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Evidence for Presence in the Cell Wall of *Candida albicans* of a Protein Related to the hsp70 Family

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We have previously reported the isolation of several clones from a cDNA expression library from *Candida albicans*, one of which was associated with a constitutively expressed 70-kDa protein. The moiety was present in the β-mercaptoethanol extracts of cell walls from both blastoconidia and germ tubes. The surface expression of this moiety was revealed by an indirect immunofluorescence assay using affinity-purified antibody to the fusion protein produced by the clone. The 0.68-kb cDNA insert was sequenced. A database search revealed extensive homology with the 70-kDa family of stress or heat shock proteins (hsps). The 77% homology with another *C. albicans HSP70* sequence suggested that this fragment represented a second member of the *HSP70* family in this organism. Homology ranging from 65 to 76% was observed with members of four subfamilies (*SSA*, *SSB*, *SSC*, and *SSD*) of the *Saccharomyces cerevisiae HSP70* gene family. The nucleic acid sequence and the deduced amino acid sequence of the open reading frame showed greatest homology with *SSA1* and *SSA2* sequences, and the gene corresponding to the cDNA clone was designated *C. albicans* SSA2. The relationship with the *SSA* family was supported by reactivity of the 70-kDa component with antibody recognizing the Ssa proteins of *S. cerevisiae*. The presence of an hsp70 in the cell wall was confirmed by two additional methods. Cell wall proteins were biotinylated with a non-membrane-permeable derivative to distinguish extracellular from cytosolic proteins. Biotinylated hsp70 was detected by Western blotting (immunoblotting) among the biotinylated components affinity purified by chromatography on streptavidin, thereby establishing its presence in the cell wall. Immunoelectron microscopy showed that the 70-kDa component was present at the cell surface as well as the outer surface of the plasma membrane and extended through the cell wall, occasionally appearing to reach the cell surface through channels. Northern (RNA) blot analysis showed that the gene was expressed in yeast cells growing in yeast extract-peptone medium at both 25 and 37°C and in Lee medium at 25°C and during formation of germ tubes in Lee medium 37°C. No obvious increase in the expression level was detected after the temperature shift. Members of the hsp70 family have been reported to be immunoreactive. The fusion protein produced by the cDNA clone was recognized by serum from healthy individuals and patients with candidiasis. Since members of the hsp70 family of eucaryotic proteins are associated with chaperone and translocation functions, in addition to being immunogenic, this protein may play a role in the assembly and function of other cell wall proteins.

The dimorphic fungus *Candida albicans* is both a commensal and opportunistic pathogen of humans. It causes a wide range of infections in individuals who are deficient in some aspect of normal host defense (6, 58). In general, the severity of the infection correlates with the severity of the host defect, and the immunocompromised host is among those at risk for the most serious life-threatening infections. Both morphological forms of the organism interact with the host. The cell wall is the structure that maintains the shape characteristic for each morphology, acts as a permeability barrier, and, due to its external localization, is also the site of the initial interaction between the organism and the environment, including host cells and tissues (11). Glucan and chitin are the polymers that provide rigidity to the cell wall, and glyco(manno)proteins are embedded in the wall, as well as being dominant in the outer surface (19).

Several reports have demonstrated a high degree of complexity in the protein composition associated with the cell wall of this organism (13, 15, 20, 59). However, our present understanding of the different aspects of the biology of these protein constituents is still incomplete. Mannoproteins are thought to play important roles in almost every aspect of the biology of this organelle and may be involved in morphogenesis (17, 51) and in modulating several cell surface properties that can be survival or virulence factors (25), such as antigen presentation, immunomodulation, and adherence (11, 12, 19, 36). Hence, identification and characterization of individual cell wall components, and specifically proteins, could lead to a better understanding of the overall functions played by this supramolecular structure, including its role in pathogenesis.

