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Identification of Two Germ-Tube-Specific Cell Wall Antigens of *Candida albicans*

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Outer cell wall layers of intact yeast- and mycelial-phase *Candida albicans* B311 were extracted with dithiothreitol. Antisera against mycelial-phase organisms were absorbed with yeast-phase organisms or yeast-phase extract and used to stain Western blots of sodium dodecyl sulfate-polyacrylamide gels loaded with yeast- and mycelial-phase extracts. Autoradiography of gels loaded with extracts from organisms surface labeled with ^{125}I was used to detect surface antigens containing proteins. Antigen bands of interest identified in Western blots were cut from the blots and used to immunize rabbits. Two antigens were identified in the mycelial-phase extract which were not present in the yeast-phase extract. The first was a 19-kilodalton protein that was present in the cell walls of germ tubes but was not expressed on their surfaces. The second was a polysaccharide-rich high-molecular-weight antigen which was expressed on the surface of the germ tube. Treatment of mycelial-phase extract with protease and endo-β-*N*-acetylglucosaminidase H demonstrated that this antigen was composed of polysaccharides linked through di-*N*-acetylchitobiose groups to proteins.

The ability of the fungus *Candida albicans* to alter its cells from ovoid, budding blastocondia (yeast phase) to continuous, septate hyphae (mycelial phase) is of interest for several reasons. First, the morphological conversion constitutes a useful experimental model of developmental change in a relatively simple eucaryote. Second, short hyphal elements (germ tubes) may be important in the adherence of organisms to the host epithelium (4, 6, 9). Finally, mycelial-phase organisms are found in infected tissues (3).

Information about the identity and distribution of antigens in the outer cell wall layers of germ tubes could be important in determining the structures responsible for the attachment of germ tubes to epithelial cells. Detection of antigens expressed preferentially in the outer cell wall layers of germ tubes or of antibodies against these antigens in the sera of infected patients could be important diagnostically. Knowledge of which germ tube antigens are involved in the pathogenesis of *C. albicans* infections could be important in devising active or passive immunization schemes for such hosts.

In a previous paper (7), we compared procedures for extracting materials from the cell walls of intact mycelial-phase and yeast-phase *C. albicans*. When extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), we found that a simple extraction with dithiothreitol (DTT) released a remarkably complex array of protein, glycoprotein, and polysaccharide molecules from outer cell wall layers of both phases of the organism. We detected a larger array of protein components in DTT mycelial-phase extract (DTT-M) than in DTT yeast-phase extract (DTT-Y). DTT-M contained a high-molecular-weight component (HMC), which was very rich in polysaccharides but which migrated consistently in SDS-PAGE gels to a location expected for proteins having a molecular mass of about 235 to 250 kilodaltons (kDa). When treated with periodic acid-Schiff (PAS) stain, gels loaded with a DTT-Y had dense, diffuse staining of their upper portions but no readily identifiable HMC. A 19-kDa protein component was present in DTT-M and not in DTT-Y. These data corroborate previous work by us (8) and other investigators (2, 10, 11) that indicated that there are antigens expressed preferentially in the germ tube cell walls of *C. albicans*. In the present paper, we present evidence from SDS-PAGE, Western blot, and fluorescent-antibody techniques that the HMC found in DTT-M was located on the surface of the germ tube and bore antigenic determinants that were not found in high-molecular-weight molecules extractable from blastoconidia cell walls. The importance of proteins and chitobiose linkages in the integrity of this HMC is explored here. Other data are reported that demonstrate that although the 19-kDa antigen was expressed exclusively in the germ tube cell wall, it was not expressed on the surface of the cell wall.

MATERIALS AND METHODS

Culture of *C. albicans*. *C. albicans* organisms were grown in the yeast or mycelial phase in slightly modified Lee medium as described previously (7, 8). Unless otherwise specified, strain B311 was used in all work reported here. For some experiments, we grew the following strains from the American Type Culture Collection, Rockville, Md.: 28366, 34133, and 28367 (serotype A); 36801 (serotype A); and 36802 (serotype B). Cells were harvested with a sintered-glass filter or by centrifugation, washed with saline, and stored at −20°C until used.

