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Identification of Wall-Specific Antigens Synthesized during Germ Tube Formation by *Candida albicans*

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Walls of the two cellular forms (blastoconidia and mycelia) of *Candida albicans* ATCC 26555 were obtained from cells metabolically labeled (6-h pulse) with ^{14}C-protein hydrolysate and [^{3}H]threonine. Walls were purified by thorough washings with buffered and sodium dodecyl sulfate solutions and digested with Zymolyase 20T. The enzymatic treatment released four major high-molecular-weight mannoproteins (HMWM), with apparent molecular masses of 650, 500, 340, and 200 kilodaltons (HMWM-650, HMWM-500, HMWM-340, and HMWM-200, respectively), from yeast cells, whereas two high-molecular-mass mannoproteins (HMWM-260 and HMWM-180) were solubilized from mycelial cells. Some additional minor low-molecular-weight species were also detected in the enzymatic digests of walls from both types of cell. Single and dual pulse-chase experiments indicated that the HMWM-260 and HMWM-180 species reflect de novo synthesis of new proteins specific for the mycelia and do not represent a topological rearrangement of blastoconidium wall components. Monoclonal antibodies were raised against the HMWM-260 species (quantitatively the predominant component in the mycelial walls), and polyclonal rabbit antibodies were obtained against yeast or mycelial cell walls. Anti-mycelial cell wall polyclonal antibodies were adsorbed to whole killed blastoconidia to remove antibodies against common blastoconidium and mycelial wall antigens. Titration by enzyme-linked immunosorbent assay revealed that the monoclonal antibodies could recognize an epitope of the protein moiety of the HMWM-260 mannoprotein. Immunoblotting and immunofluorescence techniques using these monoclonal and polyclonal antibodies confirmed that the HMWM-260 and HMWM-180 species are specific components of the envelope of the mycelial cell walls.

Candida albicans grows either by budding, leading to the formation of blastoconidia, or by production of germ tubes, which give rise to continuous septate hyphae (35). Yeast-phase *C. albicans* normally colonizes skin folds, mucous membranes, and the intestinal ducts, and antibodies against yeast-phase surface antigens are ubiquitous, even in healthy individuals (13, 20, 53). However, both morphologies are found simultaneously in infected tissues (31, 42), and formation of germ tubes is thought to be important in the adherence of fungal cells to host epithelium (21, 25, 47) and the avoidance of ingestion by phagocytic cells (46). Thus, less adherence occurs when blastoconidia devoid of germ tubes are used in adherence assays (21, 22).

The cell wall of *C. albicans* not only maintains the shapes which characterize each growth form, but is also the site of the initial interaction between the organism and the environment, playing an important role in the colonization of tissues and presentation of antigens to the host (7). However, there is no consensus on the nature of the molecules in the cell wall of the fungus that are responsible for its adherence to epithelial cells (6, 8–10, 27, 41, 43, 44, 48). Since the mycelial phase is virtually always found during tissue invasion (35), detection of antigens which are expressed preferentially in germ tube cell walls (and which may be important pathogenically) could be useful in diagnosing systemic (invasive) candidiasis and in devising immunization strategies for the immunocompromised host (16, 29, 35, 39). The existence of antigenic components specific for the mycelial cell walls has been observed by different authors using distinct extraction procedures (14, 39, 45, 49, 50). However, it remains to be established whether the presence of germ tube-specific molecules indicates the de novo synthesis of new protein species, represents a topological rearrangement of blastoconidium components, or reflects quantitative differences in components of both walls (50). In any case, some of these germ tube-specific antigens have been partially characterized as high-molecular-weight mannoproteins (14, 39, 50). Their expression and topological surface localization, depending on the growth conditions, growth state, and morphology of the cells, have been determined by immunological procedures using polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) (7, 18, 49, 52).

In this study we have characterized, by means of single and dual radioactive pulse-chase experiments, antigenic high-molecular-weight mannoproteins (HMWMs) specific for the mycelial phase, which reflects protein species incorporated de novo into the cell wall of the mycelial cells. PAbs and MAbs were prepared against such antigens. An MAb reacting against the protein moiety of a mycelial-phase-specific HMWM (migrating at a position that would correspond to a protein having a molecular mass of 260 kilodaltons [kDa]) was obtained. Immunofluorescence indicated that this antigen is expressed on the surface of mycelial cells.

MATERIALS AND METHODS

Organism and culture conditions. *C. albicans* ATCC 26555 was used throughout this work. It was maintained by subculturing every 2 to 3 weeks on slopes of Sabouraud dextrose agar. The organism was propagated as blastoconidia in the minimal medium supplemented with amino acids described by Lee et al. (26). Erlenmeyer flasks (500 ml) containing 150 ml of Lee medium were inoculated with a

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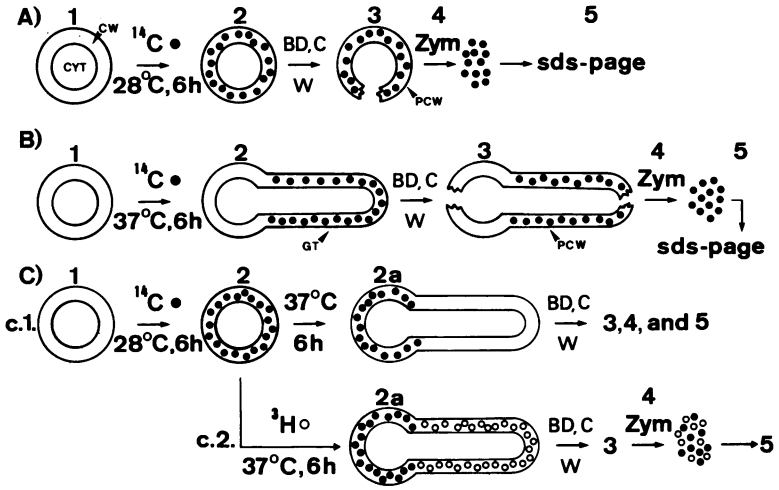