Among the proteins so far identified in the cell wall of *C. albicans* there are number of moieties with receptor-like characteristics (1, 8, 9, 16, 39, 44, 45, 50, 68) and responsible for cell surface hydrophobicity and adhesion to plastic (31, 34, 35, 43, 67); several predominantly periplasmic enzymes (57, 60); enolase and 3-phosphoglycerate kinase, which may or may not have an enzymatic function in the cell wall (3, 4); form-specific molecules (14, 49, 64, 65); and a 47-kDa moiety that is a breakdown product of the candidal heat shock protein 90 (hsp90) (52, 55). We have recently reported the isolation of several cDNA clones coding for cell surface proteins of *C. albicans* (2). One of these cDNA clones, R, was associated with a 70-kDa protein which appeared to be constitutively expressed in both yeast cells and germ tubes. In the present report, we have characterized this protein as a member of the hsp70 family of proteins and confirmed its subcellular localization by means of biotinylation of extracellular components and

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immunoelectron microscopy. In addition, we have examined the reactivity of the fusion protein produced by the cDNA clone with human sera.

MATERIALS AND METHODS

Organisms and culture conditions. *C. albicans* 3153A was maintained by subculturing every 2 to 3 weeks on Sabouraud dextrose medium with 1.5% agar. The organism was propagated as yeast cells or germ tubes as previously described (14, 45) in the minimal medium supplemented with amino acids described by Lee et al. (42). For some experiments *C. albicans* was grown in 2% yeast extract–1% peptone–2% glucose medium (YEPD) at 25 or 37°C.

Cell wall extracts. Protein and glycoprotein components of the walls were released from intact cells by treatment with 2-mercaptoethanol (βME extract) as described before (13, 15). Briefly, organisms were extracted in alkaline buffer containing βME. The supernatant was recovered, dialyzed, and lyophilized. The Zymolyase extract was obtained by treatment of βME-extracted cells with enzyme, and recovery, dialysis, and lyophilization of the supernatant were performed as previously described (13, 15). Protoplasts were lysed in the presence of a buffer containing *n*-octylglucoside as previously described (15), the homogenate was centrifuged, and the supernatant (cytosolic extract) was removed. The total sugar content in the cell wall extract was determined colorimetrically with mannose as a standard (27). The protein content in the cytosolic extracts was estimated by measurement of the A_{280}.

Biotinylation of cell surface components of *C. albicans*. *C. albicans* blastoconidia were propagated as above described, and cell surface components were labeled with biotin essentially as previously described (15), except that *N*-hydroxysuccinimide–sulfo-biotin (Pierce, Rockford, Ill.), which cannot permeate the cell membrane (21), was used as the biotinylation agent. After biotinylation, cell surface components were extracted with βME as described above. Biotin-labeled components present in the extract were adsorbed onto immobilized streptavidin (Pierce). After exhaustive washings to remove unbound (nonbiotinylated) components, the bound material was solubilized by boiling with sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below). Following electrophoresis, the separated components were transferred to nitrocellulose and detected by using the affinity-purified antibody to the fusion protein obtained as described below.

Isolation of a cDNA clone. The isolation of cDNA clone R has previously been described (2). A cDNA expression library was constructed in λgt11*Sfi-Not*, a directional vector, with mRNA from germ tubes and screened with a polyclonal antiserum against *C. albicans* cell wall preparations (45). Positive plaques detected with the polyclonal antiserum were picked up and rescreened at low densities until all plaques were positive.

Affinity-purified antibody. Affinity-purified antibody was prepared as described previously (2, 3). The fusion protein was produced by the isolated lambda clone on solid medium and transferred to a nitrocellulose filter. The filter was incubated in the presence of the pooled polyclonal antisera for 3 h. The filter was washed extensively with 10 mM Tris (pH 7.4) containing 0.9% NaCl and 0.05% Tween 20 (TBST). Bound antibody was eluted from the filter with 10 mM glycine buffer (pH 2.3) containing 0.9% NaCl, 1% bovine serum albumin (BSA), and 0.05% Tween 20 and neutralized with 2 M Tris base (18). The antibody concentration was approximately 40 μg/ml.

