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Adherence of *Candida albicans* Germ Tubes to Plastic: Ultrastructural and Molecular Studies of Fibrillar Adhesins

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Germ tubes of *Candida albicans* produced an additional fibrillar surface layer responsible for enhanced adherence to plastic. The correlation between germination of *C. albicans* and adherence of germ tubes to a plastic matrix led us to consider the existence of germ tube-specific adhesive components involved in the attachment process. Using concanavalin A-sensitized latex microspheres, we first detected extracellular molecules on the plastic surface after removal of the adherent germ tubes. Electron microscopy confirmed that fibrils of the germ tube involved in cell-substratum interconnections were retained on the plastic surface. Cytochemistry with concanavalin A-gold labeling demonstrated that these fibrillar structures contained mannoproteins. Dithiothreitol and iodoacetamide treatment of washed plastic allowed us to further characterize these fibrillar adhesins. Through analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two components with molecular weights (MWs) of 68,000 and 60,000 were detected on the plastic surface. The 68,000-MW component appeared to be one of the major constituents of the germ tube surface layers. Biosynthetic labeling experiments performed with L-[^{35}S]methionine revealed two additional proteins: a high-MW component (greater than 200,000), and a 200,000-MW component. These four proteins, strongly labeled on the plastic surface and on the germ tube cell wall layers, were in contrast slightly labeled or even nonidentified in the culture supernatant, suggesting their involvement in germ tube adherence.

The specific attachment of microorganisms to host tissues and nonbiologic materials is mediated by surface constituents called adhesins. Contrary to adhesins of bacteria and protozoa, adhesins of pathogenic fungi, and particularly those of *Candida albicans*, are poorly documented. Invasion of the human host by *C. albicans* can occur by adherence of *C. albicans* to plastic surfaces (prostheses, catheters, prosthetic valves) (20) and then dissemination by the vascular system (21). In the last few years, several authors have determined the parameters of binding of *C. albicans* to different cell or substratum systems (8, 15, 22, 23). However, the natures of the candidal ligands which promote attachment (3, 12, 14) and of their cellular receptors (4) are not yet elucidated. Indirect evidence suggests that *C. albicans* adherence is governed by extracellular mannoproteins (13, 30) and that attachment to epithelial cells involves the protein moiety of these adhesins (3). Analysis of cell wall extracts of *C. albicans* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) demonstrated that the outer cell wall layers of *C. albicans* blastoconidia and germ tubes contain a complex array of polysaccharides, proteins, and glycoproteins (18). Some of these molecules are expressed preferentially at the surface of germ tubes (6, 19, 24–27). Since germ tubes initiate filamentous forms encountered in pathogenicity (17) and since they are associated with enhanced adherence to epithelial surfaces (7), we chose this morphological form to study the adhesive surface structures involved in the adherence process.

Here we report the development of a *C. albicans* adherence assay with plastic petri dishes and concanavalin A-conjugated latex particles to detect extracellular molecules at the sites of cell-substratum contact. Furthermore, we describe the ultrastructural localization, isolation, and partial biochemical analysis of some molecules which may play a role in the attachment of *C. albicans* to plastic. Preliminary results reported here provide evidence that proteins of *C. albicans* cell wall origin are retained on the substratum and are involved in germ tube adhesion.

MATERIALS AND METHODS

Organism and culture conditions. *C. albicans* 1066 (serotype A), originally isolated from a case of human septicemia, was used throughout. It was maintained on slopes of Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) and subcultured twice a month. Blastoconidia were prepared by subculturing in modified Lee medium (11) without amino acids and containing (in grams per liter): $(NH_4)_2SO_4$, 5; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 2.5; NaCl, 5; glucose, 10; and biotin, 0.04. After incubation for 36 h at 25°C with constant shaking, cells in the stationary phase were harvested by centrifugation and washed three times in distilled water. Germ tubes were obtained in medium 199 (Flow Laboratories, Irvine, Scotland) at pH 6.7 as described by Dabrowa et al. (5).