FIG. 1. Schematic diagram summarizing the different strategies used to detect and characterize cell wall components from both blastoconidia and blastoconidia bearing germ tubes (mycelium). In all experiments blastoconidia (step 1) were previously maintained for 72 to 96 h at 4°C for starvation. Starved blastoconidia were incubated at 28°C (A) or 37°C (B) for 6 h in the presence of ^{14}C-protein hydrolysate (●). After pulse, labeled cells (step 2) were broken by ballistic disruption (BD), and the cell wall fraction was purified by repeated centrifugations (C) and washings (W). Purified cell walls (PCW) (step 3) were digested (step 4) with Zymolyase (Zym), and enzyme digests were analyzed by SDS-PAGE (step 5). In part C of the scheme, blastoconidia (step 1) were previously labeled by incubation for 6 h at 28°C in the presence of ^{14}C-protein hydrolysate and starved for 72 to 96 h at 4°C. Labeled blastoconidia (step 2) were transferred to fresh medium and incubated for 6 h at 37°C in the absence (c.1) or presence (c.2) of [^{3}H]threonine (○) for secondary labeling. Single- and dual-labeled blastoconidia bearing germ tubes (mycelium) were harvested and broken by ballistic disruption, and the radioactive molecules present in the purified cell wall fraction (step 3) were solubilized by treatment with Zymolyase (step 4) and analyzed by gel electrophoresis (step 5). Other abbreviations: CW, cell wall; CYT, cytoplasm; GT, germ tube (mycelium).

loopful of cells from a 12- to 18-h-grown slant culture and were incubated in a gyratory incubator (200 rpm) at 28°C for 12 to 14 h. After the incubation period, the A_{650} was 0.6 U (exponential growth phase), as measured with a Perkin-Elmer Coleman 250 spectrophotometer. Cells were collected from the culture medium by centrifugation at 3,000 × *g* (10 min), washed twice with sterile glass-distilled water, and resuspended at a concentration of about 1 mg (dry weight) of cells per ml in sterile glass-distilled water. The suspension was incubated at 28°C for 3 h with shaking and then stored at 4°C for 72 to 96 h (starvation period). For growth experiments, starved blastoconidia were used to obtain exponential-phase cultures by inoculating them (200 μg [dry weight] of cells per ml) in fresh Lee medium at 28°C for yeast growth or at 37°C for the formation of mycelium. After 6 h at 37°C, more than 90% of the cells exhibited well-defined mycelial tubes. The protocol followed to obtain blastoconidia and blastoconidia bearing germ tubes (defined as mycelium throughout), described above, is a modification of that previously described (15).

Labeling with radioactive precursors. The scheme in Fig. 1 summarizes all the different strategies used for radioactive labeling and characterization of protein components of cell walls from both blastoconidia and mycelium.

For single radioactive labeling of proteins, starved blastoconidia were inoculated (see above) into Lee medium supplemented with 0.2 μCi of ^{14}C-protein hydrolysate (specific activity, 56 mCi [2,072 MBq] per milliatom of carbon) per ml and incubated with shaking for 6 h at 28°C for yeast growth (Fig. 1A) or at 37°C for the formation of mycelium (Fig. 1B). After the pulse, purified cell walls were obtained from labeled blastoconidia and mycelial cells. Radioactivity incorporated into walls was determined after solubilization of wall components as described below. In other experiments, starved, radioactive-labeled blastoconidia were inoculated into Lee medium and incubated at 37°C for 6 h. Samples from this culture were taken at different times, and the radioactivity initially incorporated into the walls of the blastoconidia was chased during the germ tube formation period (Fig. 1C, part c.1).

For dual radioactive-labeling experiments (Fig. 1C, part c.2), starved radioactive blastoconidia (see above) were inoculated into Lee medium supplemented with 0.2 μCi of [^{3}H]threonine (specific activity, 5 Ci/mmol [185 GBq/mmol]) per ml and incubated for 6 h with shaking at 37°C. The radioactivity (^{14}C) initially present in the cell walls, and that incorporated (^{3}H) during the second pulse, were chased at different times during the process of germ tube formation.

Cell wall preparation. Blastoconidia or mycelium were collected by centrifugation (3,000 × *g*, 10 to 15 min) and washed twice with chilled 0.001 M phenylmethylsulfonyl fluoride in 0.01 M Tris hydrochloride buffer (pH 7.2) (buffer A). Large volumes of cells were broken by suspending the cell pellet up to a final volume of 25 ml with buffer A in a Duran flask containing 25 ml of dry Ballotini glass beads (0.45 to 0.5 mm in diameter). The suspension was shaken for two periods (2 min each) at 0°C in a Braun homogenizer. The procedure resulted in complete cell breakage. Small cell samples were broken by vortexing with glass beads (5 mg of beads per mg of cells); complete cell breakage was also obtained. The cell walls were sedimented (1,200 × *g* for 10 min) from the cell-free homogenate, washed four times with chilled buffer A, then boiled for 5 min with 2% sodium dodecyl sulfate (SDS) in glass-distilled water, and finally washed four more times with chilled 0.001 M phenylmethylsulfonyl fluoride in glass-distilled water. Cell breakage was assessed by examination of the preparations in a phase-contrast microscope. Isolated walls were freeze-dried and stored at −20°C until used.

Generation of PAbs against whole cell walls of blastoconidia and mycelium. Samples (50 mg) of walls from blastoconidia and mycelia were suspended in 1 ml of sterile 0.01 M

phosphate (pH 7.4)–0.15 M NaCl (phosphate-buffered saline [PBS]), emulsified with an equal volume of complete Freund adjuvant, and injected subcutaneously into adult female New Zealand White rabbits. Immunizations (now with incomplete Freund adjuvant) were repeated weekly for three weeks, and 7 days later the rabbits were bled. The immunoglobulin G (IgG) fraction was separated from the crude serum and tested by immunoblotting and enzyme-linked immunosorbent assay (ELISA) against material extracted by Zymolyase 20T (Miles Laboratories) from purified cell walls (see below).

Germ tube-specific antiserum (mycelium-specific PAb; mPAb) was prepared by adsorption of antiserum raised against mycelial cell walls with *C. albicans* blastoconidia. The serum was adsorbed three times with heat-killed blastoconidia (6 mg [dry weight] of cells per ml of the purified IgG fraction solution) and stored at −70°C until used.

Solubilization of wall proteins by digestion with hydrolytic enzymes. Proteins were solubilized from purified walls (either from blastoconidia or mycelium) by treatment with Zymolyase 20T (10 μg of enzyme complex per 100 to 150 μg of purified, freeze-dried cell walls, in 0.001 M sodium azide and 0.01 M phenylmethylsulfonyl fluoride in 0.010 M Tris hydrochloride buffer, pH 7.4) at 28°C for 2 h. After treatment, the wall residue was removed by centrifugation (1,200 × *g*, 15 min) and discarded. The radioactivity in the supernatant was determined in 50-μl samples adsorbed to Whatman GF/C glass fiber filters which were placed in toluene-based fluid for dried samples. Measurements were made in a Beckman LS-7500 liquid scintillation counter equipped with data reduction accessory to automatically calculate counts per minute in single- and dual-label samples. The solubilized material was concentrated by freeze-drying. The total sugar and protein contents in the lyophilized material were determined, respectively, by the methods of Dubois et al. (12) and Lowry et al. (28).