Western blotting (immunoblotting). SDS-PAGE with 5 to 15% gradient gels was performed under denaturing conditions as described previously (14, 40). Protein in the gels was detected by Coomassie blue staining as previously described (15). Electrophoretic transfer to nitrocellulose paper (Schleicher & Schuell, Keene, N.H.) was performed with a semidry electroblotter at 0.8 mA/cm^2 for 1 h (10, 45, 66). Immunodetection of proteins transferred to nitrocellulose was done as previously described (2, 3, 14), using the pooled polyclonal antiserum to cell wall proteins at a final concentration of 1:1,000 in TBST supplemented with 1% BSA (TBSTB) or the affinity-purified antibody to the fusion protein. Peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel, Organon Teknika Corp., Durham, N.C.) diluted 1:2,000 in TBST (TBSTB) was used as a secondary detector antibody. H_2O_2 and 4-chloro-1-naphthol substrates were added, and the colored bands were allowed to develop (33). In another experiment, polyclonal antibody (PAb) 1173 (at a final dilution of 1:500) generated against denatured *Saccharomyces cerevisiae* Ssa1p C1ΔB was used in immunoblots of *C. albicans* βME and cytosolic extracts. PAb 1173 was the generous gift of E. Craig.

Reactivity of human serum with the cloned protein. To investigate the presence of antibodies against the cloned protein in sera from control individuals and individuals suffering from candidiasis, an immunoblot procedure was used. Luria-Bertani agar plates were overlaid with 3 ml of Luria-Bertani medium–agarose containing 250 ml of *Escherichia coli* Y1090 previously incubated in the presence of the HSP70 phage. λgt11 was used as a negative control. The fusion protein was induced, transferred to nitrocellulose disks, and probed with antisera essentially as described above. Each human serum was used at a final dilution of 1:500 as the primary antibody preparation, and the second detecting antibody was a peroxidase-conjugated goat anti-human immunoglobulin (Cappel). Sera from 11 patients with systemic candidiasis, 1 patient with chronic mucocutaneous candidiasis, and 4 normal individuals were tested. Patient sera were obtained from H. R. Buckley (Temple University Medical School, Philadelphia, Pa.).

Indirect immunofluorescence (IIF). IIF assays were performed with germ tubes using the affinity-purified antibody and a fluorescein-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim, Indianapolis, Ind.) as the secondary antibody by following the procedure described elsewhere (2, 3, 14). Prior to the assay, the affinity-purified antibody was concentrated fivefold with Centricon-10 tubes (Amicon Inc., Beverly, Mass.). Control samples in which incubation with the first antibody was omitted were negative. Cells were examined with a Nikon Labophot equipped for epifluorescence, and digital pictures of the fluorescent samples were taken with a cooled (−45°C) charge-couple device camera (Star 1; Photometrics, Tucson, Ariz.) as reported before (45). The digital image was converted to a TIFF file, processed for brightness and contrast, and scaled (imgworks; Silicon Graphics Inc., Mountainview, Calif.) and printed (printer from Codonics Inc., Middleburg, Ohio).

Immunoelectron microscopy. Freeze substitution was used for fixation of *C. albicans* germ tubes. Briefly, cells were rapidly frozen in −196°C propane and substituted with 0.01% OsO_4 in dry acetone for 3 to 4 days. The cells were gradually brought to 4°C and infiltrated with Unicryl resin (Ted Pella Inc., Redding, Calif.) and cured under UV light. Thin sections were obtained and washed in buffer, and nonspecific binding sites were blocked with BSA in phosphate-buffered saline (PBS). The sections were incubated for 3 h at room temperature with monovalent affinity-purified antibody, washed with PBS, and incubated for 1 h at room temperature with a 1:7 dilution of colloidal gold-conjugated anti-rabbit antibody (EY Laboratories, Inc., San Mateo, Calif.). The sections were washed with PBS and counterstained with uranyl acetate and lead citrate. Samples were observed with a Hitachi H-600 microscope at 75 kV. Negative control grids in which the first incubation step with the affinity-purified antibody was omitted were processed in parallel.

cDNA sequence analysis. The 0.68-kb insert of clone R was amplified by PCR (GeneAmp PCR Reagent Kit; Perkin-Elmer Corp., Norwalk, Conn.). Both strands of the PCR product were sequenced by using the fmole DNA Sequencing system (Promega Corp., Madison, Wis.). The nucleotide sequence analysis were performed with the FASTA and Map programs of the Wisconsin Sequence Analysis Package version 8 (Genetics Computer Group, Madison, Wis.). This program was also used to access the BLAST network service of the National Center for Biotechnology Information to search for homology to the deduced amino acid sequence of the longest open reading frame. Multiple sequence alignments were obtained with the PileUp program.