Adherence assay. Blastoconidia in the stationary phase were inoculated in medium 199 at a final concentration of 10^7 cells per ml and incubated for 3 h at 37°C in 35- or 94-mm-diameter biologically neutral polystyrene petri dishes (Greiner, Nürtingen, Federal Republic of Germany) containing, respectively, 2 or 15 ml of the blastoconidium suspension. In some cases, the blastoconidium suspension was placed on petri dishes as spots of 250 or 500 μl. Under these conditions, over 90% of the organisms produced germ tubes that adhered readily to the plastic surface.

Kinetic studies of germ tube formation and adherence. Germination was quantitated by counting blastoconidia bearing germ tubes every 20 min for 2 h and 20 min with a phase-contrast microscope with a graticule mounted in the focus of the ocular. For each petri dish, 10 fields (0.64 mm^2

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each) were counted, and the mean numbers of germ tubes per field ± the standard deviations were calculated. Petri dishes were then rinsed with distilled water to dislodge all the loosely adherent blastoconidia. The fungal cells which adhered to the plastic surface were counted as described above. All results were expressed as a percentage of the whole blastoconidial population.

Detection of cell wall components associated with germ tube adherence. After a 3-h incubation, germ tubes were removed from the plastic surface with a rubber policeman. Petri dishes were then exposed to a stream of distilled water at an angle of 90° from a distance of approximately 10 cm with a squeeze bottle with a tip opening of 1 mm. Petri dishes were then incubated in phosphate-buffered saline containing 0.5% bovine serum adlbumin for 1 h at room temperature. Detection of *C. albicans* adhesins on the plastic surface was done with concanavalin A (ConA; Industrie Biologique Française, Villeneuve-la-Garenne, France)-coated latex beads (0.8 μm) sensitized as previously described for fibrinogen (31). After a 30-min incubation, the excess latex beads were withdrawn by washing, and petri dishes were observed by light microscopy. Controls included native microspheres or ConA-treated microspheres and α-methyl-D-mannopyranoside (0.2 M).

Visualization of macromolecules at the sites of cell-substratum contact. Germ tubes adherent to plastic were fixed in situ with 2.5% (vol/vol) glutaraldehyde buffered at pH 7.4 with 0.1 M sodium cacodylate for 1 h, postfixed for 1 h with 1% (vol/vol) buffered osmium tetroxide, and embedded in Epon. Sections transverse or parallel to the plastic surface in the area of cell-substratum contact, stained with uranyl acetate or uranyl acetate and lead citrate, were examined with a 100 CX JEOL microscope. The same treatment was applied to other petri dishes after physical shearing of germ tubes. Adherent germ tubes removed from the plastic surface and treated with dithiothreitol (DTT) (see below) and nonadherent germ tubes obtained in glass tubes were similarly treated for electron microscopy. In some cases, before fixation, germ tubes and washed petri dishes were treated with ConA-sensitized 10-nm gold particles (31) at a 1/10 dilution in phosphate-buffered saline for 30 min at room temperature.

Biosynthetic labeling. A homogeneous population of blastoconidia was prepared by inoculating cells at a final concentration of 10^6/ml into 10 ml of modified Lee medium. After a 36-h incubation at 25°C with constant shaking, organisms in the stationary phase were pelleted and washed three times in sterile distilled water. Washed blastoconidia were then suspended in methionine-free medium 199 (pH 6.7) containing 4 μCi of L-[^{35}S]methionine per ml (specific activity, 264 mCi/mmol; Amersham Corp., Arlington Heights, Ill.). Organisms in the labeling medium were incubated in plastic petri dishes for 3 h at 37°C. Under these conditions, no modification was observed in the percentages of blastoconidia bearing germ tubes and of germ tubes adhering to the plastic surface.

