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The Glycolytic Enzyme Glyceraldehyde-3-Phosphate Dehydrogenase of *Candida albicans* Is a Surface Antigen

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A λgt11 cDNA library from *Candida albicans* ATCC 26555 was screened by using pooled sera from two patients with systemic candidiasis and five neutropenic patients with high levels of anti-*C. albicans* immunoglobulin M antibodies. Seven clones were isolated from 60,000 recombinant phages. The most reactive one contained a 0.9-kb cDNA encoding a polypeptide immunoreactive only with sera from patients with systemic candidiasis. The whole gene was isolated from a genomic library by using the cDNA as a probe. The nucleotide sequence of the coding region showed homology (78 to 79%) to the *Saccharomyces cerevisiae TDH1* to *TDH3* genes coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and their amino acid sequences showed 76% identity; thus, this gene has been named *C. albicans TDH1*. A rabbit polyclonal antiserum against the purified cytosolic *C. albicans* GAPDH (polyclonal antibody [PAb] anti-CA-GAPDH) was used to identify the GAPDH in the β-mercaptoethanol extracts containing cell wall moieties. Indirect immunofluorescence demonstrated the presence of GAPDH at the *C. albicans* cell surface, particularly on the blastoconidia. Semiquantitative flow cytometry analysis showed the sensitivity of this GAPDH form to trypsin and its resistance to be removed with 2 M NaCl or 2% sodium dodecyl sulfate. The decrease in fluorescence in the presence of soluble GAPDH indicates the specificity of the labelling. In addition, a dose-dependent GAPDH enzymatic activity was detected in intact blastoconidia and germ tube cells. This activity was reduced by pretreatment of the cells with trypsin, formaldehyde, and PAb anti-CA-GAPDH. These observations indicate that an immunogenic, enzymatically active cell wall-associated form of the glycolytic enzyme GAPDH is found at the cell surface of *C. albicans* cells.

Candida albicans is one of the most frequently encountered etiologic agents that cause opportunistic infections such as so-called candidiasis, a disease whose systemic manifestation could prove fatal and whose incidence is increasing as a result of an expanding immunocompromised population (5, 37, 39).

The diagnosis of invasive candidiasis is usually difficult to establish by clinical criteria; therefore, culture techniques and serodiagnostic tests for antigen and antibody detection have been employed as a laboratory aid to diagnosis. Blood cultures for *Candida* species generally exhibit low sensitivity (39), and the tests for determination of marker antigens need further fine-tuning to improve their sensitivity and specificity so that they will be valuable in guiding clinical treatment decisions (38, 43, 44). Likewise, standard immunological tests to detect anti-*Candida* antibodies usually have low specificity and/or sensitivity, since they mainly recognize antibodies against *Candida* cell wall mannan, which are ubiquitous in human sera (26, 54, 58); furthermore, the crude preparations of candidal antigens cannot be standardized enough to allow good test reproducibility among laboratories (26). Despite the considerable heterogeneity of the humoral responses to antigens of *C. albicans* in humans, several cytosolic antigens have been identified. These include enolase (55), a 47-kDa fragment of a 90-kDa heat shock protein (hsp90) (34, 36), a 75-kDa heat shock protein, and a non-heat shock protein 96-kDa antigen (11), and some other glycolytic enzymes such as pyruvate kinase and alcohol dehydrogenase (52). Interestingly, some cytosolic proteins, such as enolase, members of the hsp70 family, and phosphoglycerate kinase, have been described to be bona fide components of *C. albicans* cell walls (2, 3, 31). Although some of these moieties have been used as potential marker antigens for antibody detection in invasive candidiasis (20, 26, 33, 35, 58), a simple, reliable, and easy-to-perform serological assay for the diagnosis of systemic *Candida* infections has not yet been developed.

In the present study, we have screened a *C. albicans* cDNA library for sequences that encode immunogenic proteins by using pooled sera from patients with a high level of anti-*C. albicans* antibodies in order to identify antigens potentially useful for diagnosis of candidiasis or that may play a role in infection. By this approach, a cDNA clone coding for another enzyme of the glycolytic pathway, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was obtained. The whole gene has been isolated, and the enzyme has been found to be present and enzymatically active in the cell wall as well as in the cytoplasm.

MATERIALS AND METHODS

Strains used and culture conditions. *C. albicans* ATCC 26555 was employed in this study. It was maintained by subculturing on 1.5% Bacto Agar slopes of Sabouraud dextrose medium. Cells were propagated as blastoconidia at 28°C in a minimal medium supplemented with amino acids as described by Lee et al. (29), harvested, and stored at 4°C for 72 to 96 h (starvation period) as reported previously (7). Starved blastoconidia were inoculated (200 μg [dry weight] of cells per ml) in fresh Lee medium at 28°C to obtain cultures of blastoconidia or at 37°C for the formation of blastoconidia bearing germ tubes (7). *Escherichia coli* Y1090 was used for immunoscreening of the cDNA library, and the plasmidic genomic library was in *E. coli* JM109; both strains were cultured and manipulated according to the method described by Sambrook et al. (45).