Northern (RNA) blots. Total RNA from *C. albicans* 3153A grown under different conditions was extracted by the hot-phenol method (69). Electrophoresis, blotting, and hybridization conditions were as described before (62). Briefly, after electrophoresis, RNA was transferred to a nylon membrane (Nytran Plus; Schleicher & Schuell) with a Turboblotter (Schleicher & Schuell) by following the manufacturer's instructions. A PCR product from the cDNA insert of clone R was obtained, labeled with [^{32}P]ATP by nick translation (Nick Translation Kit; Amersham Life Science Inc., Arlington Heights, Ill.), and used for detection. Hybridization was performed with Rapid-hyb buffer (Amersham Life Science) by following the manufacturer's instructions.

Nucleotide sequence accession number. The sequence of the 0.6-kb insert of clone R has been deposited with GenBank under accession number U25718.

RESULTS

Western blotting and IIF. Affinity-purified antibody to the fusion protein produced by the cDNA expression clone was used to identify the protein encoded by the cloned fragment. A single band of approximately 70 kDa was detected in βME extracts both from yeast cells and germ tubes by Western blotting (Fig. 1, lanes 3 and 4). No reactivity was detected with the Zymolyase extract from either morphology (data not shown). Several affinity-purified antibody preparations similarly reacted with only this moiety and not with any other components present in the extracts (Fig. 1, lanes 1 and 2) and thus demonstrated the specificity of the antibody preparations. This monovalent affinity-purified antibody was used in an IIF assay to assess the expression of the protein on intact fungal cells (Fig. 2A and B). The protein was expressed at the cell surface, as both yeast cells and germ tubes were stained with the antibody preparation.

Northern blotting. The expression of the gene in yeast cells and germ tubes growing in different media and at different temperatures was examined by Northern blotting (Fig. 3). A 2.3-kb transcript was detected in all conditions tested and appeared to be present independently of growth media, morphology, or temperature. It was present to a similar extent in yeast cells growing in YEPD (lanes 1 and 2) and Lee medium (lane 3) and at both 25°C (lanes 1 and 3) and 37°C (lanes 2 and 4).

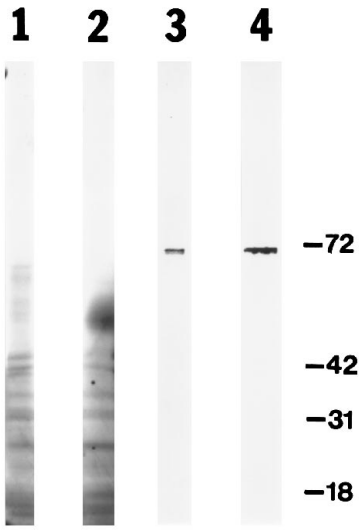

FIG. 1. Analysis of SDS-PAGE separations by staining and Western blotting. βME extracts (300 μg of sugar) from yeast cells (lanes 1 and 3) and germ tubes (lanes 2 and 4) were separated by SDS-PAGE and stained with Coomassie blue (lanes 1 and 2) or transferred to nitrocellulose and Western blotted with affinity-purified antibody to the fusion protein of the cDNA clone (lanes 3 and 4). Molecular masses of protein standards run in parallel are shown on the right in kilodaltons.

It was present both in growing blastoconidia (lanes 1 to 3) and during germ tube formation (lane 4). The transcript was also detected in the stationary-phase cells from which germ tubes were induced and at 1 and 24 h after germ tube induction (data not shown). It appeared to be present at a reduced level in the stationary-phase cells and particularly at 24 h.