Mannan purification. Mannan from *C. albicans* blastoconidia or mycelium was prepared by the procedure described by Peat et al. (38) and purified by three consecutive precipitations with Fehling solution.

PAGE. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) performed basically as described by Laemmli (24). Polyacrylamide slab gradient gels (5 to 15%; ratio of acrylamide to bisacrylamide, 30:0.2) with 3.5 to 4% polyacrylamide stacking gels were used. Two volumes of sample were mixed with 1 volume of electrophoresis sample buffer (35% glycerol, 14% 2-mercaptoethanol, 7% SDS, and 0.035% bromphenol blue as a tracking dye, in 0.5 M Tris hydrochloride, pH 6.8) and heated for 5 min in a boiling water bath prior to application onto the gels. Electrophoresis was performed at a constant current of 25 mA. The following molecular weight standards were run in parallel: thyroglobulin (330,000), ferritin (220,000), myosin (205,000), β-galactosidase (116,000), phosphorylase B (97,400), bovine serum albumin (66,000), catalase (60,000), ovalbumin (45,000), lactate dehydrogenase (36,000), pepsin (34,700), carbonic anhydrase (29,000), trypsinogen (24,000), and β-lactoglobin (18,400). Gels were stained for protein by binding of Coomassie blue. For fluorography the gels were treated with Amplify (Amersham), dried, and exposed for adequate periods of time on Kodak X-Omat films at −70°C.

Western blot (immunoblot) techniques. After SDS-PAGE, the proteins were transferred to nitrocellulose paper by using an electroblotter (Bio-Rad Trans-Blot cell with an LKB 2197 power supply) essentially as described by Burnette (4), except that the transfer buffer used was 0.025 M Tris–0.192 M glycine (pH 8.3) plus 20% (vol/vol) methanol, and the electrophoretic transfer was accomplished at 6 to 8 V/cm for 15 to 17 h at 4°C. Proteins were immunodetected by following the protocol described in the Bio-Rad Immun-Blot (GAR-HRP) assay kit based on the procedure of Burnette (4) and Towbin et al. (54), using the specific antibody at a final concentration of 1:500 (for PAbs) or 1:3,000 (for MAbs; see below) in 0.010 M Tris hydrochloride buffer (pH 7.4) containing 3% bovine serum albumin as a blocking agent, 0.9% NaCl, and 0.05% Tween 20. Diluted (1:2,000), peroxidase-labeled goat anti-rabbit or goat anti-mouse IgG (Bio-Rad) was used as an indicator antibody, with 4-chloro-1-naphthol as the chromogenic reagent. Concanavalin A (ConA) staining of nitrocellulose blots was conducted by the method of Hawkes (17) with the modifications of Millette and Scott (33).

Purification of a mycelial cell wall antigen. Walls from blastoconidia bearing germ tubes were digested with Zymolyase 20T as described above. After treatment, the wall residue was removed by centrifugation (1,200 × *g*, 15 min), and the supernatant which contained the solubilized molecules was concentrated by freeze-drying. The solubilized material was suspended in 1 ml of glass-distilled water, mixed with 0.5 ml of electrophoresis sample buffer (see above), heated for 5 min at 100°C, and subjected to SDS-PAGE on 5 to 15% slab gradient gels (1.5 mm thick), with a 3.5% acrylamide stacking gel. The stacking gel had a large well (11.5 cm wide by 3 cm high), which permitted loading of up to 5 ml of sample solution, and a small reference well (0.8 cm wide by 3 cm high) formed to one side of the resolving gel slab. Radioactive ^{14}C-labeled walls were also digested with Zymolyase (see Fig. 1B), and the solubilized material was subjected to electrophoresis. The unlabeled sample solution (1.5 ml), containing ca. 100 mg (dry weight expressed as total sugar content) of Zymolyase-solubilized material, was loaded into the large well of the stacking gel, and 200 μl (50,000 cpm) of labeled sample was added to the reference well. Both samples were run in parallel. The gel was treated for fluorography and exposed for 3 days on Kodak X-Omat film. A transverse section of the dried gel, corresponding to a polypeptide specific for the mycelial cell walls (see Fig. 2, lane 2, band d; M_r of 260×10^3) as revealed by the developed fluorographic film (this HMWM released from the cell wall by Zymolyase was barely stained by Coomassie blue), was cut out, rehydrated in acetic acid-methanol-water (10:30:60, by volume), and crushed, and the material was electroeluted (32). The purified material was concentrated by freeze-drying and suspended in PBS. The concentration of material in this solution was given as the content of total sugar per volume unit. The purity and mannoproteic nature of the isolated 260-kDa species was assessed by SDS-PAGE, blotting to nitrocellulose paper, and ConA staining.

Immunization and production of hybrid cells. Adult male BALB/c mice were immunized by intraperitoneal injections of 100 μg (expressed as total sugar content) of the purified 260-kDa mannoprotein in 0.1 ml of PBS, which was emulsified with an equal volume of complete Freund adjuvant. This procedure was repeated twice at 2-week intervals. Seven days after the third immunization, samples of blood were obtained, and the titer of serum antibody was determined by ELISA (see below). The mice were boosted with 25 μg of mannoprotein without adjuvant 3 days before they were sacrificed.

Cell fusion and selection of hybrids were performed essentially as described by Dippold et al. (11). Spleen cells from an immunized mouse were fused with $P_3 \times 63/Ag8.653$

mouse myeloma cells at a 10:1 ratio in 1 ml of fusion medium for 2 min at 37°C. Ten days after the fusion, samples of media from wells with growing hybridomas were screened for the production of antibody against the antigen. Cells from positive wells were subcloned by limiting dilution in 96-well Nunclon plates. Mouse peritoneal macrophage cells (ca. 2×10^5 per well) were used as a feeder layer. Ascites fluid was obtained by intraperitoneal injection of 10^7 hybridoma cells into pristane (2,6,10,14-tetramethylpentadecane [Sigma])-primed BALB/c mice. After 7 to 14 days, the ascites fluid was recovered with a sterile needle. MAbs were obtained either from hybridoma cultures or from ascites fluid. MAbs were purified from ascites fluid by precipitation with 50% ammonium sulfate, followed by chromatography on protein A-agarose (Sigma). The immunoglobulin class of antibodies was determined with the radial immunodiffusion plates system from Serotec Ltd.