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Screening of cDNA and genomic libraries. A λ cDNA library constructed from *C. albicans* mycelial cells (48) was immunoscreened with pooled sera from seven individuals (two patients with systemic candidiasis and five neutropenic patients) with high levels of anti-*C. albicans* immunoglobulin M (IgM) and IgG antibodies following previously described procedures (45, 48). About 60,000 PFU were plated in petri dishes, and the dishes were incubated for 3 h at 42°C for induction of the phage lytic cycle; thereafter, a Hybond-N filter (Amersham) saturated with 10 mM isopropyl-β-D-thiogalactoside was layered onto the plates to induce production of the β-galactosidase–protein fusion, and the plates were incubated overnight at 37°C. Filters were immunodetected with pooled sera (diluted 1:100) as previously described (1, 7). Peroxidase-labelled goat anti-human IgMs (diluted 1:2,000) were used as indicator antibody.

The cDNA from the positively selected clone was isolated with the λ forward and reverse universal primers to amplify recombinant phage DNA by standard polymerase chain reaction protocols (24) and subcloned into pGEM-T (Promega) following the manufacturer's instructions; this cDNA fragment was used as a probe to screen a genomic library constructed from *C. albicans* WO-1 chromosomal DNA in pEMBL Ye23 (4), according to standard procedures (45). Labelling of cDNA probes and hybridizations were performed by the digoxigenin labelling and detection kits from Boehringer Mannheim.

Southern and Northern blots and sequencing. Preparation of plasmidic DNAs from bacterial strains and chromosomal DNA from *C. albicans* ATCC 26555, digestion with restriction endonucleases, agarose gel electrophoresis, and transfer to Hybond-N filters (Amersham) were carried out according to the method described by Sambrook et al. (45). Labelling of cDNA probes and hybridization conditions were performed as mentioned above. Total RNAs from *C. albicans* yeast and germ tube cells (after 3 h of germ tube induction) were obtained as described by Denis et al. (12). RNA gel electrophoresis and transfer to Hybond-N filters (Amersham) were carried out by previously described methods (45). For hybridization and chemiluminiscence detection, the Boehringer Lumigen PPD kit was used.

Sequencing of cDNA and genomic DNA fragments was completed with universal forward and reverse primers as well as synthetic oligonucleotides (Pharmacia Biotech.) deduced from the sequence. Both DNA strands were sequenced. Sequencing reactions were performed with the *Taq* Dye Dideoxy Terminator Sequencing kit (Applied Biosystems), and the nucleotide sequences were determined with an Applied Biosystems automated DNA sequencer (model 373A). Nucleotide and amino acid sequences were analyzed by the FASTA program and the BLAST network service.

Preparation of cell extracts. Protein and glycoprotein components of the walls were released from intact cells by treatment with β-mercaptoethanol (βME) as previously described (6, 8, 9). The βME-extracted cells were treated with a β-(1,3)-glucanase complex (Zymolyase 20T) to obtain protoplasts, which were lysed, and the supernatant (cytosolic extract) was recovered following centrifugation as previously described (8, 9). The total sugar and protein contents were determined by the methods described by Dubois et al. (13) and Lowry et al. (32), respectively.

Generation of PAbs. The cytosolic extract (1 mg of protein) from blastoconidial cells was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on linear slab gels (12.5% acrylamide) with a 6% acrylamide stacking gel, basically according to the procedure previously described (28), and the protein species were transferred to nitrocellulose paper (7, 8). A small section of the paper was revealed by immunoblotting with a monoclonal antibody (MAb) to the GAPDH from chicken (MAb anti-CH-GAPDH). The immunodetected protein was solubilized from the paper and used to immunize female New Zealand White rabbits as previously described (9). Levels of anti-GAPDH antibodies in sera were determined by an enzyme-linked immunosorbent assay (ELISA) with GAPDH from *Saccharomyces cerevisiae* (Sigma) and cytosolic extracts of *C. albicans* as test antigens.

Western blotting. Proteins were separated by SDS-PAGE as already described (7, 28) and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). Immunodetection with the MAb anti-CH-GAPDH (diluted 1:1,000) was performed following procedures already described (7, 9); peroxidase-conjugated goat anti-mouse Igs (diluted 1:2,000) was used as a second antibody, and labelled proteins were detected with the enhanced chemiluminescence detection system (Amersham) according to the manufacturer's guidelines. When the PAb raised against *C. albicans* GAPDH was used (final dilution of 1:1,000), peroxidase-labelled goat anti-rabbit Igs (diluted 1:1,500) was used as the indicator antibody, and the reactive bands were developed with hydrogen peroxide and 4-chloro-1-naphthol as the chromogenic reagent. The GAPDHs from *S. cerevisiae* and from chicken muscle (Sigma) were used as control proteins.

Indirect immunofluorescence (IIF). Fungal cells (blastoconidia and germinated blastoconidia) collected from exponentially growing cultures were washed twice with 10 mM phosphate buffer (pH 7.4)–150 mM NaCl (phosphate-buffered saline [PBS]) and resuspended (10^6 cells per ml) in PAb anti-CA-GAPDH diluted 1:10 in PBS. After 1 h at room temperature with gentle shaking, the cells were washed four times with PBS and resuspended in fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Igs (1:10 dilution) in PBS, and the suspensions were incubated under the same conditions. Finally, the cells were washed again, resuspended in 100 μl of PBS, and examined (wet mountings) for epifluorescence (UV filter no. 487702; excitation line, 365/366 nm) (7). Control experiments were performed by omitting incubation of cells with the PAb anti-CA-GAPDH.