Sequence analysis. The PCR product of the cloned cDNA insert representing the 3′ end of the gene was sequenced (Fig. 4A). A database search revealed extensive homology with the 70-kDa family of stress proteins or hsps. Among the *HSP70* gene family with extensive homology were a *C. albicans* sequence and sequences from *S. cerevisiae* that represented four subfamilies, *SSA*, *SSB*, *SSC*, and *SSD* (Table 1). The homology with the *C. albicans HSP70* was 77%, suggesting that the two genes were different. The sequence showed the least homology with the *S. cerevisiae SSC1* and *SSB1* sequences and the greatest homology with *SSA1* and *SSA2*, 72 and 76%, respectively. Homology with the *SSE* subfamily was 56 to 61% extending over less than 175 bp. The deduced amino acid sequence of the longest open reading frame was also used to search for protein homology. The greatest protein homology was found with the *SSA1* and *SSA2* gene products of *S. cerevisiae*. Less homology was found with the other *C. albicans HSP70* gene protein or other members of the *S. cerevisiae* hsp70 family. The N terminus of the proteins is more conserved, and differences in the C-terminal region of Ssa1p and Ssa2p include a 9-bp (3-amino-acid) deletion in *SSA2* (63). Comparison of the deduced *C. albicans* amino acid sequence with that of *S. cerevisiae SSA1* showed the absence of the deletion near the C terminus (Fig. 4B). However, there appeared to be a likely insertion of 2 amino acids some 75 amino acids upstream compared with both Ssa1p and Ssa2p. This insertion was shared with the deduced sequence of *C. albicans HSP70*, although amino acids differed in one of two sites. Overall, *C. albicans HSP70*, which has 73% nucleotide identity with *SSA1*, has 14 insertions in the predicted amino acid sequence relative to that of *SSA1* (41). The gene corresponding to this cDNA clone R was designated *SSA2* in accordance with suggested nomenclature for stress 70 proteins (30) and homology with the *SSA* subfamily of *S. cerevisiae*.

Reactivity of antibody against *S. cerevisiae* Ssa proteins with *C. albicans* extracts. To confirm the identity of the cDNA clone R sequence as a representative of the *HSP70* family based on sequence comparisons, we further investigated the relationship between the 70-kDa component in the cell wall and members of this hsp70 family of proteins in *S. cerevisiae* (Fig. 5). Components present in the βME extracts (lane 1) and cytosolic extracts (lane 2) from *C. albicans* blastoconidia were immunoblotted with PAb 1173, a PAb generated against denatured *S. cerevisiae* Ssa1p. The antibody preparation specifically interacted with an approximately 70-kDa moiety in both extracts. This observation supported the identification of the 70-kDa component associated with cDNA clone R as a member of the hsp70 family and more specifically with the Ssa subfamily of hsps in *S. cerevisiae.*

Biotinylation of surface proteins. Since members of the hsp70 family of proteins have not been described in the cell wall, we used two methods to demonstrate that the 70-kDa protein was a bona fide cell wall component. In one approach, proteins external to the cell membrane were specifically labeled. *N*-Hydroxysuccinimide–sulfo-biotin, which cannot permeate the cell membrane (21), was used to label intact yeast cells. After extraction with βME, biotinylated components were isolated by streptavidin affinity chromatography. The purified biotinylated components were analyzed by Western blotting with the affinity-purified antibody obtained with the fusion protein of the cDNA clone (Fig. 6). The affinity-purified antibody recognized a moiety with an approximate electrophoretic mobility of 70 kDa in both the total extract (lane 1) and the purified biotinylated proteins (lane 2). Biotinylation of the moiety recognized by the antibody with *N*-hydroxysuccinimide–sulfo-biotin demonstrated that it was present outside of the cell plasma membrane and associated with the cell wall.

Immunoelectron microscopy. Immunoelectron microscopy was used as the second method to localize the 70-kDa protein. Yeast cells were fixed by freeze substitution, and the protein was detected with the monovalent affinity-purified antibody (Fig. 2C and D). The moiety reacting with the monovalent affinity-purified antibody was present at the cell surface as well as the outer surface of the plasma membrane. It was observed extending through the cell wall, occasionally appearing to reach the cell surface through channels (Fig. 2D, arrows). Most of this component was localized to the cell wall, although some reactivity was observed in the cytoplasm.

Reactivity of human sera with *C. albicans* Ssa1p. hsps are immunodominant antigens and major targets of the host immune response during different types of infection (37, 38, 48). We investigated whether the fusion protein produced by clone R was recognized by sera from patients with candidiasis and a control group of individuals. The fusion protein or the protein from a λ clone lacking an insert were induced and transferred to nitrocellulose, and the nitrocellulose filters were incubated with the test sera. None of the sera reacted with the negative control lacking the cloned insert. When tested against the fusion protein produced by clone R, a positive reaction was detected with all 16 serum samples independent of origin. These observations indicated that this protein is a target for the immune response.