ELISA. ELISA was performed as described by Voller et al. (55), with the following modifications. Wells of Nunc-Immunoplate I (A/S Nunc) plates were coated with 25 μg (expressed as total sugar) of antigen (mannan or material solubilized by Zymolyase from both morphogenetic forms of *C. albicans*) in 50 μl of 0.030 M sodium carbonate buffer (pH 9.6) and incubated at 4°C for 18 h. The plate was rinsed three times with PBS containing 1% bovine serum albumin and 0.05% Tween 20 (PBSBT). Germ tube-specific PAbs or MAbs from culture media from positive wells with growing hybridomas or from ascites fluid (see above) were diluted with PBSBT and added to the appropriate well, and the plate was incubated at 37°C for 1 h in a moist chamber. The wells were then rinsed three times with PBSBT, and 50 μl of a 1:100 dilution in PBSBT of goat anti-mouse (for MAb) or anti-rabbit (for PAb) polyvalent immunoglobulins conjugated to peroxidase (Sigma) was added to each well. After incubation for 1 h at 37°C, the wells were rinsed with PBSBT. After the addition of substrate mixture containing *o*-phenylenediamine, the plate was incubated in the dark for 10 min, and the reaction was stopped by the addition of 25 μl of 3 M H_2SO_4 to each well. The color intensity was determined at 492 nm with an automated plate reader (Easy Reader EAR 400; Institute Pasteur Laboratories).

Indirect immunofluorescence for detection of cell surface antigens. The immunofluorescence assay was carried out basically as described by Sundstrom and Kenny (49). Organisms were washed twice in PBS, fixed for 10 to 20 min with 0.5% Formalin in PBS, and stored in this suspension until used. The cell concentration was adjusted to 10^6 cells per ml by addition of PBS, and drops from this suspension were placed on the wells of microscope Microslides (BioMerieux) and allowed to air dry. Drops (10 μl) from different antiserum dilutions in PBS (dilutions of ascites fluid in the case of MAb [alternatively, undiluted culture supernatants were also used], or dilutions of the blastoconidia-adsorbed PAb against cell walls from mycelium) were dropped over the antigen. Microslides were placed in a moist chamber at 37°C for 30 min and then washed in two changes of PBS for a total of 15 min. Fluorescein isothiocyanate-conjugated goat anti-rabbit or anti-mouse IgG (10 μl; Ortho-Diagnostics Systems) was added to slides, which were incubated again at 37°C for 30 min in a moist chamber, washed with PBS as above described, rinsed with glass-distilled water, and mounted in PBS containing 90% glycerol. The cells were examined with a Zeiss Photomicroscope III equipped for epifluorescence (UV filter no. 487702 [excitation line, 365/366 nm]). Fluorescence was dependent on the reaction of the cells with PAb or MAb, since no fluorescent cells were observed if cells were incubated only with the second antibody.

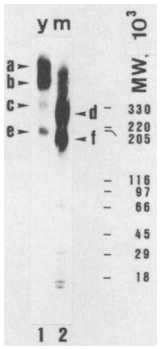

FIG. 2. Proteins extracted by Zymolyase from blastoconidium (lane 1) and mycelial (lane 2) cell walls (see legend of Fig. 1, parts A and B, for further experimental details). Solubilized molecules were analyzed by SDS-PAGE (in 5 to 15% acrylamide slab gradient gels) and fluorography (30,000 cpm per well). Major proteins extracted from the walls of yeast (y) and mycelial (m) cell walls are indicated by bold arrowheads and letters. Molecular weights (10^3) of standard proteins are listed to the right of the figure.

Miscellaneous. Gel electrophoresis and blotting reagents were from Bio-Rad. SDS-molecular weight markers were from Sigma Chemical Co. and Pharmacia. Culture medium compounds and complete and incomplete Freund adjuvants were purchased from Difco. ^{14}C-protein hydrolysate and [^{3}H]threonine were obtained from Amersham International. Unless specifically indicated, all other chemicals were from Sigma Chemical Co.

RESULTS

Identification of blastoconidium and mycelial wall antigens in radioactive ^{14}C-labeled walls. Purified walls obtained from blastoconidia and mycelial cells that had previously been grown in the presence of ^{14}C-protein hydrolysate to label the protein moiety of their mannoproteins, following the experimental protocol summarized in Fig. 1 (parts A and B), were enzymatically digested, and the solubilized material was analyzed by SDS-PAGE and fluorography. Four major highly glycosylated HMWMs with molecular masses of 650, 500, 340, and 200 kDa were solubilized by the glucanase complex from cell wall preparations of blastoconidia (Fig. 2, lane 1), whereas two major HMWM species with molecular masses of 260 and 180 kDa, along with a polydisperse material with a mass higher than 400 kDa, were detected in the enzyme digests from mycelial walls (Fig. 2, lane 2). The mannoprotein nature of the molecules solubilized by Zymolyase was confirmed by ConA staining of nitrocellulose blots (Fig. 3C). These observations were in agreement with those previously reported (14).

Identification of blastoconidium and mycelial cell wall antigens during germ tube formation. To ascertain whether the HMWM-260 and HMWM-180 species detected in the Zymolyase digests from isolated mycelial cell walls (Fig. 2, lane 2) reflect the de novo synthesis of new proteins specific for the wall of mycelial cells, and to determine whether changes in the mannoprotein pattern (HMWM-650, -500, -340, and -200 species) associated with the blastoconidium wall structure (Fig. 2, lane 1) occur during the process of germ tube formation and elongation, single and dual radioactive pulse experiments were done.