Flow cytometry analysis. Immunofluorescence assaying with the PAb anti-CA-GAPDH was performed by the procedure described above. Blastoconidia were subsequently fixed in 1% paraformaldehyde solution in PBS and analyzed by flow cytometry. Flow cytometry analyses were performed on an EPICS Elite Cell Sorter (Coulter Electronics, Inc., Hialeah, Fla.) as previously described (42). Duplicate samples were processed in the absence of PAb anti-CA-GAPDH as negative controls.

In some experiments, cells were incubated with different amounts of trypsin for 30 min at 37°C (proteolytic treatment was stopped by three washes with PBS containing 3% [wt/vol] bovine serum albumin) and assayed by IIF and flow cytometry. In other instances, cells were treated with 1% βME in sterile glass-distilled water for 1 h at 37°C, prior to being analyzed by IIF and flow cytometry. When indicated, IIF reactions were determined in the presence of increasing concentrations of GAPDH from *S. cerevisiae* (Sigma).

Determination of GAPDH and other enzyme activities. The assay for GAPDH activity was carried out according to the method originally described by Ferdinand (14), with minor modifications. An assay for intact whole cells was developed to determine whether the GAPDH on the surface of *C. albicans* cells functions as an active enzyme. Duplicate samples of different amounts of exponentially growing cells were incubated with and without glyceraldehyde-3-phosphate (G-3P) (7 μl of a solution containing 49 mg of substrate per ml; Sigma) in the presence of NAD (100 μl of a 10 mM solution; Boehringer-Mannheim) in assay buffer (40 mM triethanolamine, 50 mM Na_2HPO_4, 5 mM EDTA, 0.1 mM dithiothreitol [pH 8.6]) to a final volume of 1 ml. After incubation of the reaction mixtures at room temperature, cells were removed by centrifugation and the supernatants were analyzed for the presence of NADH by recording the absorbance at 340 nm with a Shimadzu UV-160 spectrophotometer. Activity was expressed as the concentration (micromolar) of NADH generated during the assay. Enzymatic activities on trypsinized (as described above) blastoconidia, on cells prefixed with 1% (vol/vol) formaldehyde (15 min at room temperature), and on cells preincubated with PAb anti-CA-GAPDH (1 h at 37°C) were also determined. In some experiments, yeast cells from exponentially growing cultures were preincubated either (i) with a cytosolic *C. albicans* extract or (ii) with a solution of GAPDH from *S. cerevisiae* prior to activity determination.

The amounts of carboxypeptidase Y and α-mannosidase in intact whole cells were determined basically according to the procedures described by Schwencke et al. (46) and van der Wilden et al. (53), respectively.

Nucleotide sequence accession number. The accession number to the GenBank/EMBL databases of the nucleotide and predicted amino acid sequences of the *C. albicans TDH1* gene described in this paper is U72203.

RESULTS AND DISCUSSION

Isolation of cDNA clones encoding immunogenic polypeptides. Since the most useful markers in patients with first-time *C. albicans* sepsis are IgM antibodies to blastoconidial antigens (20), we used a pool of sera prepared from two patients with proved *C. albicans* sepsis (evidenced by positive blood cultures) and five neutropenic patients (with high levels of anti-*C. albicans* IgG and IgM antibodies) to screen a cDNA library, looking for polypeptides reacting with IgM antibodies. Immunoscreening of the cDNA library with the pooled sera resulted in the isolation of seven clearly positive clones after three successive rounds of screening. The cDNA insert of the strongest immunoreactive clone was amplified by PCR, resulting in a 0.9-kb fragment which was subcloned into pGEM-T. With this cDNA as a probe, it was found that the other three positive clones initially isolated were homologous to the selected one. The reactivities of this clone against the individual serum samples of the pool were analyzed. Lysis plaques of this clone obtained under conditions of induction of the fusion protein were strongly recognized by two individual serum samples and three serum samples gave a weak reaction, whereas two serum samples did not react at all (not shown). Interestingly, the two serum samples immunoreactive against the polypeptide encoded by the selected cDNA clone were those from the patients with recognized systemic candidiasis (positive blood culture). Although a larger patient population must be tested, this preliminary result suggests a correlation between the existence of systemic *C. albicans* infection and the presence of IgM antibodies against the polypeptide encoded by the cDNA clone.