DISCUSSION

Four lines of evidence demonstrated that the 70-kDa protein associated with the cloned sequence was a bona fide compo-

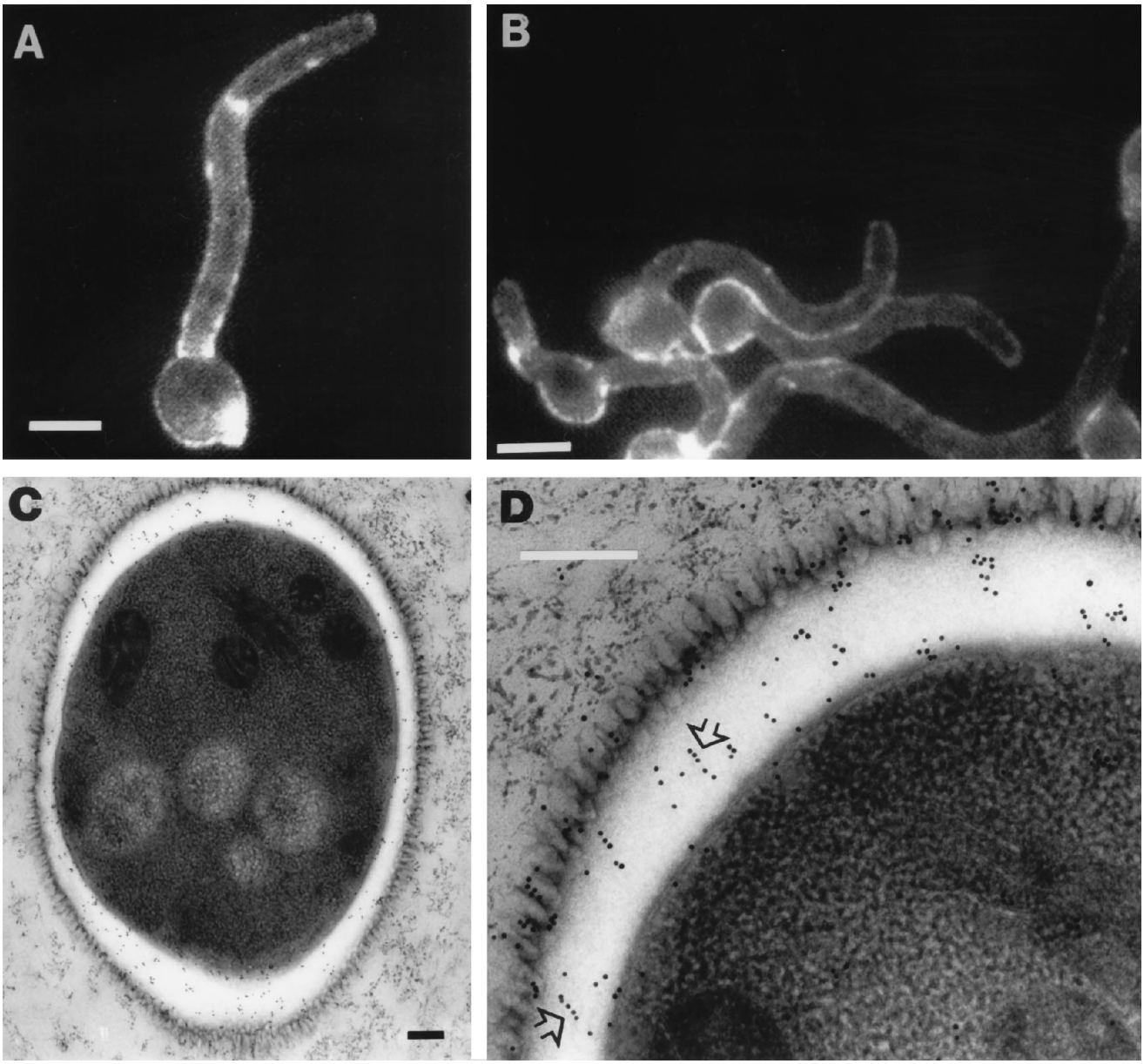

FIG. 2. IIF and immunoelectron microscopic localization. The monovalent affinity-purified antibody was used in an IIF assay with fungal cells (A and B; bars, 5 μm). Immunoelectron microscopy of *C. albicans* cells was performed using the same antibody preparation (C and D; bars, 0.2 μm). Panel D is an enlargement of the upper right portion of panel C. Arrows in panel D point towards channel-like structures.