Radiolabeling organisms with ^{125}I. Antigens on the surface of the cell wall were labeled by a modification of the method described by Sundstrom and Kenny (11). Washed microorganisms were suspended in 0.2 M sodium phosphate buffer (pH 7.2) at a concentration of 4×10^8 yeast- or 2×10^8 mycelial-phase organisms per ml. A 3-ml sample of each suspension was incubated with 4 mCi of $Na^{125}I$–250 μl of Enzobead reagent (Bio-Rad Laboratories, Richmond, Calif.)–100 μl of 1% (wt/vol) β-D-glucose (Sigma Chemical Co., St. Louis, Mo.) for 20 min at room temperature. The reaction was stopped with 30 μl of 1% (wt/vol) sodium azide, and the

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microorganisms were washed twice in 0.2 M phosphate buffer and twice in 0.05 M Tris hydrochloride (pH 7.5) containing 0.8 M mannitol. As a control, 250-μl beads were placed in 3 ml of phosphate buffer–2 mCi of ^{125}I, other labeling reagents were added, and the beads were washed in the same manner as the labeled organisms were.

Extraction, SDS-PAGE, and Western blot techniques. The DTT extracts DTT-Y and DTT-M were obtained as previously described (7, 8). The protein content of extracts was estimated by the Lowry technique with bovine serum albumin (nitrogen, 15.4%) as a standard (7). Radiolabeled DTT-Y and DTT-M or control material was dialyzed against SDS-PAGE sample buffer. Unlabeled extracts were dialyzed and lyophilized. Unless otherwise specified, unlabeled extracts were prepared for SDS-PAGE analysis by dissolving them at a concentration of a 40 mg of extract per ml of sample buffer; 10-μl samples were analyzed. Samples were separated on 6 and 12.5% SDS-PAGE slab gels as described by Laemmli (5). A sample of radiolabeled extract containing 10,000 cpm of ^{125}I activity was added to each lane of the SDS-PAGE gel. After electrophoresis, gels were stained with Coomassie blue or PAS reagent or the separate components in the gels were transferred electrophoretically to nitrocellulose as described by Towbin et al. (12), with conditions as previously described (7). Amido black or antibodies (indirect enzyme-linked antibody technique) were used to stain portions of the resultant Western blots (7). Gels were first washed and dried to perform autoradiography. They were then applied to Kodak X-Omat XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and sealed in a light-tight container for 3 days at −20°C. Dried gels were removed, and the film was developed as recommended by the manufacturer.

Production of antisera. New Zealand White rabbits were immunized by weekly subcutaneous injections with 5×10^8 Formalin-killed mycelial-phase organisms in 0.5 ml of complete Freund adjuvant or with 10^8 viable yeast-phase organisms. Some rabbits were immunized with antigens isolated on Western blots of SDS-PAGE gels. Strips of each blot were developed with suitable antisera to determine the location of antigens in the lanes of the blot. Pieces of nitrocellulose containing the desired antigens were cut from the remainder of the blot, dissolved in dimethyl sulfoxide, and injected subcutaneously into rabbits after the nitrocellulose particles were emulsified in 0.5 ml of complete Freund adjuvant. Injections were given weekly for 6 weeks and then every 2 weeks.

Human sera. With informed consent, patients with documented severe *Candida* infections or normal human subjects provided serum samples.

Antibody absorption and indirect fluorescent-antibody staining. Most antisera were absorbed with heat-killed yeast-phase organisms as previously described (7, 8). Some sera were absorbed three times by mixing equal volumes of a serum specimen and a solution containing 10 mg of DTT-Y per ml of saline. Antisera and absorbed antisera were evaluated for their ability to bind to blastoconidia or germ tubes by an indirect fluorescent-antibody technique, which has previously been described (8). Indirect fluorescent-antibody staining showed that each anti-mycelial-phase antiserum absorbed with yeast-phase organisms or DTT-Y stained only the germ tubes of mycelial-phase organisms.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed as described by Weeke (13) with Gel-bond plates (5 by 7 cm) (FMC Corp., Marine Colloids Div., Rockland, Maine) and 1% agarose type I (Sigma Chemical Co., St. Louis, Mo.) in barbital buffer (pH 8.6). First-dimension electrophoresis was conducted at 10 V/cm for 45 min, and second-dimension electrophoresis was conducted at 2 V/cm for 18 h. Indicator gels contained 40 μl of antiserum per cm^2. Gels were washed, pressed, and dried and then stained with Coomassie blue.

