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Partial Biochemical Characterization of Cell Surface Hydrophobicity and Hydrophilicity of *Candida albicans*

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Hydrophobic yeast cells of *Candida albicans* are more virulent than hydrophilic yeast cells in mice. Results of experiments performed in vitro suggest that surface hydrophobicity contributes to virulence in multiple ways. Before definitive studies in vivo concerning the contribution of fungal surface hydrophobicity to pathogenesis can be performed, biochemical, physiological, and immunochemical characterization of the macromolecules responsible for surface hydrophobicity must be accomplished. This report describes our initial progress toward this goal. When hydrophobic and hydrophilic yeast cells of *C. albicans* were exposed to various enzymes, only proteases caused any change in surface hydrophobicity. Hydrophobic cell surfaces were sensitive to trypsin, chymotrypsin, pronase E, and pepsin. This indicates that surface hydrophobicity is due to protein. Papain, however, had no significant effect. The hydrophobicity of hydrophilic cells was altered only by papain. The proteins responsible for surface hydrophobicity could be removed by exposure to lyticase, a β1-3 glucanase, for 30 to 60 min. When 60-min lyticase digests of hydrophobic and hydrophilic cell walls were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12.5% resolving gel, each protein population contained a single unique protein that was not evident in the other protein population. However, when the cell wall surface proteins of hydrophobic and hydrophilic cells were first labeled with ^{125}I and then removed by lyticase and analyzed by SDS-PAGE, at least four low-molecular-mass (<65 kilodaltons) proteins associated with hydrophobic cells were either absent or much less abundant in the hydrophilic cell digests. This result was seen for both *C. albicans* strains that we tested. When late-exponential-phase hydrophilic cells were treated with tunicamycin, high levels of surface hydrophobicity were obtained by stationary phase. These results indicate that the surface hydrophobicity of *C. albicans* reflects changes in external surface protein exposure and that protein mannosylation may influence exposure of hydrophobic surface proteins.

The opportunistic pathogenic fungus *Candida albicans* is capable of expressing surface hydrophobicity when grown in either the yeast or hyphal form (22, 33). Associated with expression of surface hydrophobicity is enhanced virulence. In mouse survival experiments, hydrophobic yeast cells of a standard laboratory *C. albicans* strain, designated LGH1095, caused more rapid mortality than hydrophilic cells (1). A second isolate of *C. albicans*, designated LGH870, gave similar results (P. P. Antley and K. C. Hazen, unpublished results). The enhanced virulence of hydrophobic cells may be attributable to the hydrophobic surface influencing several steps in the pathogenic process. These steps include adherence to host epithelium, avoidance of polymorphonucleated neutrophil killing mechanisms, and germ tube formation (1, 9, 18, 20–22). The last step appears especially important in causing candidiasis (40). In *Candida* septicemia, in which colonization of indwelling catheters or prosthetic devices serves as the nidus, expression of surface hydrophobicity again appears to play a central role. Several groups of investigators have demonstrated that hydrophobic yeast and hyphal cells attach more than hydrophilic cells to various types of plastics used in prostheses and catheters (27, 28, 33, 38).

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These various observations suggest that fungal surface hydrophobicity expression is pivotal to the success of parasitizing *C. albicans* in causing serious infection. The expression of surface hydrophobicity by yeast cells in vivo may, however, be transient. Hydrophilic cells, obtained by growth at 37°C in vitro, exposed hydrophobic surface macromolecules for only a short period when subcultured into fresh medium (18). This result suggests that antigenic variation occurring during pathogenesis of candidiasis could involve surface hydrophobic molecules in addition to mannoprotein antigens (4, 36, 37).

Further knowledge about the biochemistry and biology of hydrophobicity expression by *C. albicans* is needed before definitive studies concerning cell surface hydrophobicity (CSH) in pathogenesis can be performed. Paramount to this endeavor is the identification and characterization of the surface macromolecules responsible for hydrophobicity expression by yeast and hyphal cells. The description of surface hydrophobic molecules and their expression kinetics in vivo will provide important needed information for development of potential vaccines. Here, we report our initial studies concerning the surface hydrophobicity biochemistry of *C. albicans* and demonstrate that CSH expression involves the participation of surface proteins. The results also suggest that changes in protein mannosylation levels influence exposure of hydrophobic surface molecules.

MATERIALS AND METHODS

Organisms and culture conditions. *C. albicans* LGH1095 was used throughout this study. This strain, which was originally isolated from a blood culture of a septicemic patient, has been used as our standard strain in previous studies (1, 17, 21, 22, 25). *C. albicans* LGH870, originally obtained from an oral lesion, and a stable, hydrophobic variant derived from isolate LGH1095 were also used in some experiments. The hydrophobic variant, designated O31-E10, was obtained by a repeated single-cell isolation technique involving limiting dilutions (19). Yeast cells of these isolates were subcultured three times at 24- to 26-h intervals in Sabouraud dextrose broth, unless otherwise indicated, at 23 and 37°C as previously described (18, 23). At 23°C, isolates LGH1095 and LGH870 express surface hydrophobicity, and at 37°C, they express surface hydrophilicity (22, 25). The variant O31-E10 expresses surface hydrophobicity at both growth temperatures (19).