nent of the cell wall. Affinity-purified antibody to the fusion protein identified the 70-kDa moiety in the βME extracts containing cell wall components from both morphologies of the organism (Fig. 1, lanes 3 and 4). IIF demonstrated the localization of some of the component at the cell surface of both morphologies (Fig. 2A and B). Biotinylation of intact cells demonstrated that the 70-kDa moiety was present outside the cell membrane and associated with the cell wall. Immunoelectron microscopy showed that the component was found throughout the cell wall, although it was concentrated at both the outer and inner surfaces (Fig. 2). In some places there appeared to be a channel containing the component connecting the inner and outer cell wall surfaces. Similar channel-like connections have previously been reported (5, 55). The last two lines of evidence provided additional evidence that the βME extract contains bona fide cell wall proteins.

The 0.68-kb cDNA insert from the 3′ end of the gene was sequenced, and a database search revealed extensive homology to members of the 70-kDa family of hsps. The homology (65 to 76%) with some members of the *S. cerevisiae HSP70* gene family was similar to the intraspecies homology (Table 1) (47). The 77% homology with another *C. albicans* mRNA sequence, *HSP70*, suggested that this fragment represented a second member of the *HSP70* family in this organism. The homology to the *SSA* subfamily of *S. cerevisiae* and the apparently constitutive nature of expression detected (Fig. 3) suggested that clone R was most closely related to *SSA1* and *SSA2*. These genes are more closely related to each other than to other

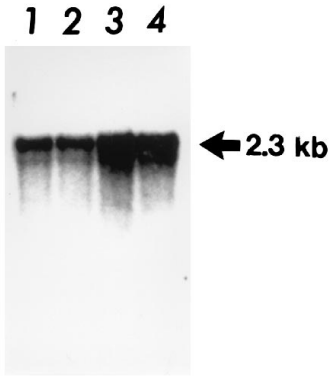

FIG. 3. Northern blot analysis. RNA was obtained from *C. albicans* 3153A growing as blastoconidia in YEPD medium at 25 or 37°C (lanes 1 and 2, respectively) or as blastoconidia in Lee medium at 25°C (lane 3) and 6 h after induction of formation of germ tubes in Lee medium at 37°C (lane 4). Hybridization was performed as described in Materials and Methods. The size of the transcript was calculated by regression analysis of standards run in parallel.

members of the family (61). The relationship of the cell wall component to the *SSA* family was further supported by reactivity of antibody to Ssa proteins with the *C. albicans* cell wall component (Fig. 5). Since both of the *HSP70* genes identified in *C. albicans* show the greatest homology with the SSA subfamily of *S. cerevisiae*, we have chosen also to call this family *SSA* in *C. albicans*. We propose that the *HSP70* designation of La Valle et al. (41) for the first member of the family reported be replaced with *SSA1*. We have named the gene corresponding to clone R *SSA2*.

Proteins responding to heat shock or stress have previously been reported in *C. albicans* (26, 70). Several of these appeared to correspond in size to those associated with the general heat shock response, including a protein of 70 kDa. Proteins belonging to the stress 70 family are highly conserved proteins that contribute to the protection and repair of cells after exposure to stress conditions and are found in different cellular locations, but so far they have not been described in the cell wall (47). As noted, the 70-kDa moiety was demonstrated to be present in the cell wall by four experimental protocols. The experimental design of biotinylation cannot distinguish between protein specifically secreted to the cell wall and hsp70 that reached the cell surface by surface binding following release to the culture medium by lysed cells. However, the immunoelectron microscopy observations clearly demonstrated that the protein was located throughout the cell wall, not just at the periphery. Since proteins that bind to the intact cell do not penetrate beyond the periphery of the cell wall (7, 9, 56),