Enzyme treatments. For some experiments, DTT-M and DTT-Y were treated with endo-β-*N*-acetylglucosaminidase H (Endo-H) (Sigma) as described by Zlotnik et al. (14). To digest extracts, we used a citrate-phosphate buffer prepared by titrating 0.2 M Na_2HPO_4 to pH 5.0 with 0.1 M citric acid. When radiolabeled material was being analyzed, samples of the labeled extract containing 100,000 cpm were incubated at 37°C for 24 h with an equal volume of buffer containing 0.02 U of Endo-H and 10 μl of toluene. A second volume of Endo-H solution was added, and incubation was continued for another 24 h. Endo-H digestion of unlabeled extracts was done by dissolving 2.5 mg of extract in 25 μl of buffer, adding an equal volume of enzyme solution (0.2 U), and incubating the preparation at 37°C for 18 h. For digestion with protease VII (Sigma), 2.5 mg of extract was dissolved in 25 μl of 0.1 M Tris (pH 8.9). Next, 42 μg of protease in the same volume of buffer was added, and the solution was incubated for 18 h at 37°C.

RESULTS

Screening antisera for reactivity with DTT-M protein antigens. The availability of antisera containing antibodies against the largest possible number of individual antigens of DTT-M was essential in searching for antigens expressed preferentially or exclusively in germ tube cell walls. Previous work had shown that SDS-PAGE gels containing low percentages of acrylamide (6 to 7.5%) were useful in identifying polysaccharide-rich components of cell wall extracts, whereas gels containing higher amounts of acrylamide (10 to 12.5%) demonstrated the largest number and best separation of protein antigens (7). Therefore, we screened antisera for use in identifying protein antigens of DTT-M extracts with 12.5% SDS-PAGE gels. Antisera were obtained from rabbits injected with viable yeast-phase organisms (yeast-phase organisms would transform to mycelial-phase organisms at the site of localized infection) and from rabbits injected with Formalin-killed mycelial-phase organisms. Antisera were absorbed with yeast-phase organisms and used to stain Western blots of gels loaded with DTT-M and DTT-Y. Antisera obtained after a limited number of injections of viable yeast-phase organisms recognized antigens in DTT-M spanning a wide range of molecular weights, whereas antisera obtained after multiple injections of viable organisms tended to recognize larger antigens (>35 kDa). Antisera produced by immunization with Formalin-killed mycelial-phase organisms recognized antigens spanning a wide molecular weight range; however, the pattern of antigens recognized by a given antiserum was variable. In view of these findings, antisera from rabbits given a limited number of injections of viable yeast-phase organisms and from rabbits hyperimmunized with Formalin-killed mycelial-phase organisms were pooled. These pooled antisera were used to probe extracts for antigens expressed preferentially in germ tube cell walls. A pooled anti-mycelial-phase antiserum was absorbed extensively with DTT-Y to identify antigens present only in DTT-M. Blots of DTT-M gel tracks stained with this absorbed antiserum demonstrated an intensely stained band of 19 kDa that was not evident in stained blots of DTT-Y tracks. In previous work (7), we had found a 19-kDa component that was stained by Coomassie blue in SDS-

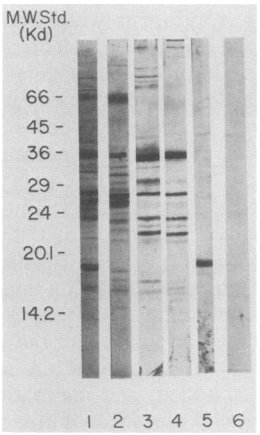

FIG. 1. Reactivity of antisera against selected components of DTT-M. Western blots of 12.5% gels loaded with DTT-M (lanes 1, 3, and 5) or DTT-Y (lanes 2, 4, and 6) were stained with heat-killed yeast-absorbed antisera. Lanes: 1 and 2, pooled anti-mycelial-phase serum; 3 and 4, anti-36-kDa component of DTT-M; 5 and 6, anti-19-kDa component of DTT-M.

PAGE tracks loaded with DTT-M but that did not appear to be present in tracks loaded with DTT-Y. Thus, this 19-kDa component was thought to be a protein antigen that might be expressed only in the germ tube cell wall.

Demonstration that the 19-kDa antigen resided exclusively within the germ tube cell wall. With blots of 12.5% SDS-PAGE gels loaded with DTT-M and stained with yeast-absorbed anti-mycelial-phase serum specimens, two bands were selected for use in immunizing rabbits. The first was the 19-kDa antigen which appeared to be present in stained blots of DTT-M and absent from blots of DTT-Y (Fig. 1). A thick band corresponding to a molecular mass of 36 kDa in DTT-M blots was also selected as a control. Since a deeply stained band doublet was present in DTT-Y blots at a position corresponding to 36 kDa, we thought that there would be a 36-kDa component in DTT-M and DTT-Y that shared antigenic determinants.