Microsphere hydrophobicity assay. The surface hydrophobicity of *C. albicans* populations was assessed by using a microsphere hydrophobicity assay which has been described in detail elsewhere (17, 18, 22). Briefly, washed yeast cells were adjusted to 2×10^6 cells per ml in sodium phosphate buffer (0.05 M, pH 7.2, PB), and 100 μl of the cell suspension was mixed with 100 μl of blue polystyrene microspheres (0.801-μm diameter; Serva Fine Biochemicals, Westbury, N.Y.) which had been prepared at approximately 9×10^8 spheres per ml in PB. The working concentration of microspheres can be easily obtained by diluting 6 μl of a 10% solids stock suspension of microspheres in 2 ml of buffer. After equilibration to room temperature, the cell-microsphere mixture is vigorously vortexed for 30 s. The percentage of cells having three or more attached microspheres is considered the CSH value of the yeast cell population. At least 100 cells were counted per sample, and each cell population was assayed in triplicate.

Throughout the assay, strict attention was given to ensure that the glassware was free of contaminants which could lead to spurious levels of CSH. We have found that the following protocol works well. Glassware is immersed in 2% (vol/vol) Contrad 70 (Polysciences, Warrington, Pa.) and heated to 121°C for 5 min in an autoclave. While warm, the glassware is then rinsed five times with distilled water. The glassware is then immersed in 1% HCl in deionized water for at least 2 h and rinsed with deionized or triple-distilled water until no acidity is detectable. The glassware is dried at 140°C overnight before use in experiments.

Enzymatic and chemical treatments. *C. albicans* yeast cells were prepared for enzymatic and chemical treatments by first washing the cells with ice-cold deionized water and then adjusting the cell concentration to 2×10^8 cells per ml of deionized water. Two milliliters of yeast cell suspension was mixed with 2 ml of a twofold-concentrated chemical or enzymatic reagent. The mixture was incubated for 60 min at 37°C with occasional shaking. The enzymes and appropriate buffers used in these experiments are based on those described by Lee and King (32) and are listed in Table 1 along with the dithiothreitol (DTT) conditions.

Each proteolytic enzyme was determined as active by using the colorigenic substrate azocoll (Sigma Chemical Co.), which is composed of insoluble, powdered cowhide particles. Azocoll (25 mg) was suspended in 5 ml of proteolytic enzyme solution and incubated at 37°C for 15 min. The proteolytic activity was determined by monitoring the change in A_{520} of the azocoll-enzyme mixture (Lambda 3B spectrophotometer; The Perkin-Elmer Corp., Oak Brook, Ill.). Lipase activity was detected by using a standardized test (Sigma Diagnostic Serum Lipase, Sigma Diagnostics Kit 800-A).

TABLE 1. Enzymatic and chemical treatments used to characterize cell surface hydrophobicity and hydrophilicity of *C. albicans* LGH1095[a]

Enzyme	Concn	Buffer	pH
Lipase	1.0 mg/ml	0.1 M sodium acetate	5.0
α-Phospholipase D	2.0 mg/ml	0.2 M sodium acetate–0.08 M $CaCl_2$	5.6
Phospholipase C	100 U/ml	0.05 M $NaPO_4$–1 mM β-mercaptoethanol–0.4 mM $ZnCl_2$	7.0
C_4 esterase	1.0 mg/ml	0.05 M Tris–0.5 M NaCl	8.0
C_4 esterase	0.5 mg/ml	0.05 M Tris–0.5 M NaCl	8.0
Papain	3.0 mM	0.01 M $NaPO_4$–5 mM cysteine–2 mM EDTA	8.0
Trypsin	0.4 mM	0.01 M $NaPO_4$	8.0
Pepsin	0.3 mM	0.07 M $NaPO_4$	7.5
α-Chymotrypsin	0.4 mM	0.01 M $NaPO_4$	8.0
Pronase E	1.0 mg/ml	0.04 M KPO_4	7.5
Lyticase	500 U/ml	0.1 M Tris hydrochloride	8.1
DTT	50 mM	0.1 M Tris hydrochloride–5 mM EDTA	8.6

[a] Based on treatment protocols used by Lee and King (32).

