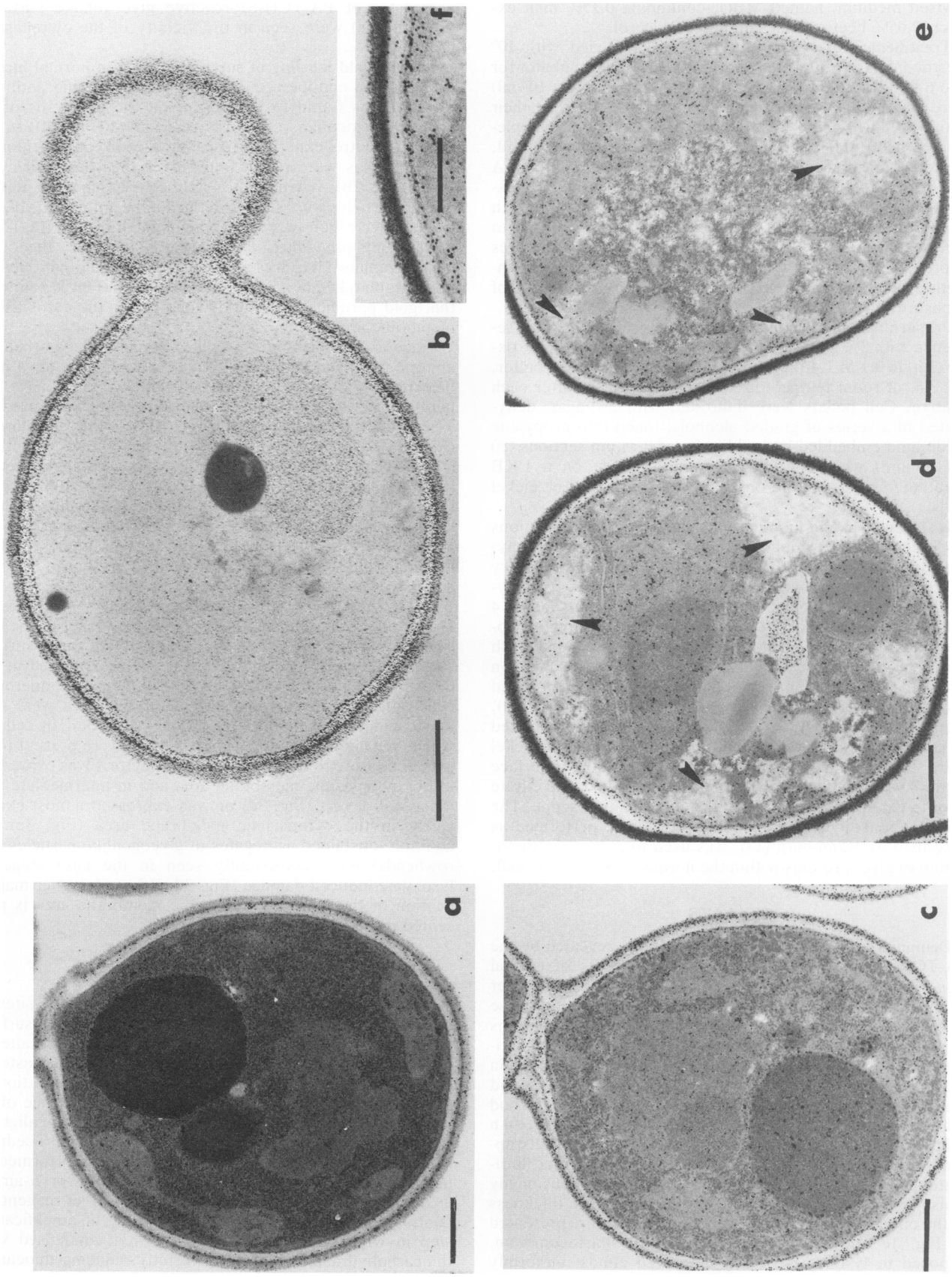
**Immunogold labeling of surface factor 4 in normal blastoconidia.** The epitope recognized by monospecific antibody factor 4 was found to be present on the cell walls of all normal blastoconidia of *C. albicans* 4454M (Fig. 2a). In the negative control experiment (protein A-gold complex alone), gold particles were rarely found over the yeast cell cytoplasm (Fig. 2b). When factor 4 antibody was applied, immunogold particles were seen to delineate the amorphous highly dense layer which was starkly highlighted by the PA-TCH-SP staining procedure. On tangential sections, the outer fibrillogranular layer was also labeled with gold particles. A micrograph taken at a higher magnification (Fig. 3a) showed that gold particles were rarely found over the cytoplasmic membrane area.

Since a previous study provided some evidence that the epitope recognized by monospecific antibody factor 4 may be expressed on an extractable cell wall glycoprotein (4), the periodic acid-silver methenamine procedure was used to assess the distribution of glycoproteins within the normal *Candida* cell wall. As revealed by this histochemical method (Fig. 3b), cell wall glycoproteins were found to be located in the amorphous highly dense layer delineated by immunogold labeling of surface factor 4 (Fig. 3a) and in the intermediate layer in which discrete silver grains were deposited (Fig. 1a), as seen with the PA-TCH-SP staining. The outer brushlike fibrillogranular layer was hardly delineated by the periodic acid-silver methenamine procedure.