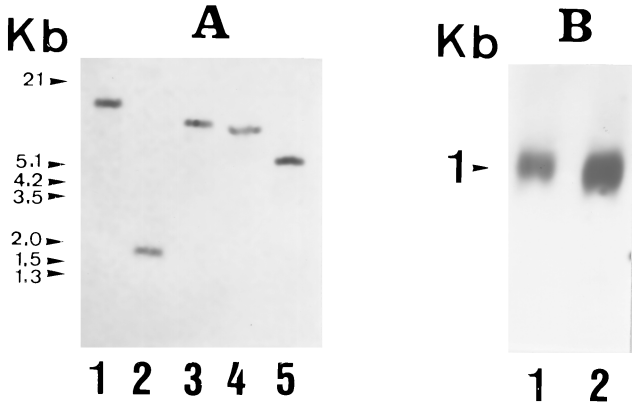

FIG. 1. (A) Southern blot analysis of chromosomal DNA obtained from *C. albicans* ATCC 26555. Each lane contains 10 μg of DNA digested with *Sal*I (lane 1), *Hin*dIII (lane 2), *Bam*HI (lane 3), *Eco*RI (lane 4), or *Pst*I (lane 5). The 0.9-kb cDNA clone selected by the immunoreactivity of the encoded fusion protein against the pooled sera was used as a probe. Molecular size markers are indicated. (B) Northern blot analysis. Total RNAs were obtained from yeast cells (lane 1) and germinated blastoconidia (lane 2) after 3 h of germ tube induction of *C. albicans* ATCC 26555, 10 μg of RNA was loaded onto each well, and the 0.9-kb cDNA fragment selected by the immunoscreening of the cDNA library with the pooled sera was used as a probe.

Characterization of cloned cDNA. Southern blot analysis of *C. albicans* genomic DNA probed with the 0.9-kb cDNA fragment revealed, for all five cutting enzymes used, one single hybridizing band (Fig. 1A), although with *Hin*dIII, the only enzyme that cuts within the probe, a small DNA fragment (0.2 kb) was not detected. The high-stringency conditions used suggest that one copy of the gene homologous to the cDNA probe is present in the *C. albicans* genome, although additional genes displaying partial sequence homology with the probe may also encode GAPDH proteins. Northern blot analysis with the same probe revealed the presence of one relatively abundant mRNA species of about 1 kb present both in yeast and germ tube cells (Fig. 1B). Sequencing of this cDNA was performed, and comparison of the nucleotide sequence with the EMBL/GenBank database sequences showed significant homology (about 78%) to the *S. cerevisiae TDH1* to *-3* genes coding for GAPDH (21–23). The cDNA sequence (884 bp) contains the 3′ end of the gene and an open reading frame showing high identity (76%) to the yeast GAPDH but lacks the 5′ end of the coding region.

The identification of glycolytic enzymes as immunogens during candidiasis is well documented. Enolase is known to be an abundant immunodominant antigen in *C. albicans* infection (55). Antibodies to candidal pyruvate kinase and alcohol dehydrogenase in sera from patients suffering from superficial candidiasis have also been reported (52). In addition, IgE antibodies present in sera from allergic patients are reactive with alcohol dehydrogenase (49) and with enolase, phosphoglycerate kinase, and aldolase (25). Therefore, our data extend the above list of immunogenic glycolytic enzymes to GAPDH. The fact that four of the seven isolated clones were homologous indicates that GAPDH is a major antigen in *C. albicans*, although it should be noted that highly expressed genes are also highly abundant in the cDNA libraries and, thus, that their cloning is statistically favored.

Cloning of the *C. albicans* gene coding for GAPDH. The screening of a genomic library with the cDNA as a probe resulted in the isolation of five positive clones. Restriction and Southern blot analysis demonstrate that all five clones were identical (not shown); thus, only one single clone was obtained. This clone contains a 6.5-kb fragment of the *C. albicans* genome which contains the whole gene homologous to the cDNA probe. This gene was sequenced, and it was found to contain a coding sequence of 993 nucleotides coding for a 331-amino-acid polypeptide. The coding region shows significant homology (78 to 79%) with the *S. cerevisiae TDH1-3* genes and also with the chicken GAPDH gene (67%) (50). Comparison of the amino acid sequences indicates a high degree of identity to the *S. cerevisiae* (76%) and the chicken (67%) proteins. Thus, we have cloned a *C. albicans* gene coding for GAPDH, which was designated *C. albicans TDH1* (where TDH is triose phosphate dehydrogenase).

Reactivities of MAb anti-CH-GAPDH and PAb anti-CA-GAPDH with the protein encoded by the cDNA clone and with *C. albicans* extracts. To confirm that the cloned cDNA encodes the *C. albicans* GAPDH, we investigated whether the fusion protein produced by the phage recombinant clone was recognized by the MAb anti-GAPDH from chicken. The results showed that this MAb recognized the fusion protein present in the plaque from the recombinant phage containing the cloned cDNA, since no immunoreactivity was detected in a control plaque from a phage lacking the cDNA insert (Fig. 2A). Afterward, we performed an immunoblot analysis with this MAb to further confirm that it recognizes the GAPDH from *C. albicans* (Fig. 2B). The MAb reacted with the control protein (GAPDH from chicken) (Fig. 2B, lane 1) and immunodetected a major band with the same apparent molecular weight as that of the control protein (33 kDa) in the cytosolic extracts from blastoconidia (Fig. 2B, lane 4) and germinated blastoconidia (Fig. 2B, lane 5). These results confirmed that the cloned cDNA codes for the *C. albicans* GAPDH.