Cell wall digestion and protein preparation. Cell walls of hydrophobic and hydrophilic cells were partially or completely digested by exposure to lyticase (a β1-3 glucanase; Sigma) for different times as described elsewhere (20). Yeast cells were washed three times with cold saline (0.85% NaCl in distilled water) and suspended to 2×10^8 to 6×10^8 cells per ml of digest buffer (Tris hydrochloride, 0.1 M, pH 8.1) containing 1 mM phenylmethylsulfonyl fluoride. To prevent protoplast lysis, 0.5 M $MgSO_4$ was added to the digest buffer. Cell wall digestion was initiated by mixing the cell suspension with an equal volume of lyticase solution (1,000 U/ml of digest buffer) and incubation at 35°C with occasional shaking. At various times (15, 30, 60, and 120 min) a portion of the cell suspension was removed and washed twice with digest buffer minus phenylmethylsulfonyl fluoride, and CSH was determined. Protoplast production was determined by lysis with 10% (wt/vol) sodium dodecyl sulfate (SDS) (20). Samples (50 μl) of the lyticase-treated cell suspension were placed in glass test tubes (12 by 75 mm) containing 450 μl of 10% SDS and mixed thoroughly. The concentration of intact cells was determined with the aid of a hemacytometer and compared with untreated controls to obtain the percent protoplast formation.

Cell wall proteins released by lyticase digestion were obtained by consecutive centrifugations and retention of the supernatant liquid. In preparations of cell wall proteins obtained by limited lyticase digestion (1 h), $MgSO_4$ was not added to the digest buffer. The first centrifugation ($400 \times g$, 5 min, 4°C) removed most of the cells. To ensure that all cells were removed, the supernatant liquid was transferred to a glass centrifuge tube and subjected to centrifugation at $10,000 \times g$ (10 min, 4°C). The protein concentration was determined based on the method of Bradford (3; Bio-Rad Protein Assay Kit; Bio-Rad Laboratories, Richmond, Calif.). The proteins in the supernatant were precipitated with ethanol (final concentration, 80% [vol/vol]; 0 to 4°C) overnight. The precipitated proteins were pelleted by centrifugation ($10,000 \times g$, 60 min, 4°C), and the pellet was washed twice with cold (−20°C) ethanol. The pellet was then dried

TABLE 2. Effect of enzymatic treatments on CSH of *C. albicans* LGH1095 yeast cells grown at 23 and 37°C

Treatment[a]	CSH (%) at:							
	23°C				37°C			
	Initial[b]	No enzyme	With enzyme	*P* value[c]	Initial	No enzyme	With enzyme	*P* value
Lipase	84.2 ± 0.5[d]	83.2 ± 14.4	81.8 ± 13.4	NS	6.4 ± 3.0	10.0 ± 9.2	6.8 ± 0.2	NS
α-Phospholipase	95.9 ± 0.4	96.8 ± 1.6	97.8 ± 0.7	NS	0.8 ± 0.2	0.9 ± 0.2	0.7 ± 0.6	NS
Phospholipase C	98.2 ± 1.5	97.8 ± 1.9	97.1 ± 2.1	NS	0.2 ± 0.1	0.2 ± 0.2	0.3 ± 0.3	NS
C_4 esterase	93.7 ± 1.2	97.4 ± 1.1	98.6 ± 1.3	NS	0.3 ± 0.4	0.8 ± 0.4	0.8 ± 0.4	NS
Papain	97.5 ± 0.5	97.7 ± 0.5	83.5 ± 7.1	NS	1.7 ± 0.3	2.9 ± 1.1	86.1 ± 12.2	<0.02
Trypsin	88.6 ± 2.0	87.2 ± 1.2	4.3 ± 4.2	<0.01	3.2 ± 1.0	3.3 ± 1.4	2.9 ± 0.9	NS
Pepsin	94.8 ± 0.7	93.3 ± 0.4	1.0 ± 0.4	<0.001	4.0 ± 2.0	4.4 ± 3.3	0.5 ± 0.7	NS
α-Chymotrypsin	96.6 ± 1.6	96.9 ± 0.3	0.2 ± 0.2	<0.001	3.2 ± 1.3	2.7 ± 2.6	0.0 ± 0.0	NS
Pronase E	94.8 ± 0.7	94.9 ± 1.0	0.8 ± 0.7	<0.001	4.0 ± 2.0	6.9 ± 0.1	0.2 ± 0.2	<0.01
DTT	97.4 ± 0.7	96.9 ± 0.8	98.3 ± 2.1	NS	1.1 ± 0.7	2.3 ± 0.2	98.7 ± 1.6	NS

[a] All treatments were for 1 h. The conditions are presented in Table 1.
[b] Initial refers to yeast cell populations prior to treatment.
[c] Significance was determined with the two-tailed Student *t* test. NS, Not significant.
[d] Values represent the mean from at least two experiments with triplicate sample ± the standard deviation.

by pervaporation with nitrogen. Further processing of the samples depended on the intended subsequent analysis. The ethanol supernatant fluid was tested for the presence of proteins (39). It was dried under vacuum (Speed-Vac; Savant Instruments, Hicksville, N.Y.), solubilized in electrophoresis sample buffer (see below), and subjected to electrophoresis. No proteins were detectable from the ethanol supernatant fluids of either hydrophobic or hydrophilic cell wall preparations.