Isolation of germ tube cell wall components and adhesins. Adherent germ tubes removed from plastic petri dishes and the washed petri dishes were separately treated with DTT and iodoacetamide (Fluka, Buchs, Switzerland) as described by Smail and Jones (24). Briefly, pelleted organisms were suspended in 10 volumes of 0.05 M Tris hydrochloride (pH 7.5) containing 0.8 M mannitol. Washed petri dishes were filled with this buffer (~15 ml each). DTT (0.5M) in Tris-mannitol buffer was added to a final concentration of 12 mM. After a 2-h incubation at 37°C with constant shaking, 0.5 M iodoacetamide in the same buffer was added to a final concentration of 17 mM, and, finally, all materials were left for 1 h at room temperature. DTT extracts, as culture supernatants, were dialyzed against four changes of deionized distilled water for 48 h at 4°C in dialysis tubing with an 8,000-molecular weight (MW) exclusion and finally lyophilized.

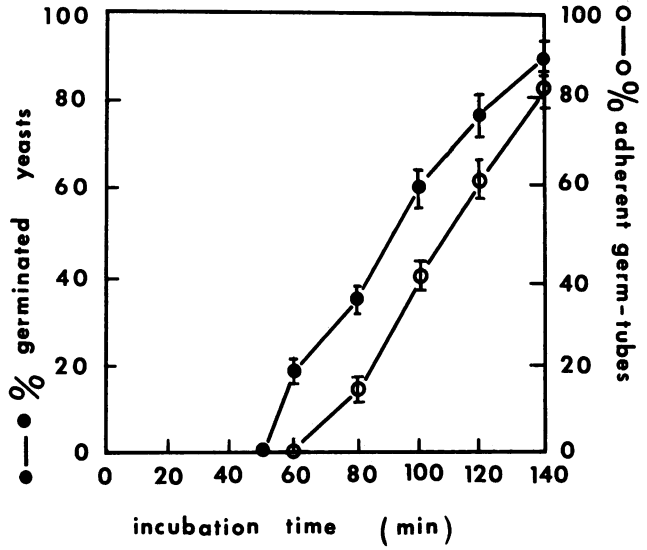

FIG. 1. Kinetics of germination (●) and adherence (○) of *C. albicans* to the plastic surface. Bars represent standard deviations.

SDS-PAGE, autoradiography, and Western blotting. Samples were dissolved in a buffer containing 62.5 mM Tris hydrochloride, 2% (wt/vol) SDS, 10% (wt/vol) glycerol, and 5% (vol/vol) 2 mercaptoethanol (pH 6.8). After being boiled for 2 min, 40 μl of each sample (75 μg of proteins or 100,000 cpm) was placed on a 1.5-mm-thick slab gel of 12.5% polyacrylamide with a 3% polyacrylamide stacking gel and electrophoresed as described by Laemmli (10). Gels were stained with Coomassie brilliant blue, and apparent protein MWs were interpolated from the migration of ferritin (MW, 220,000), phosphorylase *b* (MW, 94,000), bovine serum albumin (MW, 67,000), hen egg albumin (MW, 43,000), carbonic anhydrase (MW, 30,000), soybean trypsin inhibitor (MW, 20,000) and α-lactalbumin (MW, 14,400).

For identification of radiolabeled proteins, stained gels were treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), dried, and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for 3 days before being developed.

Transfer of proteins from gels to nitrocellulose paper and detection of the mannoproteins with ConA and peroxidase were carried out as described by Sundstrom and Kenny (26).

RESULTS

Kinetics of germ tube formation and adherence. Germination and adherence were quantitated every 20 min (Fig. 1). The first germ tubes appeared 50 min after inoculation of blastoconidia in medium 199 (pH 6.7). At 1 h, only 18% of the blastoconidia had initiated germ tube formation as compared with 90% of blastoconidia bearing germ tubes at 2 h and 20 min. These results imply great differences from one germ tube to another in the length of hyphae and, consequently, in cell wall expansion. Adherence to plastic oc-

curred readily after germination. The similar evolution of the two curves indicated that germination and adherence to plastic were closely related. Blastoconidia did not adhere or did so weakly.