Because the GAPDH enzyme has been described to be present on the surface of cells of several microbial species (15–17, 40) and two other glycolytic enzymes from *C. albicans* (enolase and 3-phosphoglycerate kinase) have been detected in the cell wall (2, 3), we searched for the presence of a cell wall-associated form of GAPDH in βME extracts obtained from intact *C. albicans* cells, since it is well established that such extracts contain bona fide cell surface molecules without

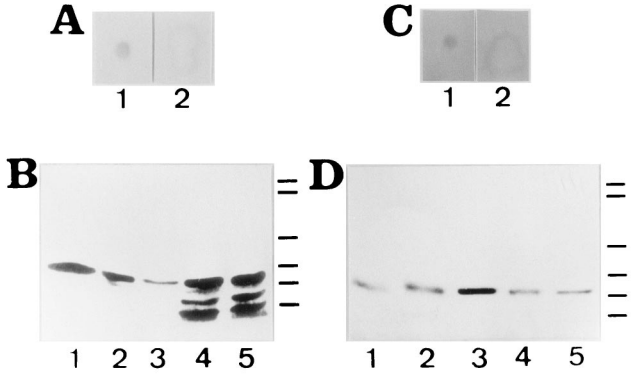

FIG. 2. Immunoblot analysis with the MAb anti-CH-GAPDH (A and B) and with the PAb anti-CA-GAPDH (C and D). (A and C) Reactivities of the antibodies with the fusion protein present in the plaques produced by the selected λ recombinant cDNA clone (lane 1) or with plaques from a λgt11 clone lacking a cDNA insert used as a negative control (lane 2). (B and D) Western immunoblot analysis of βME extracts (lanes 2 and 3; samples applied to each well contained 600 μg of material, expressed as total sugar content) and cytosolic extracts (lanes 4 and 5; 50 μg of protein per well) from blastoconidia (lanes 2 and 4) and germinated blastoconidia (lanes 3 and 5). Lanes 1, control GAPDH proteins from chicken (0.01 μg [B]) and from *S. cerevisiae* (2 μg [D]). Migration of proteins used as molecular mass standards (101, 83, 50.6, 35.5, 29.1, and 20.9 kDa [from top to bottom]) is shown.

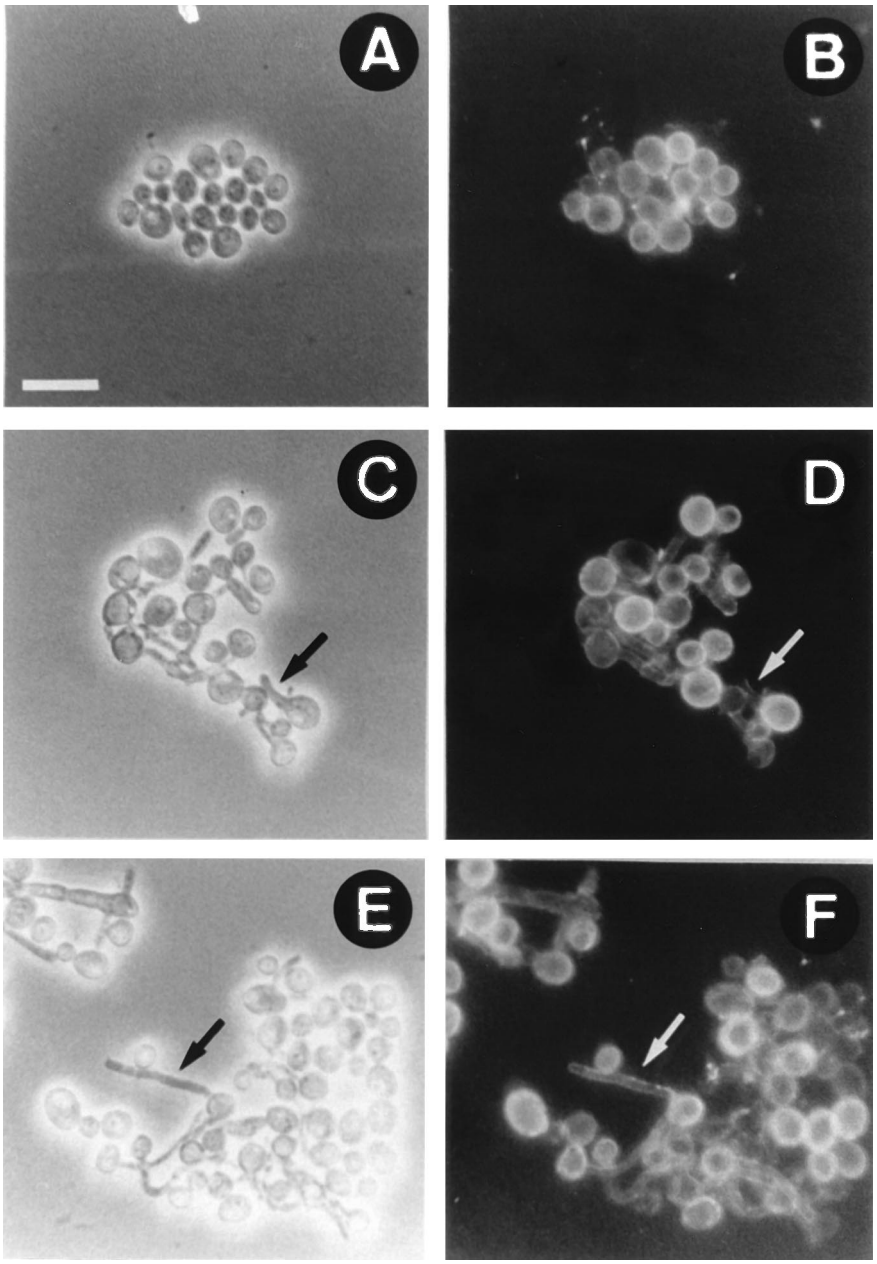

FIG. 3. Surface localization of GAPDH by IIF with PAb anti-CA-GAPDH. Blastoconidia (A and B) and germinated blastoconidia (C to F) were incubated with the PAb anti-CA-GAPDH and FITC-conjugated goat anti-rabbit Ig as described in Materials and Methods. (A, C, and E) Phase-contrast microscopy; (B, D, and F) UV illumination (fluorescence). Note the fluorescence at the blastoconidial surface (B). The germ tube surface visualized by phase-contrast microscopy (C and E [arrows]) exhibited a weak fluorescence (D and F [arrows]). Bar, 10μm.