Radioiodination of cell wall surface proteins. To radioiodinate surface proteins of hydrophobic and hydrophilic yeast cells of *C. albicans*, the method of Judd (26) was used with minor modifications. The method utilizes iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglucouril; Sigma) to catalyze iodination and is based on the original observations of Fraker and Speck (15). Yeast cells were washed three times with cold sodium phosphate-saline buffer (0.05 M phosphate, 0.85% NaCl, pH 7.4; PBS) and adjusted to 5×10^7 cells per ml in cold PBS. From the suspension, 1.5 ml was transferred to a 1.5-ml microcentrifuge tube. The cells were pelleted by brief (30 s) centrifugation (approximately 11,000 × *g*). The cells were suspended in 150 μl of cold PBS, and 2 μl of KI (10 μM) was added to the suspension. After being mixed, the suspension was transferred to a 1.5-ml microcentrifuge tube which contained iodogen (15). To the cell suspension, 4 μl of $Na^{125}I$ (25 μCi/μl; carrier free; approximate specific activity, 17 Ci/mg; ICN Pharmaceuticals, Irvine, Calif.) was added and mixed. The suspension was incubated at 0 to 4°C for 30 min with gentle mixing every 10 min. The cell suspension was transferred to a 1.5-ml microcentrifuge tube, and the cells were washed five times with cold PBS.

The ^{125}I-labeled cell wall surface proteins were removed from the cells by lyticase treatment. The cells (suspended in 0.15 ml of digest buffer) were mixed with an equal volume of digest buffer containing 1,000 U of lyticase per ml and phenylmethylsulfonyl fluoride (1 mM) and incubated for 60 min at 35°C. After cell wall digestion, the cells were pelleted and the supernatant was transferred to a 1.5-ml microcentrifuge tube. No further processing of the sample was performed. The samples were stored for no longer than 3 weeks at −20°C prior to analysis.

SDS-PAGE. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the discontinuous buffer system of Laemmli (31) was used, but the recipes for the sample, resolving, stacking, and reservoir buffers presented by Hames (16) were followed with one exception. EDTA (0.2 M) was substituted for SDS in the stacking and resolving gel formulations. Unless otherwise stated, the resolving gel contained 12.5% T–2.6% C. Proteins were solubilized in PBS, mixed with an equal volume of 2× sample buffer (final concentrations, 0.0625 M Tris hydrochloride, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue [pH 6.8]), and heated at 100°C for 3 to 5 min. When gels were run for analysis of ^{125}I-labeled proteins, each lane was loaded with 10^6 cpm of the appropriate sample. All gels were run at constant voltage (150 V). Low-molecular-weight protein standards (British Drug Houses, Poole, England) were also run with each gel.

After electrophoresis, the gels were stained with Coomassie brilliant blue G250. ^{125}I-labeled proteins were detected by autoradiography after destaining and drying the gels onto cellophane. For autoradiography, Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) and Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Wilmington, Del.) were used.

TM treatment. Yeast cells were grown in yeast nitrogen base containing amino acids (Difco Laboratories, Detroit, Mich.) plus 50, 250, or 500 mM glucose under the same conditions described above for Sabouraud dextrose broth. At late exponential phase (ca. 16 h for cells grown at 37°C and 20 h for cells grown at 23°C), tunicamycin (TM) (125 μl of a stock solution containing 1 mg/ml) was added to the cultures to achieve a final concentration of 5 μg/ml. The TM stock was prepared by dissolving TM in a minimum amount of 1 M NaOH and diluting with sterile deionized water. The cells were incubated for 8 h after the addition of TM. The CSH of the cells was then determined by the microsphere assay. Preliminary experiments were conducted in which the incorporation of ^{14}C-labeled amino acids into protein was monitored during TM treatment in order to ascertain whether the concentration used to affect surface protein glycosylation did not also inhibit protein synthesis (2). No difference in protein synthesis between treated and control cells was detected.

RESULTS

Effect of enzymes and DTT treatment. Surface hydrophobicity was susceptible to various protease treatments but not to lipases (Table 2), indicating that surface hydrophobicity is

TABLE 3. Effect of lyticase treatment on CSH of 23°C and 37°C-grown yeast cells of *C. albicans* LGH1095

Growth temp	Treatment period (min)	CSH level (%)	Released cell wall protein (μg of protein/mg [dry wt] of cells)[a]
23°C	0	98.4 ± 2.1	23.0 ± 1.4
	15	31.7 ± 1.7	0.91 ± 0.06
	30	16.3 ± 2.8	1.11 ± 0.13
	60	2.9 ± 0.7	2.6 ± 0.13
	120	1.8 ± 3.4	5.89 ± 0.24
37°C	0	4.2 ± 2.4	27.9 ± 0.2
	15	9.0 ± 6.0	1.45 ± 0.09
	30	19.7 ± 16.2	1.45 ± 0.05
	60	26.9 ± 10.6	3.54 ± 0.29
	120	45.1 ± 12.2	6.93 ± 0.14

[a] Protein concentrations were determined as described in Materials and Methods. The dry weight of cells is based on the cellular dry weight just prior to exposure to lyticase.

due to proteins. Hydrophilic cells were unaffected by the enzyme treatments with one exception (Table 2). Papain caused hydrophilic cells to appear hydrophobic. Similarly, hydrophobic cells remained hydrophobic after treatment with papain, but other proteases caused the cells to appear hydrophilic. When hydrophobic and hydrophilic cells were treated with DTT, only the hydrophobic status of hydrophilic cells was affected. These cells became substantially hydrophobic (Table 2).