The anti-19-kDa component antiserum produced intense staining of only the 19-kDa antigen in DTT-M blots and failed to reveal any corresponding antigen in DTT-Y blots (Fig. 1). Thus, it was concluded that the 19-kDa protein of DTT-M represented an antigen expressed exclusively in the germ tube cell wall of *C. albicans*.

Sera obtained by immunization with the 36-kDa component produced intense staining of a component of about 36 kDa in both DTT-M and DTT-Y blots (Fig. 1). This verified that an appropriate antigen obtained from DTT-M blot segments could stimulate an antibody response against antigens shared by DTT-M and DTT-Y. Thus, the findings with respect to the 19-kDa antigen just described were not artifacts related to the immunization procedure. Surprisingly, the antiserum raised against the 36-kDa blot segment also produced rather intense staining of several other bands in both DTT-M and DTT-Y blots. This could have been produced by contamination of the blot segment used for immunization with traces of other components.

Both the anti-36- and anti-19-kDa antisera failed to stain blastoconidia or germ tubes of mycelial-phase *C. albicans*. Control slides prepared with anti-mycelial-phase antiserum stained both the blastoconidia and germ tubes. Anti-36- and anti-19-kDa sera each produced distinctive, clearly discernible precipitin arcs in crossed immunoelectrophoresis against DTT-M, indicating that the sera contained high titers of antibody against the 36- and 19-kDa components, respectively (Fig. 2). Together, these data indicated that both antigens cannot be expressed on the surface of the cell wall.

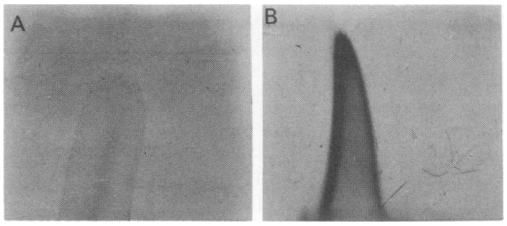

FIG. 2. Crossed immunoelectrophoresis of DTT-M against anti-19-kDa (A) and anti-36 kDa (B) antiserum. First-dimension anode is to the right and second-dimension anode is at the top of each gel.

Evidence for an extractable HMC expressed on the surface of *C. albicans*. Previously (7) we found SDS-PAGE gels having acrylamide content of <7% were useful in identifying polysaccharide-rich HMC in DTT-M and DTT-Y. Moreover, with a PAS stain, a gel loaded with DTT-M had a discrete, densely staining HMC that was absent or faintly visible in a DTT-Y gel. To determine whether HMC in DTT-M and DTT-Y contained protein and whether the extracts contained components expressed on the surface of *Candida* organisms, intact yeast- and mycelial-phase organisms were labeled with ^{125}I. Autoradiographs of SDS-PAGE gels loaded with extracts of these organisms had patterns similar to those seen by PAS staining. It was clear that the polysaccharide-rich HMC in DTT-M and DTT-Y contained small amounts of proteins that could be labeled with ^{125}I. Both extracts contained labeled material forming a discrete band corresponding to a location expected for a 235- to 250-kDa protein; however, this band was very intense in autoradiographs of DTT-M gel tracks and faint in autoradiographs of DTT-Y tracks (Fig. 3A).

Evidence that HMC in DTT-M bore germ-tube-specific antigenic determinants. Pooled anti-mycelial-phase rabbit serum which was absorbed with yeast-phase organisms stained blots of DTT-M 6% SDS-PAGE tracks in the region corresponding to the HMC identified in PAS-stained gels and autoradiographs. By contrast, this absorbed antiserum failed to stain any bands on blots of DTT-Y gel tracks (Fig. 3B). Similar results were obtained when the rabbit anti-mycelial-phase serum was absorbed with DTT-Y before use in staining blots. Yeast-absorbed pooled normal human serum did not stain the HMC on DTT-M blots. However, yeast-absorbed sera from two patients with visceral candidiasis stained the HMC in DTT-M blots and did not stain DTT-Y blots. Together, these data indicated that the HMC extractable from mycelial-phase *C. albicans* had antigenic determinants not expressed on any component extractable from the yeast-phase organism. All of the absorbed sera used to stain the extract blots brightly stained the germ tubes of mycelial-phase *C. albicans* but not their blastoconidia. The HMC was cut from blots of DTT-M gels and used to immunize rabbits. The solubilized antigen was poorly immunogenic. However, when sera obtained after 10 injections of blot segments were absorbed with blastoconidia, these sera stained the germ tubes (but not blastoconidia) of mycelial-phase organisms. It