Detection of germ tube components on polystyrene petri dishes. For localization of extracellular molecules at sites of cell-substratum contact, a specific probe was required. Since mannoproteins are the main components of the cell wall outer surface, we used ConA-conjugated latex particles to detect these components. Fig. 2a shows a portion of a spot of latex particles covering a plastic petri dish from which adherent germ tubes were removed. This result suggested that some mannoproteins were retained on plastic. Moreover, in these areas, latex particles appeared to be arranged in lines or patches of 4 to 10 particles (Fig. 2b) which seemed to correspond to the adherence sites of germ tubes. Control petri dishes with α-methyl-D-mannopyranoside or with native particles were free of microspheres.

Electron microscopy. Electron microscopy of thin sections of adherent *C. albicans* clearly demonstrated the presence of a fibrillar structure densely arranged all around the wall. This cell wall coat was composed of thin branched filaments (~30 nm long) arranged perpendicularly to the cell surface (Fig. 3a and b and 5). Numerous small granules appeared to be scattered on these filaments. The proliferation of this coat during adherence to plastic was evident in comparison with the cell walls of blastoconidia (Fig. 3b) or of nonadherent germ tubes obtained in glass tubes (Fig. 4), on which fibrils were not detected. Interconnections between the cell and the substratum were formed with fibrils (Fig. 5). In some areas, the cell wall was flat and closely apposed to the plastic surface. Fibrillar structures of germ tubes showed numerous ConA-binding sites (Fig. 6a and b), suggesting that they might be rich in mannoproteins, whereas blastoconidia were poorly labeled (Fig. 6a).

When germ tubes were removed, prints of cell wall material, and particularly fibrils, were identified on the plastic surface (Fig. 7) and labeled with ConA. After DTT-iodoacetamide treatment of germ tubes, all of the external flocculent layer was released, and fibrils were detected in the surrounding medium (Fig. 8).

Protein patterns of DTT extracts of germ tubes and adhesins. Since DTT treatment resulted in a release of fibrils from the outer cell wall layers of germ tubes, we studied the protein patterns of cell walls of adherent germ tubes and adhesins obtained by DTT extraction. A germ tube-DTT extract run on a 12.5% polyacrylamide slab gel had, after Coomassie brillant blue staining ~30 polypeptide chains with MWs ranging from 15,500 to 80,000 (Fig. 9A, lane 1). Among this large number of molecular species, only two components were revealed by SDS-PAGE analysis of the material extracted by DTT treatment from washed petri dishes (Fig. 9A, lane 3). One of them, with an apparent MW of 68,000, seemed to be one of the major components of the germ tube-DTT extract, and the other had an estimated MW of 60,000. These two components were not detected in the culture supernatant (Fig. 9A, lane 2). After being blotted and treated with ConA-peroxidase, the two components were labeled, demonstrating their mannoproteinaceous nature (Fig. 9A, lane 4).

Autoradiographic patterns of DTT extracts of metabolically labeled germ tubes and adhesins. Biosynthetic labeling experiments allowed us to identify two other adhesive components. In addition to the 60,000- and 68,000-MW components, two bands were revealed in the DTT extract from washed petri dishes (Fig. 9B, lane 3): a high-MW component (greater than 200,000) and a 200,000-MW component. These proteins, heavily labeled in the germ tube-DTT extract, were in contrast slightly labeled or not detected in the culture supernatant (Fig. 9B, lanes 1 and 2).