significant intracellular contamination (2, 8, 30, 31). Among the numerous proteins which are removed from the cell wall by βME extraction of intact cells (6, 8, 9), the MAb anti-CH-GAPDH detected a major protein species of 33 kDa in cell wall extracts from both blastoconidia and germinated blastoconidia (Fig. 2B, lanes 2 and 3), which indicates that GAPDH is also associated with the *C. albicans* cell wall.

PAbs generated against cytosolic GAPDH from blastoconidial cells were used to investigate the immunoreactivity of the fusion protein produced by the cDNA clone. PAb anti-CA-GAPDH reacted against this fusion protein (Fig. 2C) as previously described for the MAb anti-CH-GAPDH (Fig. 2A). Afterward, we performed a Western blot analysis with different extracts from *C. albicans* cells. The PAb preparation recognized a major protein species of 33 kDa present in cytosolic extracts from both blastoconidia and germinated blastoconidia (Fig. 2D, lanes 4 and 5) and also in βME extracts from blastoconidia (Fig. 2D, lane 2) and germ tubes (Fig. 2D, lane 3), thus indicating the presence of GAPDH both in the cytosol and the cell wall. The PAb anti-CH-GAPDH also recognized the GAPDH from *S. cerevisiae* that was used as a control (Fig. 2D, lane 1). The specificity of the reaction was further supported by the fact that the presence of soluble GAPDH from *S. cerevisiae* (assayed at a concentration of 1 mg/ml) in the PAb

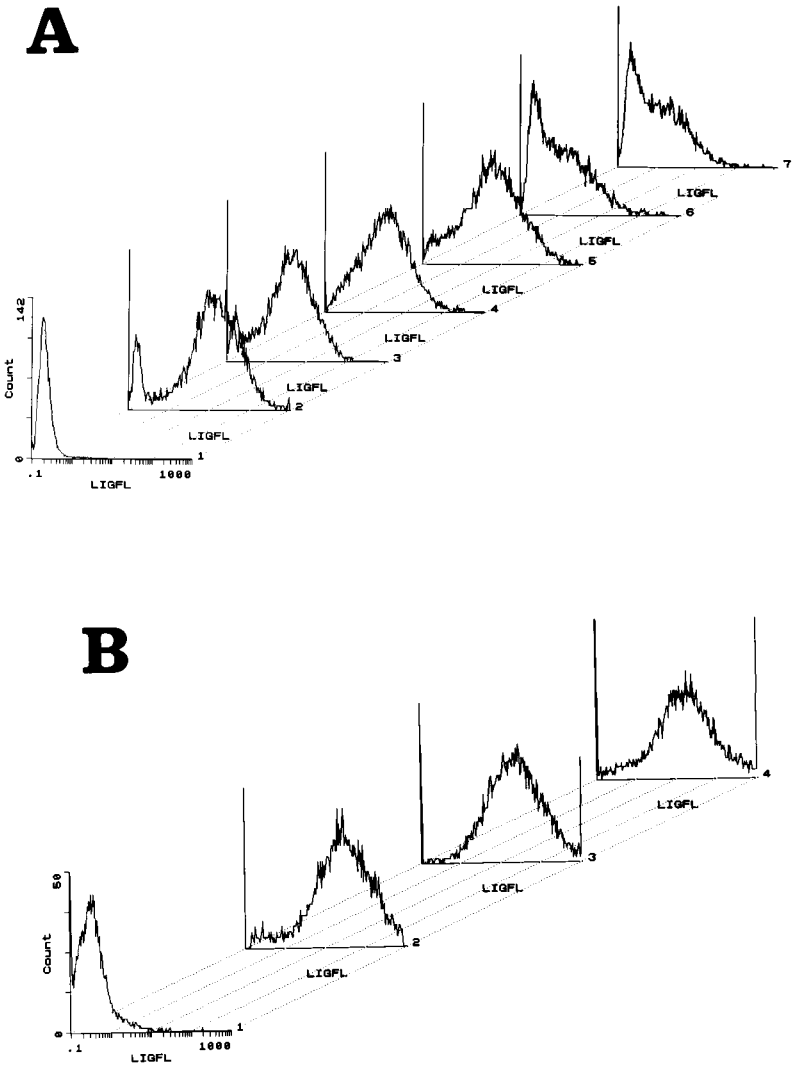

FIG. 4. Flow cytometry analysis with PAb anti-CA-GAPDH. The assay determines the extents of reactivity of PAb anti-CA-GAPDH to the yeast cell surface exposed protein before (A and B, assays 2) and after trypsin (A, assay 3), βME (A, assay 4), 2 M NaCl (B, assay 3), and 2% SDS (B, assay 4) treatments. IIF assaying was also performed in the presence of 0.1, 0.5, and 1 mg of GAPDH from *S. cerevisiae* per ml (A, assays 5 to 7). Control assays were performed in the absence of PAb anti-CA-GAPDH (A and B, assays 1). *x* axis, log of fluorescence intensity (LIGFL); *y* axis, number of fluorescent cells.

preparation used to develop the Western blot inhibited immunodetection of the 33-kDa protein (not shown).