Effect of lyticase digestion on cell wall hydrophobicity. Hydrophobic and hydrophilic yeast cells were treated with lyticase for various times, and their hydrophobicity status was monitored. The amount of protein released by the cells (on a per dry cell weight basis) of the cell pool was also monitored throughout the digestion treatment. A gradual increase in the total amount of released protein was obtained with both cell populations during the 120-min treatment period (Table 3). More protein was obtained from initially hydrophilic cells. By 60 min, hydrophobic cells (initial CSH value was greater than 90%) became hydrophilic (CSH, <10%). Hydrophilic cells became hydrophobic, but the final CSH level varied from experiment to experiment. These results demonstrate that the hydrophobic proteins and hydrophilic surface macromolecules were released by digesting cell walls with lyticase for 60 min.

SDS-PAGE analysis of lyticase digests. Hydrophobic and hydrophilic yeast cell walls were digested with lyticase for 60 min, at which point hydrophobic cells had apparently released the hydrophobic proteins (Table 3), and the released proteins were subjected to SDS-PAGE analysis with 12.5% resolving gels. The gels were stained by a combined Coomassie brilliant blue-silver process (10). Each protein population contained one protein that was not apparent in the other population (Fig. 1). For hydrophobic cells, the protein had an apparent molecular mass of 38 kilodaltons (kDa). The unique protein associated with hydrophilic cells was 34 to 35 kDa.

SDS-PAGE analysis of ^{125}I-labeled surface proteins. The cell wall surface proteins of hydrophobic and hydrophilic yeast cells of *C. albicans* LGH1095 were labeled with ^{125}I prior to limited (60 min) lyticase treatment. The released proteins were then subjected to SDS-PAGE analysis, using 12.5% resolving gels. At least seven proteins (apparent molecular masses of 16, 26, 30, 33, 40, 44, and 49 kDa) appeared to be either more abundant in or unique to hydrophobic cells versus hydrophilic cells (Fig. 2). Similar results were obtained when these experiments were repeated with two different batches of cells and different lots of Na^{125}I over a 6-month period. No difference in protein profiles were obtained when the cell wall digestion was increased to 150 min. The surface proteins of moderately hydrophobic cells (CSH, 45%), which were obtained in a single experiment, appeared to show enhanced exposure (versus hydrophilic cells) of several of the proteins associated with hydrophobic cells (data not shown). These results suggest that the low-molecular-weight proteins are responsible for surface hydrophobicity.

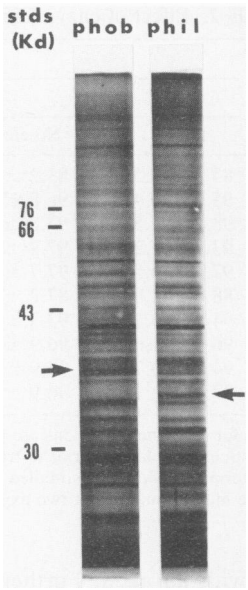

FIG. 1. SDS-PAGE analysis of cell wall extracts of hydrophobic (phob) and hydrophilic (phil) yeast cells of *C. albicans* LGH1095. Viable yeast cells were exposed to lyticase digestion for 1 h. Each lane was loaded with 20 μg of protein, and the separated proteins were visualized with a combined Coomassie brilliant blue-silver general protein stain. Unique protein bands are indicated by the arrows. Kd, Kilodaltons; stds, standards.

To detect the surface proteins associated with hydrophobic cells by autoradiography within 48 h, it was necessary to load each lane with 10^6 dpm. When fewer disintegrations per minute (10^5) were loaded, only high-molecular-weight proteins and the 36-kDa protein were detectable. Nonspecific labeling by ^{125}I of hydrophobic and hydrophilic surface proteins was evaluated by incubating cells in the presence of Na^{125}I without iodogen. Some labeling did occur, but the total number of cell-associated counts was less than 3% of the number associated with cells labeled in the presence of iodogen. SDS-PAGE analysis demonstrated that the nonspecifically labeled cell wall material had a high molecular mass (>100,000 kDa, data not shown). Whether it is polysaccharide was not determined.