In the first set of experiments (Fig. 1C, part c.1), starved blastoconidia previously labeled with ^{14}C-protein hydroly-

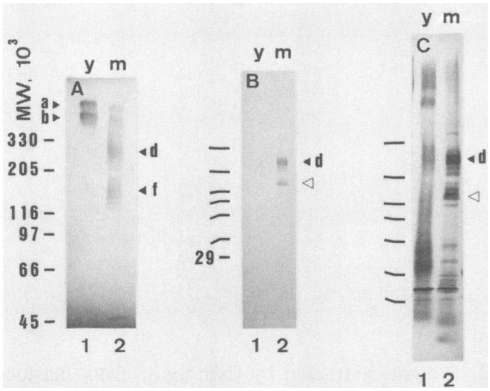

FIG. 3. Western blots of 5 to 15% slab gradient gels loaded with Zymolyase-released material from blastoconidia (y) or from mycelial cells (m; from starved blastoconidia grown for 6 h at 37°C), stained either with blastoconidium-adsorbed (germ tube-specific) PAbs (A), with MAbs raised against the HMWM-260 species specific for the mycelial cell walls (B), or with ConA (C), as described in Materials and Methods. Molecular weights (10^3) of standard proteins are indicated to the left of each panel.

sate were incubated at 37°C in Lee medium. At different times (0, 3, and 6 h), similar volumes of the culture were withdrawn and the enzymatically released wall proteins were analyzed by SDS-PAGE and fluorography. The pattern of major mannoproteins (HMWMs) associated with the cell walls of blastoconidia growing at 28°C (Fig. 2, lane 1) remained unmodified during the 72-h starvation period to which blastoconidia were subjected before being incubated under germination conditions (Fig. 4, lane 1), as well as during germ tube formation (Fig. 4, lanes 2 and 3). This observation suggests that the HMWM-650, -500, -340, and -200 species of the blastoconidium cell wall are not subjected to a detectable turnover rate, at least under our experimental conditions.

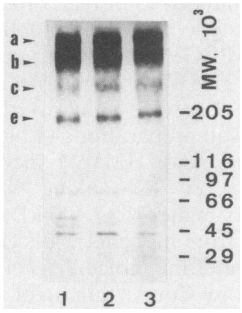

FIG. 4. Cell wall digests (Zymolyase) from mycelium originated from ^{14}C-labeled blastoconidia that were transferred to fresh medium and incubated at 37°C to induce germ tube formation (see Fig. 1, part c.1). At different times samples of cells were withdrawn, and the radioactive proteins present in the cell wall fraction were analyzed by SDS-PAGE and fluorography (30,000 cpm per well). Lane 1, Cell wall digest from blastoconidia grown at 28°C for 6 h in the presence of ^{14}C-protein hydrolysate and subjected to a 72-h starvation period in sterile distilled water as described in Materials and Methods (time zero). Lanes 2 and 3, Radiolabeled cell wall digest from blastoconidia bearing germ tubes after incubation at 37°C for 3 h (lane 2) or 6 h (lane 3). Mycelium formation was assessed by phase-contrast microscopy; by 1.5 h of incubation at 37°C, about 85% of the cells showed a well-defined germ tube, which gave rise to mature hyphae by 3 to 6 h. Molecular weights (10^3) of standard proteins are listed to the right of the gel.

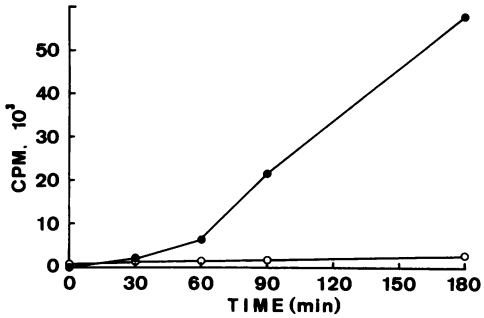

FIG. 5. Incorporation of [^{3}H]threonine (●) into material enzymatically solubilized from walls of mycelia obtained from blastoconidia previously labeled with ^{14}C-protein hydrolysate (○; see Fig. 1, part c.2, for further experimental details). At different times, samples of cells were withdrawn and the radioactivity (for both isotopes) present in Zymolyase digests obtained from the purified cell wall fraction was determined. Values shown are with reference to cells (cell walls) present in 1 ml of culture suspension.

For dual-pulse experiments (see Fig. 1C, part c.2), 72-h-starved, radioactively labeled (^{14}C) blastoconidia (see above) were incubated at 37°C in Lee medium containing [^{3}H]threonine to induce germ tube formation. Samples of the culture were withdrawn at different times, and purified cell wall preparations were obtained. Walls were digested with Zymolyase, and the ^{14}C- and ^{3}H-labeled molecules released were analyzed. The ^{3}H/^{14}C ratio in the solubilized material rose from 0 to about 20 at the end of the second pulse period (Fig. 5), thus indicating that newly synthesized protein molecules were incorporated into the cell wall structure during germ tube formation and elongation. The increase in the amount of ^{14}C label observed in the enzymatic digests after 3 h of incubation at 37°C (twofold with respect to the basal value measured at the beginning of the second pulse with [^{3}H]threonine [time 0; Fig. 5]) might represent proteins synthesized during the metabolic labeling of blastoconidia with ^{14}C-protein hydrolysate at 28°C, which were incorporated into the wall structure after the switch to germination conditions.

The material solubilized by Zymolyase from dual-labeled cell walls was analyzed by SDS-PAGE and fluorography. To detect components that might reflect the de novo synthesis of new protein species (specific for germ tubes) during the pulse with [^{3}H]threonine at 37°C, wall preparations were obtained from samples of cells taken at the indicated times (Fig. 6). The enzymatic wall digests were suspended in equal volumes of 0.010 M Tris hydrochloride buffer (pH 7.4). Appropriate volumes of these suspensions, containing constant ^{14}C radioactivity and increasing amounts of ^{3}H-labeled components, were subjected to SDS-PAGE, and the molecules were revealed by fluorography. By 1.5 h, two new radioactive-labeled polypeptides with molecular masses of 260 and 180 kDa (Fig. 6, bands d and f) were detected in the enzymatically released material, along with the HMWM species found specifically in the cell wall from blastoconidia (Fig. 6, bands a, b, c, and e). These mannoproteins had the same electrophoretic mobility as the two major HMWM species found in walls of mature hyphae (Fig. 2, lane 2). Since the same amount of ^{14}C radioactivity was loaded into each well, the increase noticed in the intensity of the HMWM specific to the blastoconidium cell wall could be due to one of two reasons: either (i) these species are also synthesized and incorporated into the wall structure, even under conditions that induce germ tube formation (compare