DISCUSSION

The results reported in this study show a strong correlation between germination and adherence to plastic of *C. albicans*. Other studies also reported evidence for greater adherence of germinated than of nongerminated *C. albicans* to buccal epithelial cells (7), suggesting that germ tube formation is accompanied by a rearrangement of or chemical changes in the cell wall components of the mother cell. Using electron microscopy and ConA-gold labeling, we demonstrated that germ tubes adhering to plastic develop an additional outermost fibrogranular layer containing mannoproteins, whereas this network was not detected on the surfaces of either the parent blastoconidium or nonadherent germ tubes obtained in glass tubes. Using conventional fixation and cytochemical markers, we (29) and others (2) previously found a cell wall coat and demonstrated that it was continuous from the mother cell along the germ tube. More recently, Tokunaga et al. (28), using rapid freezing techniques, recognized a similar fibrillar layer on germ tubes and blastoconidia. If our fixation technique with chemical treatment could modify the native state of the fibrillar cell wall coat, such modification should take place on the surfaces of the germ tube and the mother cell. We suppose that the fibrillar structures reflect a spatial reorganization of the glycoproteins of the cell wall coat in response to contact with plastic and that they perform a specialized function.

If fungal fibrils promote attachment to plastic surfaces, material isolated from the outermost layers of germ tubes and from plastic petri dishes must contain the fibrillar adhesins. Removal of germ tubes from petri dishes leaves superficial cell wall material at the sites of cell-substratum contact. Since the cell wall of *C. albicans* is coated with mannoproteins, ConA-treated microspheres seemed to be an excellent marker for detecting extra-cell wall molecules associated with the substratum. This method allowed us to identify some of the components of *C. albicans* origin which were associated with adherence. Further evidence was obtained by electron microscopic detection of fibrils retained on the plastic and by ConA-gold labeling, which confirmed the mannoproteinaceous nature of the fibrils.

Whereas SDS-PAGE analyses of cell wall surface components of *C. albicans* have been described (6, 18, 24, 26, 27), few of them have provided descriptions of adhesins (3, 14). In the present work, cell wall components were obtained by DTT-iodoacetamide treatment, which has been described as the most useful procedure for releasing outer cell wall components (18). This treatment allowed us to detect on the plastic surface some cell wall proteins with MWs of 60,000, 68,000, and 200,000 and a high-MW component (greater than 200,000). Contrary to the first two components, which were revealed by Coomassie brillant blue staining and by Western blotting and Con A-peroxidase staining, the high-MW and 200,000-MW components were detected only after autoradiography, a more sensitive technique. This result could be explained by the small amount of heavy components extracted.

These four components should be related to proteins previously described. Glycosylated proteins with MWs of 62,000 and 70,000 were reported to be present in pseudohypha but not yeast forms of *C. albicans* as receptors for C3d