The surface proteins of the hydrophobic variant, O31-E10, were also subjected to SDS-PAGE analysis after ^{125}I labeling. Unlike its parent strain, this variant is hydrophobic when grown at 23 and 37°C. Like the parent strain, the cells grown at 23°C have several low-molecular-weight proteins that are exposed on the surface (Fig. 2). The cells grown at 37°C, however, also displayed at least two proteins (19 and 44 kDa) not detectable in the extracts from the parent strain grown at 37°C and a third protein (34 kDa) that was more

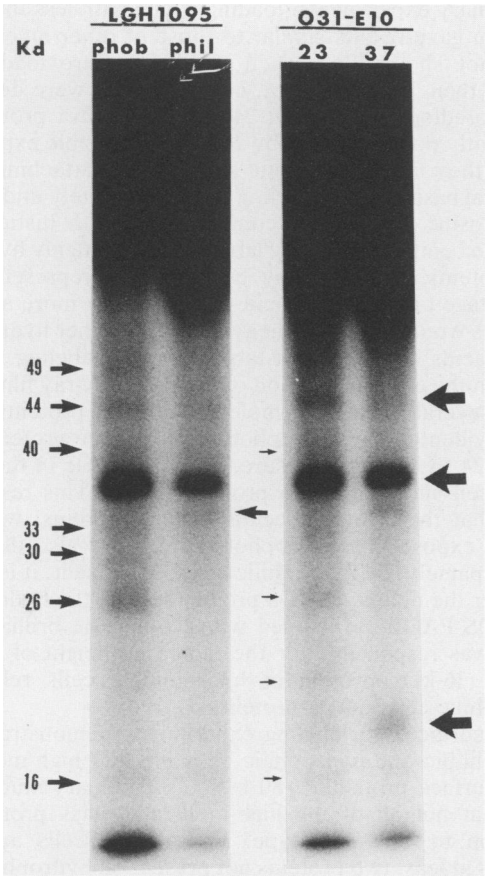

FIG. 2. Autoradiograms of SDS-PAGE gels of surface ^{125}I-labeled proteins of hydrophobic (phob) and hydrophilic (phil) *C. albicans* LGH1095 yeast cells and its hydrophobic variant O31-E10. Hydrophobic and hydrophilic cells of isolate LGH1095 were obtained by growth to stationary phase at 23 and 37°C, respectively. The cell wall proteins were released from intact cells by limited (1 h) lyticase digestion, and 10^6 cpm was loaded onto each lane. Autoradiograms were exposed for 24 h. The arrows for isolate LGH1095 indicate protein bands that were either more intense or unique to the protein profile. The arrows for variant O31-E10 indicate bands that were present in the profiles of cells grown at 23 and 37°C but not in the profile for hydrophilic cells of isolate LGH1095. The small arrows point to proteins unique to the protein profile of the variant cells. Kd, Kilodaltons.

abundant. For the analysis shown in Fig. 2, the cells grown at 23°C were >98% hydrophobic but the cells grown at 37°C were 81% hydrophobic. Regardless of the growth temperature the cells were not strongly hydrophobic, as evidenced by a low number of attached microspheres (typically no more than 5—strongly hydrophobic cells have more than 10). Also, the cells grown at 37°C appeared hydrophobic on the buds, while the mother cells were typically hydrophilic. Thus, a high amount of hydrophilic material and low amount of hydrophobic proteins were likely present in the cell wall extracts from the 37°C cells of variant O31-E10.

When the surface proteins from a second isolate of *C. albicans*, designated LGH870, were analyzed by SDS-PAGE, a pattern similar to the one obtained with LGH1095 was seen (Fig. 3). That is, several low-molecular-mass proteins (less than 55 kDa) were evident in the hydrophobic cell wall extract but not in the hydrophilic cell extract. The proteins appeared to have nearly the same molecular weight as those obtained from isolate LGH1095. Although 10^6 dpm

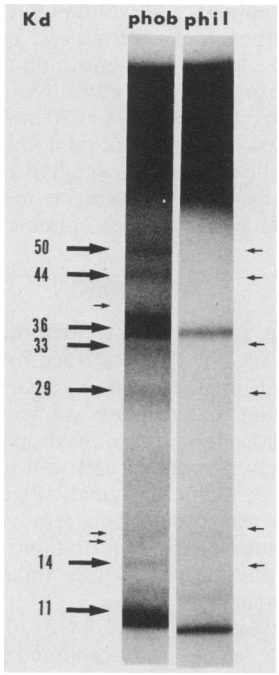

FIG. 3. Autoradiogram of SDS-PAGE gel of surface ^{125}I-labeled proteins of hydrophobic (phob) and hydrophilic (phil) *C. albicans* LGH870 yeast cells. The cell wall proteins were released from intact cells by limited (1 h) lyticase digestion, and 10^6 cpm was loaded onto each lane. Autoradiograms were exposed for 24 h. The large arrows indicate proteins which were unique to the protein profile or were more abundant than in the opposing protein profile. Protein bands that became evident after an exposure period of 19 days are indicated by the small arrows.

of the cell wall extracts was loaded into each lane, it is clear that most of the counts for the hydrophilic cells were restricted to the high-molecular-weight range. Long-term exposure (19 days) of the gel to X-ray film revealed that hydrophilic cells had the low-molecular-weight proteins exposed on their surface (indicated by the small arrows in Fig. 3), but the proteins were present in low amounts.