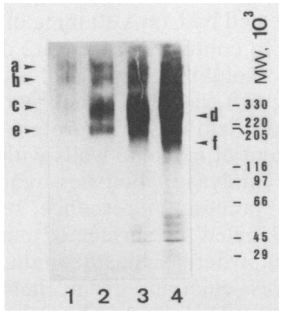

FIG. 6. Cell wall digests (Zymolyase) from mycelium originated from ^{14}C-labeled blastoconidia that were transferred to fresh medium and incubated at 37°C to induce germ tube formation in the presence of [^{3}H]threonine (see Fig. 1, part c.2). At different times, samples of cells were collected and the radioactive-labeled proteins present in the cell wall fraction were analyzed by SDS-PAGE and fluorography (constant ^{14}C radioactivity [10,000 cpm] was loaded into each well). Lane 1, Cell wall digest from blastoconidia grown at 28°C for 6 h in the presence of ^{14}C-protein hydrolysate at the beginning of the second pulse period (time zero control). Lanes 2 through 4, radiolabeled cell wall digest from blastoconidia with germ tubes after incubation at 37°C in the presence of the second radioactive precursor (^{3}H) for 1 h (lane 2), 1.5 h (lane 3), or 3 h (lane 4). Ratio of ^{3}H to ^{14}C loaded into each well: lane 1, 0; lane 2, 5; lane 3, 12; lane 4, 24. Mycelium formation was assessed by phase-contrast microscopy, as described for Fig. 3. Molecular weights (10^3) of standard proteins are listed to the right of the gel.

lanes 2, 3, and 4 with lane 1 in Fig. 6), or (ii) the increase in intensity is only apparent because of a partial overlapping of the two ^{3}H-labeled mycelia-specific HMWM species with the HMWM species from blastoconidia (previously labeled with ^{14}C), due to the polydisperse character of all these mannoproteins (13).

Generation of mycelium-specific polyclonal antisera. Antisera against cell walls from blastoconidia or mycelium (blastoconidia with germ tubes) reacted with whole yeast or mycelial organisms when tested by immunofluorescence (not shown). Antiserum which recognized only mycelial forms (mPAb; Fig. 7B) was obtained by adsorption of antiserum against mycelial walls with heat-killed blastoconidia (see Materials and Methods). The blastoconidia either were not stained or showed slight fluorescence after reaction with mPAb (Fig. 7B, arrows). The titer of this antiserum was found to be 1:1,000 when tested by immunoblotting (Fig. 3A), or 1:200 by immunofluorescence (Fig. 7B). Adsorption with blastoconidia did not completely remove antibodies against blastoconidium wall components, since the mPAb still reacted with the HMWM-650 and HMWM-500 mannoprotein species present in the cell wall of blastoconidia (Fig. 2, lane 1, bands a and b) when tested by immunoblotting (Fig. 3A, lane 1, bands a and b). The HMWM-650 and HMWM-500 species were also detected after reaction of mPAb with mycelial tube wall extracts (Fig. 3A, lane 2), yet the intensity of the binding reaction was low. However, the mPAb preparation contained immunoglobulins specific to the HMWM-260 and HMWM-180 species from mycelial cell walls (Fig. 2, lane 2, bands d and f), as revealed by immunostaining (Fig. 3A, lane 2, bands d and f).

Mycelium-specific antiserum (mPAb) was also tested by ELISA against both enzymatically released material and mannan from mycelial cell walls. Although mPAb reacted with both antigens, the intensity of the reaction was about 100 to 150% higher (A_{492}, 1.87 versus 0.82) with the enzymatic digest as antigen than with the mannan preparation, using (in both cases) the same amount of antigen (20 μg/ml, expressed as total sugar) against a 1:1,000 dilution of the mPAb serum.

Isolation of a hybridoma producing an antibody against the HMWM-260 species from mycelial cell walls. An IgG1 MAb (4C12) was isolated which was specific for the material released by Zymolyase from mycelial cell walls, but did not react with the homologous enzymatic digest from blastoconidia or with mannan that was chemically purified from blastoconidia or mycelium, when tested by ELISA (Fig. 8). By immunoblotting, MAb 4C12 recognized the HMWM-260 species (Fig. 3B, lane 2, band d), along with a minor band with an apparent molecular mass of 180 kDa (Fig. 3B, lane 2, open arrowhead), among all the mannoproteins present in

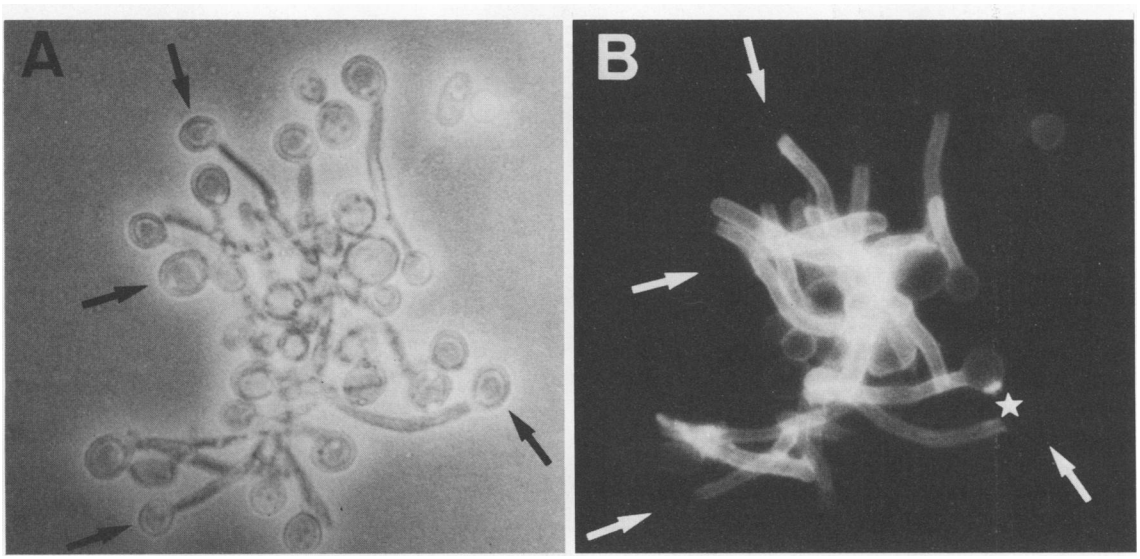

FIG. 7. Phase-contrast (A) and immunofluorescence (B) photographs of filamentous growth of *C. albicans* ATCC 26555 stained with blastoconidium-adsorbed (germ tube-specific) antiserum (1:100 dilution); arrows point to the location of blastoconidia that exhibited no fluorescence under UV illumination. Magnification, ×800.