Immunodetection of GAPDH on the surface of *C. albicans* cells. PAb anti-CA-GAPDH was used in an IIF assay to assess the presence of the protein on the surface of intact cells (Fig. 3). The strong fluorescence observed in most of the blastoconidial cells indicated that the GAPDH is exposed on their surfaces (Fig. 3B). When the IIF assay was performed with germinated yeast cells (Fig. 3D and F), most of the mother blastoconidia from which germ tubes originate were again strongly labelled, whereas the hyphal filaments showed only a faint fluorescence (Fig. 3D and F [arrows]). The reactivity appeared to depend on the previous interaction of the specific antibody (PAb anti-CA-GAPDH) with cells, since fluorescence was not observed when the cells were reacted with the second FITC-labelled marker antibody alone.

To confirm the surface location of the protein, blastoconidia subjected to different treatments were analyzed by IIF and flow cytometry (Fig. 4). Trypsin treatment of intact cells resulted in a marked reduction (65%) in their reactivities to PAb anti-CA-GAPDH (Fig. 4A, assays 2 and 3). The inability to remove the GAPDH protein from the cell surface after treatment with 2 M NaCl or 2% (wt/vol) SDS (Fig. 4B) indicates that the protein is tightly bound to the cell wall and is not weakly associated with this structure as a contaminant intracellular species that may rebind to the cell surface after being released by lysed or dying cells. Finally, treatment with βME also reduced the fluorescence intensity (65% inhibition) (Fig. 4A, assay 4), which is in accordance with the presence of the protein in the βME extract (Fig. 2B and D, lane 2). Binding of PAb anti-CA-GAPDH to cells was found to be specific as the fluorescence intensity decreased (40, 88, and 89% of inhibition) when the IIF assays were performed in the presence of different concentrations (0.1, 0.5, and 1 mg/ml, respectively) of soluble GAPDH from *S. cerevisiae* (Fig. 4A, assays 5 to 7).

Cell wall-associated forms have been described for two other candidal glycolytic enzymes; enolase has been found in the culture supernatant and in the inner layers of the cell wall but

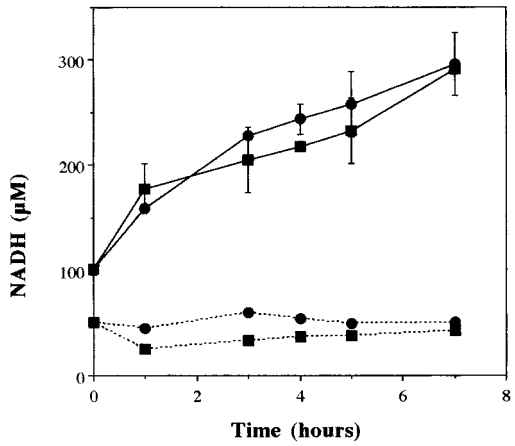

FIG. 5. GAPDH activity in intact cells. Enzyme activity was measured by determining the conversion of NAD to NADH in the presence (continuous line) or absence (dotted line) of G-3P. Activity is expressed as the concentration (micromolar) of NADH in the supernatants after incubation of the assay mixture. Activity was determined in whole cells that were incubated after the starvation period (see Materials and Methods) at 28°C to obtain yeast morphology (■) and at 37°C to induce germ tube formation (●); cells (2.5 ml of culture) were collected periodically as indicated, and the time of the enzymatic reaction was 5 min.

is not exposed at the cell surface (3, 51), and phosphoglycerate kinase has been detected at the cell surface of *C. albicans* cells and has also been detected to extend through the cell wall (2).

GAPDH activity in intact cells. To determine whether the surface GAPDH protein was active, enzymatic studies were carried out with intact cells. In the first set of experiments, activities in intact whole blastoconidia and germinated blastoconidia were determined during their growth either at 28 or 37°C, respectively, after starvation (see Materials and Methods). The results (Fig. 5) revealed a dose-dependent GAPDH activity catalyzed by both growth forms, yeasts and hyphae, which increased proportionally to cell growth. The intact cells did not catalyze the formation of NADH in the absence of the substrate (G-3P). The enzymatic activity was significantly decreased when trypsinized or formaldehyde-treated blastoconidia were used in the reaction mixture (Table 1). The enzymatic activity of intact blastoconidia was also found to be partially (by roughly 30%) but specifically inhibited by the PAb anti-CA-GAPDH (Table 1).