Effect of TM treatment on surface hydrophobicity expression. *C. albicans* LGH1095 was grown at 23 and 37°C in yeast nitrogen base containing different concentrations of glucose and in the absence or presence of TM. The CSH levels of 23°C-grown cells was high regardless of the glucose

TABLE 4. Effect of TM on CSH of *C. albicans* LGH1095 grown in yeast nitrogen base containing 50, 250, or 500 mM glucose at 23 and 37°C

Growth temp	Glucose concn (mM)	CSH (%)	
		Control	Treated
23°C	50	90.0 ± 7.6[a]	93.4 ± 1.6
	250	93.6 ± 3.3	95.0 ± 3.2
	500	96.3 ± 3.3	97.1 ± 2.8
37°C	50	17.9 ± 3.6	25.8 ± 4.1
	250	11.1 ± 4.1	20.8 ± 5.3
	500	34.2 ± 3.1	100.0 ± 0.0[b]

[a] Values represent the mean from at least three experiments with triplicate samples ± the standard deviation.
[b] Significant at $P < 0.001$ (Student's t test).

concentration (Table 4). TM had no effect on the hydrophobicity status of these cells. However, cells grown at 37°C were affected by glucose concentration and by TM. At 500 mM glucose, these cells were more hydrophobic than when grown in medium containing 50 or 250 mM glucose (Table 4). TM added to yeast nitrogen base containing 500 mM glucose caused 100% of the cells to become hydrophobic, suggesting that inhibition of surface protein mannosylation results in exposure of surface protein hydrophobic sites.

DISCUSSION

In our previous studies (18), we demonstrated that hydrophilic cells convert rapidly to surface hydrophobicity upon dilution into fresh medium. The results from the DTT and lyticase extraction experiments indicate that the rapid change from hydrophilicity to hydrophobicity is due to removal of loosely associated cell wall material that masks the hydrophobic proteins. Enzymatic digestion of the hydrophobic cell wall was required to remove the hydrophobic proteins, indicating that the hydrophobic proteins are tightly associated and possibly covalently linked to the cell wall polysaccharide matrix.

Various investigators (5, 7, 11, 14, 34, 35; for a review, see reference 6) have successfully removed glycoproteins from the *C. albicans* cell wall by using hot SDS but noted that some proteins remained associated with the cell wall. These tightly associated proteins were not extensively mannosylated. Our results as well as those of other investigators (5, 7) suggest that these proteins have low molecular masses (less than 80 kDa). We have also tried to remove the hydrophobic and hydrophilic surface macromolecules with SDS but have obtained equivocal results (data not shown). Despite extensive washing, SDS appears to remain associated with the cells, causing them to appear highly hydrophilic in the microsphere assay.

Approximately 42 proteins were revealed in SDS-PAGE gels of whole cell wall extracts of *C. albicans* yeast cells (7, 14). A similar number of proteins were seen in wall extracts of hydrophobic and hydrophilic cells that had been exposed to limited (1 h) lyticase digestion when the gels were treated with the general protein combination stain of Coomassie brilliant blue and silver. With the exception of the single proteins unique to each cell wall preparation, no qualitative differences between hydrophobic and hydrophilic cell protein profiles were seen. However, ^{125}I surface labeling revealed that hydrophobic and hydrophilic cells differed in which proteins were exposed to the extracellular milieu and that these proteins were not restricted to the unique proteins seen in the Coomassie brilliant blue-silver-stained gels. By surface labeling yeast cells with ^{125}I, it was evident that only approximately 25% of the cell wall proteins were exposed to the extracellular milieu by hydrophobic cells. This was not an artifact of the labeling procedure as an alternate method involving Enzymobeads (Bio-Rad Laboratories; 35) yielded similar results (data not shown). Our results, however, differ from those of other investigators (35, 41) who used ^{125}I to label surface proteins of *C. albicans* in that our autoradiographs revealed more proteins. The disparities are likely due to differences not only in growth conditions and strains but also in the amount of radiolabeled material loaded onto each gel lane. We loaded each gel lane with 10^6 cpm, while other investigators used less than 1.5×10^4 cpm per lane. As a result of the relatively low amount loaded on the lanes, the other investigators detected only four or fewer proteins, three of which were >100 kDa. In our preliminary experiments, loading a lane with less than 5.0×10^4 cpm gave results similar to those of other investigators (data not shown) but when the lanes were loaded with greater than 3.0×10^5 cpm, other proteins were detectable by autoradiography within 24 h. These extra proteins are apparently poorly labeled by $Na^{125}I$. A possible explanation is that they lack the specific sites for ^{125}I attachment. The chemical basis for ^{125}I labeling is not completely understood, but tyrosine is the most common site, with histidine and cysteine being occasionally labeled (29). In highly hydrophobic proteins, tyrosine may be absent or represent a low percentage of the amino acid residues. The more abundant residues would likely be phenylalanine or other hydrophobic amino acids which are not targets for ^{125}I labeling.