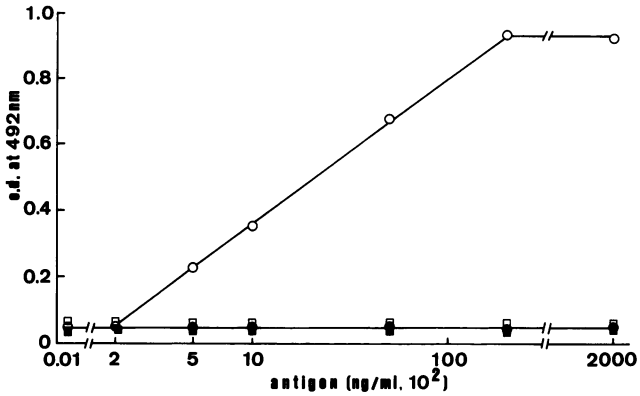

FIG. 8. Standard curve generated by ELISA (shown on semilogarithmic scale) with the MAb raised against the 260-kDa mannoprotein (HMWM-260; see Fig. 2, band d) purified from mycelial cell walls (see text), using various concentrations of mannan from blastoconidia (■) or mycelium (□) and of material released by Zymolyase from cell walls of blastoconidia (●) or mycelium (○).

the sample as revealed by ConA staining of the Western blot (Fig. 3C, lane 2). In contrast, MAb 4C12 did not react with the enzymatically solubilized material from cell walls of blastoconidia (Fig. 3B, lane 1), although this material contained a large assortment of mannoprotein species (Fig. 3C, lane 1); pretreatment of mycelial walls with pronase prior to digestion with Zymolyase abolishes reactivity (data not shown). Indirect immunofluorescence revealed that the MAb specifically labeled the surface of mature hyphae (Fig. 9D, thick arrows), whereas blastoconidia and short germ tube-like structures emerging from them (Fig. 9D, thin arrows) exhibited no fluorescence.

DISCUSSION

The identification of antigens expressed on the surface of the mycelial-phase cells of *C. albicans* should provide the basis for a better understanding of the cell wall structure and biogenesis and its role in biological functions such as host recognition of the fungus, changes in antigenicity, and pathogenesis (1, 3, 5, 7, 40, 52).

We have extended previous observations from our laboratory on two major mannoproteic components (HMWMs)

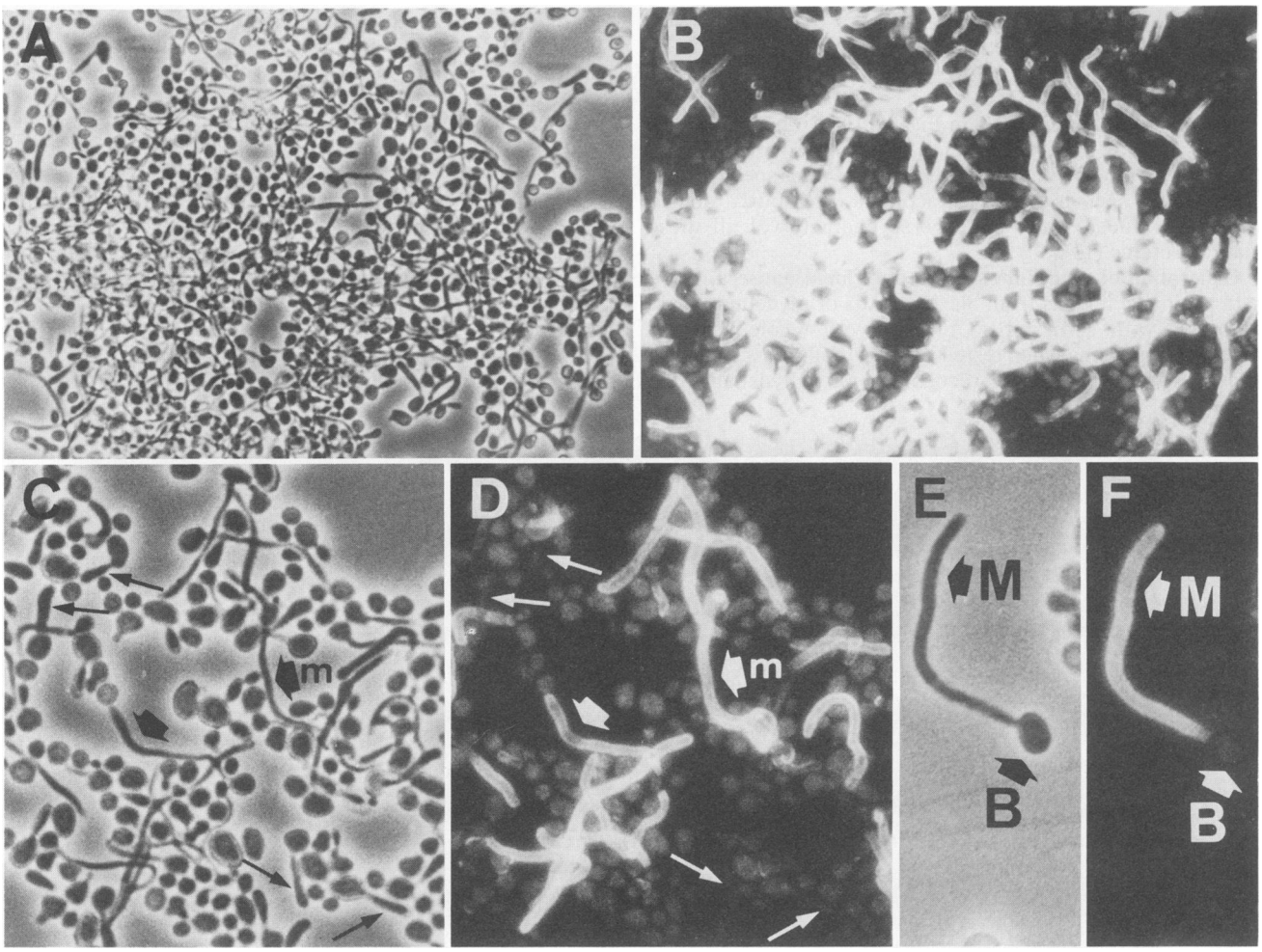

FIG. 9. Phase-contrast microscopy (panels A, C, and E) and immunofluorescence (panels B, D, and F) of intact filamentous cells of *C. albicans* ATCC 26555, stained with an MAb (1:400 dilution) raised against the HMWM-260 species specific to the mycelial cell walls. Thin arrows in panels C and D point to short germ tube-like structures that do not stain with the MAb, which, in contrast, strongly binds to mature hyphae (m; thick arrows). Magnification: Panels A and B, ×295; panels C and D, ×590; panels E and F, ×740. M, Mycelium; B, blastoconidia.

of the mycelial-phase walls, which have an electrophoretic mobility corresponding to apparent molecular masses of 260 and 180 kDa (HMWM-260 and HMWM-180), that were not detected in blastoconidium cell walls. These mannoproteins were previously characterized as molecular species with molecular masses of 160 and 205 kDa by using non-gradient acrylamide gel slabs (14). By gel chromatography they behave as a highly polydisperse population of mannoproteins with molecular masses ranging from higher than 600 kDa down to about 150 kDa (13). Results presented here confirmed previous studies (14, 39, 45, 49–51) indicating the existence of components expressed preferentially in the mycelial walls of *C. albicans*.