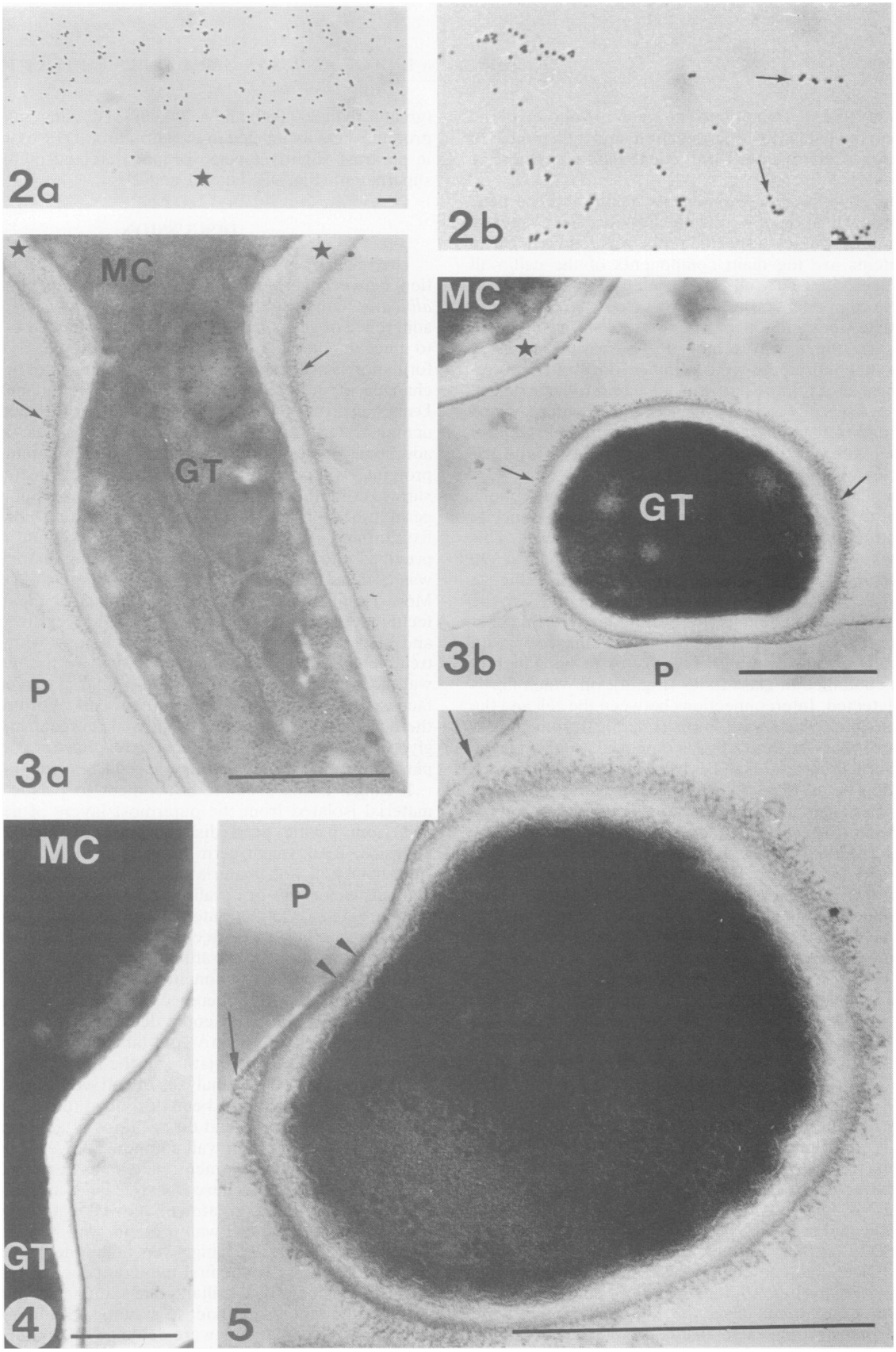

FIG. 2. Phase-contrast photomicrographs of ConA-latex microspheres binding to cell wall components retained on plastic. (a) Low-power view showing a spot of attached microspheres after the removal of germ tubes. Note the clear background around the spot (star). (b) High-power view revealing the arrangement of particles (arrows) at the sites of germ tube-substratum contact. Bars, 10 μm.

FIG. 3–5. Electron micrographs of 2-h-old germ tubes. FIG. 3. Longitudinal (a) and transverse (b) sections of a germ tube adhering to a plastic petri dish. The cell wall of the parent blastoconidium was typical, with a smooth surface (stars). Note the rough surface of the germ tube with a distinct fibrogranular layer (arrows). FIG. 4. In contrast, a nonadherent germ tube obtained in a glass tube had a uniform smooth surface, continuous from the mother cell along the germ tube. FIG. 5. High magnification of a section of an adherent germ tube. Note the interconnections between the fibrils and the substratum (arrows) and the very closely apposed surfaces (arrowheads) at the site of cell-substratum contact. GT, Germ tube; MC, mother cell; P, plastic. Bars, 0.5 μm.