**Altered expression of surface factor 4 in lysozyme-treated blastoconidia.** In cells in which only the layering of the cell wall was abnormal (2 h of exposure to lysozyme), surface factor 4 antigen was found to be strongly expressed in the disorganized fibrillogranular layer (Fig. 3c). The innermost cell wall layer and cytoplasmic membrane area were also delineated with immunogold particles (Fig. 3c). In cells in which degenerative cytoplasmic changes were present (4 to 6 h of exposure to lysozyme), surface factor 4 was expressed in the cytoplasmic membrane area and in intermediate and inner wall layers (Fig. 3d) or was expressed almost exclusively in the cytoplasmic membrane area (Fig. 3e). In addition, unstained aggregates of glycogenlike particles (arrowheads) were consistently seen in the later stage of lysozyme-induced damage (Fig. 3d and e). A higher magnification of the labeled cytoplasmic membrane area is presented in Fig. 3f.

## DISCUSSION

Histochemical staining for vicinal-glycol-reactive sites of complex carbohydrates and immunogold labeling of surface factor 4, a major *Candida* antigen, demonstrated an altered organization of the cell wall in lysozyme-treated blastoconidia: (i) disruption of the regular brushlike organization of the outer fibrillogranular layer and (ii) disappearance of an amorphous highly dense layer which was heavily highlighted by the periodic acid-silver methenamine staining procedure. With this histochemical method, aldehydic groups formed by periodic acid oxidation of adjacent hydroxyl or α-amino alcohol groups directly reduce the alkaline silver reagent. In contrast, the Thiéry procedure (16) includes an amplification step in which aldehydic groups are first condensed with thiocarbohydrazide to form powerfully reducing thiocarbohydrazone groups which deposit the silver proteinate. Accordingly, the specificity of the periodic acid-silver methe-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e
b
d
a
c

FIG. 3. Periodic acid-silver methenamine staining of a normal blastoconidium (b) and labeling of surface factor 4 in normal (a) and lysozyme-treated (c through f) blastoconidia of *C. albicans*. In normal blastoconidia (a and b), the cell wall layer immediately underneath radially oriented bundles of filaments is delineated by immunogold particles and deposited silver grains. The cytoplasmic membrane area is also labeled by a few immunogold particles. In lysozyme-treated blastoconidia, surface factor 4 is sequentially expressed in the outer fibrillogranular layer and cytoplasmic membrane area (c), in the intermediate and inner wall layers and cytoplasmic membrane area (d), and almost exclusively in the cytoplasmic membrane area (e and f). Duration of exposure to lysozyme (in hours): 2 (c), 4 (d), and 6 (e and f). Bars, 0.5 μm for panels a to e and 0.2 μm for panel f.

namine procedure is restricted to glycoprotein molecules (14), while the PA-TCH-SP reaction presumably highlights the mannans and β-1→6-glucans in the cell wall of *C. albicans* (3, 11). Staining of blastoconidia with anti-mannan antibodies and peroxidase-conjugated antiimmunoglobulins supports this interpretation of the PA-TCH-SP reaction (9, 12).

It was previously shown by flow cytometric analysis of indirect fluorescence antibody staining that all *Candida* cells expressed surface factor 4 to the same extent and independently of growth conditions, growth phase, and germination (4). In this study, we confirmed by microscopy the constant level of expression of surface factor 4 on all normal blastoconidia (Fig. 2a). This major *Candida* antigen was successfully located by immunogold staining in a glycoprotein-rich cell wall layer. We also provide evidence that the expression of surface factor 4 was dynamically altered by exposure to lysozyme. Early on, immunogold particles were concentrated in the fibrillogranular layer and the cytoplasmic membrane area was clearly delineated (Fig. 3c). Later on, either the intermediate and inner wall layer as well as the cytoplasmic membrane area were highlighted (Fig. 3d) or only the cytoplasmic membrane area was delineated with immunogold particles (Fig. 3e and f).

With prolonged exposure to lysozyme, we noted the appearance of aggregates of PA-TCH-SP-reactive glycogen-like particles in the yeast cytoplasm (Fig. 1e) and a tremendous increase in the number of stainable vicinal-glycol-reactive sites in the cell wall (Fig. 1f). These aggregates of glycogenlike particles, presumably newly synthesized wall polymers, were not demonstrated with the methods used in our previous study (7). This finding provides a clear indication of an ongoing reparative process and a highly plastic architecture of the cell wall in *C. albicans*. Previous studies demonstrated that growth conditions, starvation, and the age of culture strikingly altered the PA-TCH-SP staining of the cell wall of *C. albicans* (3, 11). Moreover, the production of the outer fibrillogranular cell wall layer was shown to be markedly influenced by the nutritional composition of the growth medium, i.e., stimulated by enrichment with galactose and inhibited by biotin deprivation (8, 18). The fact that the Thiéry reaction became strong and uniform across the wall can be explained by changes in chemical composition, i.e., preferential synthesis and deposition of reactive mannans and β-1→6-glucans over unreactive β-1→3-glucans and chitin, or a greater intermixing of these complex carbohydrates in the cell wall. We previously postulated that lysozyme could slowly hydrolyze chitobiose residues which provide N-glycosidic linkages between inner core mannans and the glycopeptide portion of mannoproteins (7). The observed disorganization and reduction in thickness of outer cell wall layers, in which mannoproteins are believed to be located (11, 12), provide evidence in favor of this hypothesis.

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