The fact that specific mannoproteins are expressed during germ tube formation suggests that changes in cell surface (cell wall) molecules may be related to differences in wall architecture and properties. However, it remains to be established whether the presence of mycelial-phase-specific wall components (i) reflects differences in the carbohydrate moiety of mannoproteins of the two growth phases, (ii) indicates quantitative differences in components of both (blastoconidium and mycelium) phases, (iii) represents a topological rearrangement of blastoconidium components, or (iv) reflects the de novo synthesis of new protein species (50, 51). In support of the last hypothesis, we have shown by means of dual-label experiments that the HMWM-260 and -180 species of mycelial walls represent new components, which are synthesized and incorporated into the cell wall structure concomitantly with germ tube formation. In addition, the mannoprotein composition of the cell wall of the blastoconidia phase (HMWM-650, -500, -340, and -200 species) remains unaltered during morphogenetic change. This observation is in agreement with our previous reports indicating that in another yeast species (*Saccharomyces cerevisiae*) intrinsic wall mannoproteins are stable for long periods of time (36, 37).

Analysis of the material released by Zymolyase suggested that the yeast-phase wall HMWMs are also present as intrinsic (but minor) components of the mycelial walls. This suggestion was reinforced by the observation that the mPAb (see Methods) still reacted, when tested by immunoblotting, with components of mycelial-phase walls. These components have an electrophoretic mobility similar to that of the major HMWM species found in blastoconidium walls (HMWM-650 and HMWM-500). The importance of these observations has to be stressed here because the existence of common antigens for both yeast and mycelial walls, along with the possibility that several human serum proteins and mannoproteins may share determinants with *C. albicans* antigens (30), is an additional factor contributing to the difficulty in distinguishing, by means of immunological techniques, colonization from invasive (systemic) disease (19, 23).

By indirect immunofluorescence with the mPAb, specific staining of hyphae was observed, whereas no fluorescence was observed in blastoconidia, thus indicating that germ tube-specific antigens are associated with the cell surface of filamentous growth. Nevertheless, sharply defined sectors of the surface in some (5 to 10%) of the blastoconidia showed strong fluorescence (Fig. 7B, star), suggesting that germ tube antigenic determinants recognized by the mPAb may be present at the cell surface even before germ tubes are morphologically identifiable. A similar observation has been reported by Sundstrom and Kenny (49), and the possibility exists that accumulation of these molecules points to the place where the germ tube will emerge. Interestingly, the mPAb contained antibodies directed against the two major HMWM species (HMWM-650 and HMWM-500) of the yeast-phase walls, as revealed by immunoblotting. Hence, the absence of fluorescence in blastoconidia might be due to the fact that (i) the immunodetection techniques employed in each case (immunoblotting versus immunofluorescence) have different sensitivity levels, or (ii) antigenic determinants in the HMWM-650 and HMWM-500 species could be masked by some other cell wall molecules (the HMWM-340 and HMWM-200 species?).

The properties of MAbs make them useful tools for examining the localization and characteristics of the antigenic determinants that are present on the surface of cells. In this study, a MAb (4C12) against the HMWM-260 species enzymatically solubilized from mycelial-phase walls was obtained. MAb 4C12 was of the IgG1 class; it did not react at all with a mannan fraction obtained from either yeast- or mycelial-phase cells, or with Zymolyase digests from *S. cerevisiae* or yeast-phase *C. albicans* isolated walls, although it strongly reacted with material solubilized by Zymolyase from mycelial-phase cell walls. In contrast to several studies involving production of MAb against cell wall extracts of *C. albicans*, which suggest that carbohydrates are the principal antigenic determinants on *Candida* cell surfaces (1–3, 7, 34, 40, 52), our observations indicated that MAb 4C12 could be directed against the peptide moiety of the mannoprotein. The procedure we employed to purify the antigen did not apparently alter the immunodeterminants present in the native molecule, since the MAb reacted with the isolated HMWM-260 species by immunoblotting (denatured antigen), and it specifically labeled the surface of intact mycelial-phase cells (native antigen) when tested by immunofluorescence. In any case, the results shown here are more in line with other reports suggesting that germ tube-specific epitopes are the result of new protein determinants, and not the consequence of a modification of the preexisting carbohydrate structure (49–51). Additional evidence reinforcing this suggestion was the observation that the intensity of the reaction (tested by ELISA) of the mPAb was 1- to 1.5-fold higher with the material enzymatically solubilized from mycelial walls as antigen than with the mannan preparation.

Immunofluorescence staining with MAb 4C12 suggested that expression of HMWM-260 was dependent on the morphology of the cells. Thus, no fluorescence was observed in blastoconidia or in short germ tube-like structures, whereas the HMWM-260 species appeared to be present on the surface of long mycelial filaments. Nevertheless, the lack of any reactivity of the MAb towards blastoconidia by immunofluorescence, in contrast to that occasionally observed in blastoconidia prior to germ tube formation upon reaction with the mPAb (see above), may indicate either the lack of the component containing the determinant in the yeast-phase walls, or the different orientation of the component at the germ tube emergence site. By immunoblotting, MAb 4C12 reacted with its homologous antigen (the HMWM-260 species), yet at least two other mannoproteins (as revealed by their reactivity towards ConA in nitrocellulose blots) were recognized by the MAb. These species had an electrophoretic mobility that would correspond to proteins having apparent molecular masses of 180 to 185 kDa, that is to say, the same as the other mycelium-specific mannoprotein (HMWM-180). The origin and nature of these mannoproteins sharing one common immunodeterminant with the HMWM-260 species are unknown, but they may represent cleavage products of the latter or differences in the amount (and type of linkages?) of carbohydrate bound to similar protein moi-

eties. In any case, further characterization of the antigenic determinants present in the HMWM-260 molecule, along with analysis of the occurrence of this and other related mannoproteins, such as the HMWM-180 species (and preparation of MAbs against these species), in different *C. albicans* strains (either from culture collections or clinical isolates), is currently being carried out in our laboratory in order to get new clues that allow a better understanding of the immunogenic surface of this fungus.

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