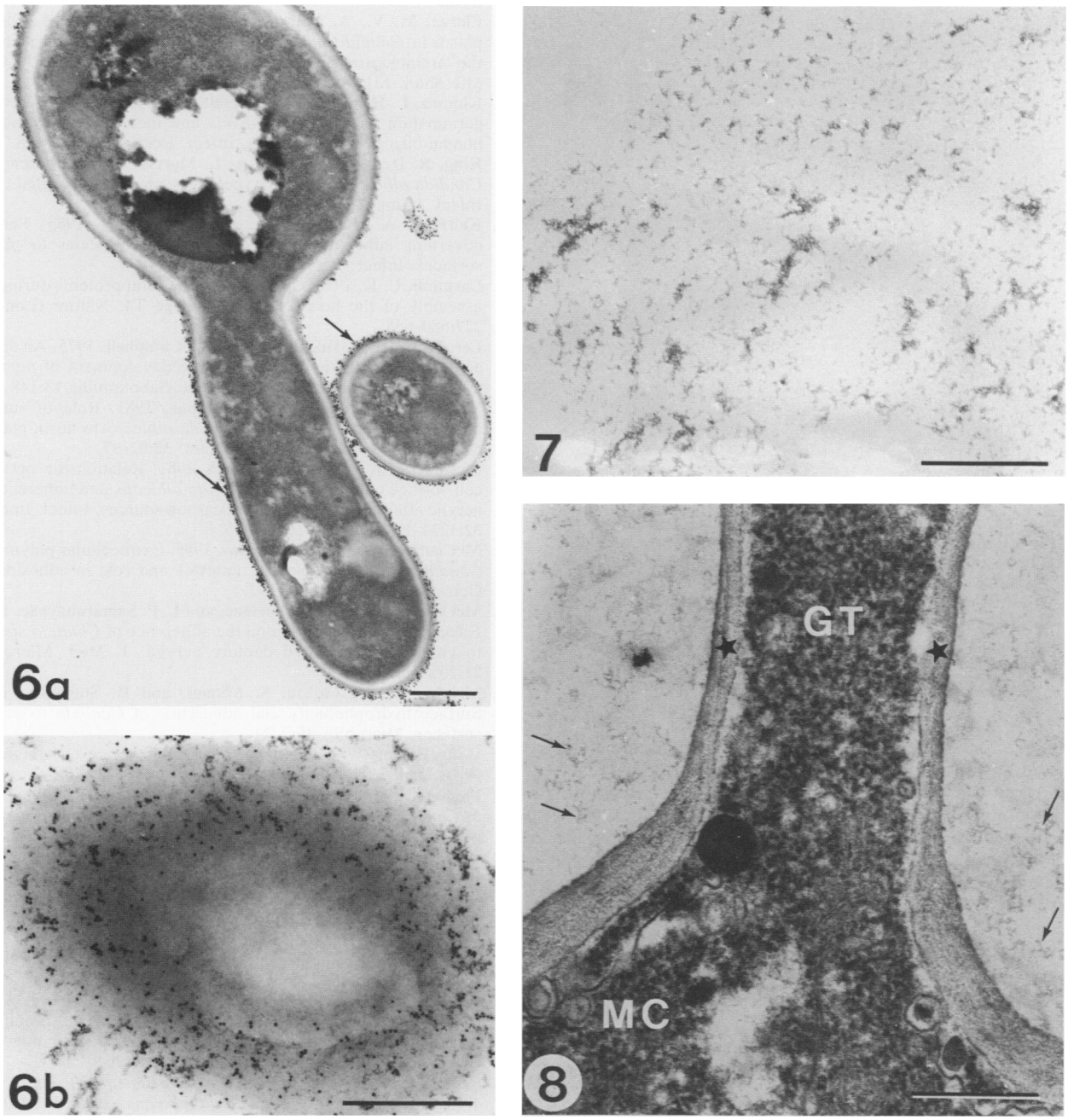

FIG. 6. Cells treated with ConA-gold showing the intense labeling of the external fibrillar layer of the germ tube (a, arrows), as compared with the parent blastoconidium. (b) Section tangential to the fibrillar cell wall coat showing the labeling of fibrillar structures. Bars, 0.5 μm.