Although the results reported above indicate that active GAPDH is an autochthonous cell surface protein, we further investigated the possibility that rebinding to intact *C. albicans* cells of cytosolic enzyme released following cell lysis occurs. Incubation of *C. albicans* cells with cytosolic extracts and soluble GAPDH protein prior to determination of activity (see Materials and Methods) did not result in any increase in the amount of NADH generated during the assay. During the assay, yeast cells (3×10^8) from an exponentially growing culture were resuspended in 0.1 ml of PBS buffer containing 0.1 mg of protein of a *C. albicans* cytosolic extract and 0.1 mg of GAPDH from *S. cerevisiae*, and the suspensions incubated at room temperature for 30 min. After incubation, the cells were pelleted and washed, and GAPDH activities were determined. In this assay, the results (expressed as mean concentrations [micromolar] of NADH generated in the assay mixtures from triplicate determinations ± standard deviations) were as follows: control, 260 ± 4; yeast cells plus cytosolic extract, 265 ± 6; yeast cells plus GAPDH, 257 ± 13; cytosolic extract, 311 ± 10; and GAPDH, 560 ± 18. For cytosolic extract and GAPDH values, activity was determined for samples containing 10 μg of total protein. These results indicate that cytosolic GAPDH does not stick to the surface of intact cells.

Determination of other intracellular activities, such as carboxypeptidase Y and α-mannosidase associated with the yeast tonoplast and with vacuolar content, respectively (18, 46, 53, 56), was used to check cell lysis. Both activities were not detected when they were assayed in whole cells after overnight incubation of the assay mixtures, whereas significant levels of activity were detected in cell extracts (obtained by ballistic disruption of cells) used as positive controls (not shown), thus indicating the absence of lysed cells in the assay mixtures.

Quantification of the percentage of GAPDH present in the cell wall is difficult to assess, since the measurement of intracellular enzyme requires the preparation of cell extracts, whereas that of the cell wall GAPDH is determined in intact cells, and, in addition, it is not known whether the latter retains the same level of activity as the cytosolic counterpart. However, from the activity data, we estimated that the cell wall-bound GAPDH roughly represents 20 to 35% of the total GAPDH protein present in yeast cells; this percentage decreases (5 to 15%) when quantitation is made from Western blots. Yet this value is most likely underestimated, because processing of βME extracts prior to immunoblotting may cause partial loss of protein. The presence of GAPDH in the cell wall and the absence of a conventional N-terminal signal sequence suggest the involvement of a nonclassical secretory pathway in the targeting of this protein, as described for a number of prokaryotic, fungal, and higher eukaryotic proteins (27). Whether the cell surface GAPDH activity is the product of the *C. albicans TDH1* gene or is encoded by a different *TDH* gene remains to be determined.

On the other hand, although the mRNA levels appear to be higher in mycelial cells, IIF assaying indicates that yeast cells are more strongly labelled, suggesting either that there is less GAPDH protein in hyphal cell walls or that it is topologically less accessible to the probe; this second possibility is supported by the fact that enzymatic activities in both yeast and germ tube *C. albicans* cells are similar. A different topological location of the protein in the cell wall may account for the lack of correlation between IIF and GAPDH activity, and it is in accordance with the contention that changes in the cell wall organization occur during the morphogenetic transition (10, 19, 47); taking into account that GAPDH is a surface antigen, as

TABLE 1. Effect of trypsin, formaldehyde, and PAb anti-CA-GAPDH on GAPDH activity in *C. albicans* yeast cells

Treatment[a]	Activity (μM)[b]	Inhibition (%)[c]
None (control)	265 ± 7	0
+Trypsin	52 ± 8	80
+Formaldehyde	83 ± 9	69
None (control)	303 ± 3	0
+PAb (1:50 dilution)	221 ± 20	27
+PAb (1:10 dilution)	214 ± 3	30

[a] Yeast cells (3×10^8) from exponentially growing cultures were collected and treated as indicated (see Materials and Methods for further details) prior to determination of enzymatic activities.

[b] Expressed as the concentration of NADH generated in the assay mixture; the time of the enzymatic reaction was 5 min, and the results shown are the mean values of triplicate determinations with standard deviations.

[c] Percentage of inhibition with reference to the respective control values.

described here, these changes may help hyphal cells to evade the host immune system.

GAPDH has also been associated with a surface location and different functions in microbial species. First, the enzyme has been reported as a major surface protein on group A streptococci (*Streptococcus pyogenes*), where it is enzymatically active, binds various mammalian proteins such as lysozyme, fibronectin, actin, myosin, and plasmin (40, 57), and also acts as an ADP-ribosylating enzyme (41). Second, in *Kluyveromyces marxianus*, the same enzyme protein has been identified as a constitutive protein of cell wall putatively involved in the interactions between cells leading to flocculation (15, 16). Third, Goudot-Crozel et al. (17) presented evidence for an active GAPDH on the surface of *Schistosoma mansoni* cells associated with human resistance to schistosomiasis, proposing that GAPDH may be a putative vaccine against human schistosomiasis. These observations raise the question of whether GAPDH in *C. albicans* may also have other nonglycolytic functions associated with noncytosolic forms of the enzyme.

In summary, we have shown that the GAPDH of *C. albicans* is another immunogenic glycolytic enzyme; although a larger patient population must be tested, the preliminary results presented here indicate that IgM antibodies against GAPDH may represent a specific marker of fungal invasiveness. Besides its cytoplasmic localization, this protein is also an integral protein of the cell wall exposed at the cell surface, where it is enzymatically active, although its role in the cell wall has not yet been determined.

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