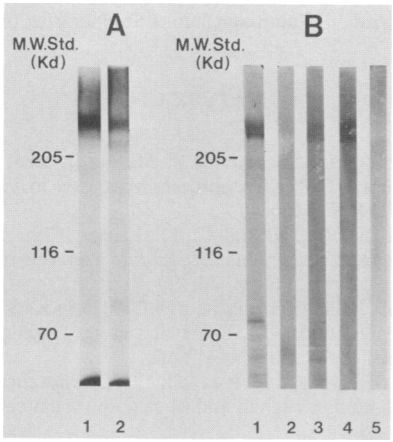

FIG. 3. Demonstration of an HCM bearing germ-tube-specific surface antigenic determinants. (A) Autoradiographs of a 6% SDS-PAGE gel loaded with DTT-M (lane 1) and DTT-Y (lane 2) of organisms surface labeled with ^{125}I. Note the presence of a dense HMC in lane 1 and a less evident band of similar molecular weight in lane 2. (B) Western blots of a 6% gel loaded with DTT-M (lanes 1, 3, and 4) and DTT-Y (lanes 2 and 5) stained with sera that had been absorbed with heat-killed yeast-phase cells. Lanes 1 and 2 were stained with pooled rabbit anti-mycelial-phase serum. Lane 3 was stained with a serum specimen from a patient with *C. albicans* hepatitis, and lanes 4 and 5 indicate a serum sample from a patient with a *C. albicans* intraabdominal abscess. The staining of bands in DTT-M in lanes 1, 3, and 4 corresponds to the HMC seen in the DTT-M autoradiograph. Lanes 2 and 5 show that the HMC in the DTT-Y autoradiograph did not bear antigenic determinants recognized by the yeast-absorbed antisera.

was apparent that the HMC was expressed on the surface of germ tubes and bore antigenic determinants not expressed on blastoconidia surface structures. It constituted an antigen unique to the germ tube surface.

Evidence that HMC was expressed in *C. albicans* strains other than B311. We prepared DTT-M and DTT-Y from five ATCC strains of *C. albicans* and analyzed them along with extracts of strain B311 by 6% SDS-PAGE gels and Western blots. Gels were run in which the same amount of each extract was analyzed (400 μg [dry weight] per lane). Other gels were run in which the amount (dry weight) of loaded extract ensured that the amount of protein in each lane was constant (100 μg per lane). Irrespective of conditions of analysis, each PAS-stained gel showed evidence of an HMC in the DTT-M of each strain, which was not evident in the DTT-Y of the strain. In Coomassie blue-stained gels loaded with constant amounts of protein, a band could be seen in each lane loaded with DTT-M corresponding to the position of the HMC in PAS-stained gels. Such a band was not seen in lanes loaded with DTT-Y. Blots were stained with yeast-absorbed rabbit anti-mycelial-phase serum raised with *C. albicans* B311. All the isolates showed a HMC in DTT-M that was not present in DTT-Y.

Sera from infected humans stained the mycelial-phase HMC in blots of gels loaded with DTT-M from strain B311. This suggested that the antigenic determinants stimulating the antibody response were expressed in the mycelial phase of wild strains of *C. albicans*, as well as the laboratory strains used for our work.

Characterization of linkages within the mycelial-phase HMC. We believed that the HMC was made up of polysaccharides that were linked together by small amounts of protein. DTT-M and DTT-Y were digested with protease and with endo-H (Fig. 4) to test this hypothesis. Endo-H is an endosaccharidase that is known to split di-*N*-acetylchitobiose moieties (1), which have been implicated in linking together polysaccharide and protein portions of *Saccharomyces cerevisiae* mannoproteins (1, 14). Protease-treated DTT-Y produced the same pattern in PAS-stained SDS-PAGE gels as did untreated DTT-Y. Faint, diffuse banding in positions expected for proteins with molecular masses of <200 kDa was seen in gels loaded with DTT-Y treated with endo-H; however, the appearance of the deeply stained material of higher molecular weight remained unchanged. In contrast, all densely staining material (including the HMC) was absent in gel tracks loaded with protease- or endo-H-treated DTT-M. Only a poorly defined, faint banding was seen in the lower portions of these tracks. Autoradiographs of gels loaded with labeled DTT-M and DTT-Y that had been treated with endo-H were also performed. It was evident that for both extracts, treatment led to a loss in labeled material at gel positions expected for proteins of >235 kDa and led to the appearance of a diffuse banding pattern due to components having lower molecular weights.