When the exposure period of the gels to X-ray film was 19 days, several of the low-molecular-weight proteins, which were evident in the hydrophobic cell surface protein profile within 24 to 48 h of exposure, became visible in the hydrophilic cell surface protein profile (Fig. 3). This result indicates that these low-molecular-weight proteins, which are readily exposed by hydrophobic cells, are also displayed, albeit sparsely, by hydrophilic cells. At present, it is unclear whether the unique 38-kDa protein seen in the hydrophobic cell SDS-PAGE gel stained with Coomassie brilliant blue-silver was responsible for the increased height of the ^{125}I-labeled 36-kDa protein of hydrophobic cells relative to hydrophilic-cell 36-kDa protein.

The surface ^{125}I labeling experiments demonstrated that hydrophobic and hydrophilic cells possess high-molecular-mass surface proteins (>80 kDa). Preliminary studies suggest that not all of the high-molecular-mass proteins are common to each cell type. Hydrophilic cells appear to possess at least two proteins not present on hydrophobic cell surfaces. Whether these proteins are the ones that are removed from the cell surface by the papain, lyticase, and DTT treatments, allowing exposure of the hydrophobic proteins, requires further investigation. The high-molecular-mass proteins are likely highly mannosylated, as shown by preliminary concanavalin A-peroxidase-stained Western blots and periodic acid-silver-stained gels. The latter stain allows visualization of glycoproteins and polysaccharides that contain 1,2 diol groups (13).

Conversion of hydrophilic cells to hydrophobicity by treatment with TM, an inhibitor of N mannosylation of secreted proteins, suggests that changes in mannosylation levels of surface proteins influences surface hydrophobicity status. Such a mechanism implies that the same external surface proteins of *C. albicans* can cause surface hydrophobicity and hydrophilicity depending on their level of glycosylation. Alternatively, removal of glycosyl groups from one surface protein allows exposure of the hydrophobic regions of a neighboring surface protein. If a single protein is accountable, then its cell surface concentration is expected to be higher than that of other surface proteins. It is noteworthy that *C. albicans* LGH1095 and LGH870 appear to expose similar surface proteins when the cells are hydrophobic. However, isolate LGH870 exhibits additional proteins (compare Fig. 2 with Fig. 3). When the hydrophobic avidities of these isolates were tested, they were found to possess a family of hydrophobic sites of equal avidity (24). However, isolate LGH870 had an additional family of hydrophobic sites which were of higher avidity. The additional proteins of isolate LGH870 may account for the appearance of the second family of hydrophobic sites.

Surface hydrophobicity has also been shown to participate in adherence of *C. albicans* to epithelial cells and whole

tissues (21; K. C. Hazen, D. L. Brawner, M. A. Jutila, and J. E. Cutler, manuscript submitted). A mannoprotein adhesin has also been shown to be involved in adhesion of *C. albicans* to epithelial cells. Numerous reports (for reviews, see references 6, 12, and 27) have demonstrated that adherence of *C. albicans* to epithelial cells is mediated by a mannoprotein, with the mannosyl moiety acting as the specific adhesin. The evidence is based primarily on the ability of concanavalin A, a lectin that recognizes mannose and glucose groups, to block adherence. Critchley and Douglas (8), however, have suggested that the protein moiety of the mannoprotein adhesin is actually responsible for adherence to epithelial cells. If the same surface molecule has hydrophobic characteristics and has the specific mannosyl ligand (adhesin), then exposure of such a mannoprotein would represent an efficient mechanism for adhesion.

Tronchin et al. (42) reported that four proteins (60, 68, 200, and >200 kDa) were involved in attachment of germ tubes to polystyrene petri dishes. We have shown that germ tubes are hydrophobic (18). These proteins remained attached to the dishes after the germ tubes were scraped off the dishes with a rubber policeman. It is likely that one or more of these proteins are involved in hydrophobic interactions between the plastic and the germ tubes, as CSH is clearly involved in yeast cell attachment to unmodified polystyrene such as bacteriological petri dishes (for a review, see K. C. Hazen, *in* R. J. Doyle and M. Rosenberg, ed., *Microbial Cell Surface Hydrophobicity*, in press). However, whether all of the proteins detected by Tronchin et al. (42) participate in the hydrophobicity-mediated attachment or whether one or more are anchoring proteins made in response to contact with the plastic surface (30) is not known.

Our results suggest the intriguing possibility that external cell wall protein changes are responsible for expression of surface hydrophobicity and hydrophilicity. These changes may be related to the presence or orientation of protein glycosyl groups. Further investigation is needed to determine whether the highly glycosylated high-molecular-weight proteins present on both hydrophobic and hydrophilic cells are directly responsible for hydrophobicity and hydrophilicity, depending on which regions of the glycoproteins are exposed to the environment, or whether they indirectly affect expression of hydrophobicity by masking low-molecular-weight hydrophobic proteins.

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