FIG. 7. Fibrils retained on plastic at the site of germ tube-plastic contact. Compare with Fig. 5 and 8. Bar, 0.5 μm.

FIG. 8. Cell wall (stars) of a *C. albicans* germ tube after DTT treatment. The outermost fibrillar wall components were removed (compare with Fig. 5), and some fibrils appeared to be free in the medium (arrows). GT, Germ tube; MC, mother cell. Bar, 0.5 μm.

by Calderone et al. (1). A high-MW component and a 200,000-MW component were found in a germ tube-Zymolyase-DTT digest by Sundstrom and Kenny (26) and by Elorza et al. with the same Zymolyase-DTT treatment (6). These molecules were labeled with L-[^{35}S]methionine and by surface iodination (26, 27). They were mannoproteinaceous in nature, as demonstrated by their ability to bind ConA and by the endoglycosidase H sensitivity of the 200,000-MW component (27). This last component was described as a germ tube-specific antigen present in mycelial-phase extract but not yeast-phase extract (27), a fact which seems to reflect de novo synthesis of protein during germination.

It has been demonstrated that the binding of macromolecules on solid surfaces results in hydrophobic interactions (16) or in modifications of electrostatic forces (9). However, compared with the large variety of polypeptide molecules detected at the surfaces of adherent germ tubes or in culture supernatants, detection of these four components at the plastic surface implies that they have a role in the attachment process to plastic. However, the results of blocking experi-

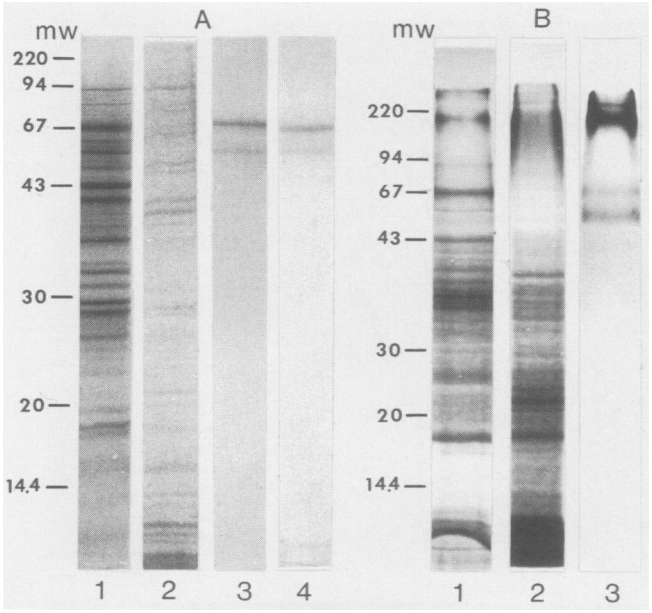

FIG. 9. SDS-PAGE electrophorograms of germ tube and adhesin-DTT extracts. (A) Lanes 1 to 3 were stained with Coomassie brilliant blue, and lane 4 was stained with ConA-peroxidase. Lanes: 1, germ tube-DTT extract; 2, culture supernatant; 3 and 4, DTT extract from washed petri dishes. (B) Results of an L-[^{35}S]methionine labeling experiment performed during germination (100,000 cpm per lane). Lanes: 1, germ tube-DTT extract; 2, culture supernatant; 3, DTT extract from washed petri dishes. MWs (mw) of standard proteins (in thousands) are shown on the left.

ments testing the effects of the adhesin extracts revealed no change in the adherence of germ tubes to plastic. This result suggests either that the adherence capacity of the fibril-adhesin complex is not due only to the proteins described here or that this capacity is altered or lost during the extraction procedures.

In conclusion, the description of these cell wall adhesins underlines the complexity of the interactions between *C. albicans* and surfaces. Owing to their possible involvement in adherence to nonbiologic surfaces, these molecules might also play a role in the induction of adherence to host cells. We are now investigating this possibility.

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