Together, these data indicated that in both DTT-M and DTT-Y, there were polysaccharides linked through chitobiose units to proteins. Polysaccharides were more highly polymerized by this mechanism in DTT-M. The HMC in DTT-M that bore germ-tube-specific antigenic determinants represented polysaccharides that had been cross-linked to produce a readily discernible collection of molecules that migrated consistently at a discrete location in the SDS-PAGE gel.

DISCUSSION

In the work described in this paper, we were seeking to identify antigens expressed in the outer cell wall layers of germ tubes but not in such layers of the blastoconidia. We identified two components which expressed germ-tube-specific antigenic determinants. These components were very different in their structure and the way in which they

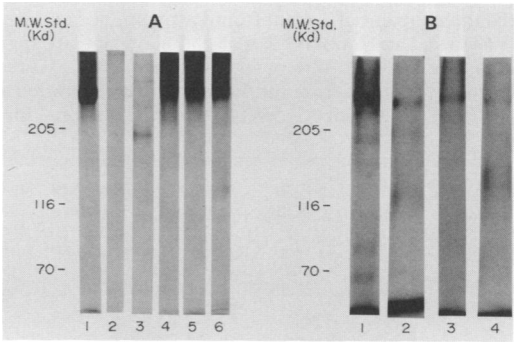

FIG. 4. Effect of protease and endo-H treatment on polysaccharide rich HMC of DTT-M and DTT-Y. (A) PAS-stained tracks of a 6% SDS-PAGE loaded with cell wall extracts and extracts treated with enzymes. Lanes: 1, DTT-M; 2, DTT-M treated with protease; 3, DTT-M treated with endo-H; 4, DTT-Y; 5, DTT-Y treated with protease; 6, DTT-Y treated with endo-H. (B) Autoradiographs of 6% SDS-PAGE loaded with extracts of surface-labeled organisms and the same extracts treated with endo-H. Lanes: 1, DTT-M; 2, DTT-M treated with endo-H; 3, DTT-Y; 4, DTT-Y treated with endo-H. The change in staining pattern in panel A shows the effect of treatments on sugar-bearing moieties, and the autoradiographs demonstrate their effect on labeled protein moieties.

expressed germ-tube-specific antigenic determinants. The 19-kDa protein antigen was not exposed to any extent on the cell wall surface. There did not appear to be a component of corresponding molecular mass in extracts of yeast-phase cell walls that simply lacked the antigenic determinants that the 19-kDa protein expressed. By contrast, the HMC found in DTT-M was rich in polysaccharides and was expressed at the cell surface. DTT-Y possessed a lower concentration of a component migrating at the same location in SDS-PAGE gels; however, this component lacked the antigenic determinants expressed by the mycelial-phase antigen.

Our finding of a polysaccharide-rich HMC bearing antigenic determinants specific to the germ tube is similar to that of Sundstrom and Kenny (11). These investigators labeled the surface of mycelial-phase *C. albicans* with ^{125}I. These organisms reacted with a rabbit anti-mycelial-phase serum that had been absorbed with yeast-phase organisms. The labeled organisms with their bound antibodies were treated with zymolase. The extracted material was precipitated with *Staphylococcus aureus* cells bearing protein A. Antigens were released by the boiling of precipitated material in SDS-PAGE sample buffer. Radiolabeled antigens were located in SDS-PAGE gels by autoradiography. The investigators found that two closely spaced antigens (with estimated molecular masses of 200 and 155 kDa) could be recovered by this technique. These antigens were shown to react with concanavalin A by a nitrocellulose blotting technique. Zymolase contains a β-1, 3-glucanase and a protease. It is conceivable that this enzyme treatment and the steps used in recovering radiolabeled antigen could have acted upon the HMC that we identified to yield these lower-molecular-weight components.

Clearly, more study will be required to determine whether the germ-tube-specific antigens described here are expressed in germ tubes of numerous *C. albicans* isolates. If they are expressed universally in this species, they could be useful in the study of the pathogenesis of candidiasis, as markers for events in the regulation of the transition from the yeast to the mycelial phase, and for construction of serodiagnostic tests.

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