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  1  CTTGTTGGGTAAATTCGAATTATCTGGTATTCCACCAGCTCCAAGAGGTGTTCCACAAATTGAAGTTACTTTCGATATTGATGCTAATGGTATCTTGAAT 100
101 GTTTCTGCTTTAGAAAAAGGTACCGGTAAAACTCAAAAGATTACTATTACTAACGATAAAGGTAGATTATCTAAAGAAGAAATTGAAAAGATGGTCAGTG 200
201 AAGCTGAAAAGTTCAAAGAAGAAGATGAAAAGGAAGCTTCTAGAGTCCAAGCTAAGAACCAATTGGAATCTTATGCTTACTCTTTAAAGAACACTTTGGG 300
301 TGAAGAACAATTCAAATCTAAATTGGATGCTTCAGAAATTGAAGAAGTCACTAAAGCTGCTGATGAAACTATTGCTTGGTTAGATTCCAACCAAACTGCT 400
401 ACTCAAGAAGAATTTGCTGATCAACAAAAAGAATTAGAATCTAAAGCTAACCCAATCATGACCAAAGCTTACCAAGCTGGTGCTACTCCTTCTGGTGCTG 500
501 CTGGTGCTGCTCCAGGTGGTTTCCCAGGTGGTGCTGCCCCAGAACCATCTAACGATGGTCCAACTGTTGAAGAAGTTGATTAAATGTATTAATAAT'TAT 600
601 ATATATATTAGAGATATTTATAATGTAATACTTTATAGACAGTAATAAAACGCATATTAATTTGAGATAAGAAAAAAAAAAAAAA  686
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B

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ScSSA1     452 LLGKFELSGIPPAPRGVPQIEVTFDVDSNGILNVSAVEKGTGKSNKITITNDKGRLSKEDIEKMVAEAEK 521
ScSSA2     451 LLGKFELSGIPPAPRGVPQIEVTFDVDSNGILNVSAVEKGTGKSNKITITNDKGRLSKEDIEKMVAEAEK 521
ScSSA3     452 LLGKFELSGIPPAPRGVPQIDVTFDIDANGILNVSALEKGTGKSNKITITNDKGRLSKDDIDRMVSEAEK 522
ScSSA4     453 LLGKFELSGIPPAPRGVPQIEVTFDIDANGILNVSAVEKGTGKSNKITITNDKGRLSKEDIDKMVAEAEK 522
CaHSP70    454 LLGKFELSGIPPAPRGVPQIEVTFDIDANGILNVSALEKGTGKTQKITITNDKGRLSKEEIDKMVSEAEK 523
CaSSA1       1 LLGKFELSGIPPAPRGVPQIEVTFDIDANGILNVSALEKGTGKTQKITITNDKGRLSKEEIEKMVSEAEK  70
Consensus      LLGKFELSGIPPAPRGVPQI:VTFD:D.NGILNVSA:EKGTGK..KITITNDKGRLSK::I:KMV.EAEK

ScSSA1     522 FKEEDEKESQRIASKNQLESIAYSLKNTISEAG..DKLEQADKDTVTKKAEETISWLDSNTTASKEEFDD 589
ScSSA2     522 FKEEDEKESQRIASKNQLESIAYSLKNTISEAG..DKLEQADKDAVTKKAEETIAWLDSNTTATKEEFDD 589
ScSSA3     523 YRADDEREAERVQAKNQLESYAFTLKNTINEASFKEKVGEDDAKRLETASQETIDWLDASQAASTDEYKD 592
ScSSA4     523 FKAEDEQEAQRVQAKNQLESYAFTLKNSVSENNFKEKVGEEDARKLEAAAQDAINWLDASQAASTEEYKE 572
CaHSP70    524 FKEEDEKEAARVQAKNQLESYAYSLKNTINDGEMKDKIGADDKEKLTKAIDETISWLDASQAASTEEYED 573
CaSSA1      71 FKEEDEKEASRVQAKNQLESYAYSLKNTLGEEQFKSKLDASEIEEVTKAADETIAWLDSNQTATQEEFAD 140
Consensus      F:.:DE-E:-R:..KNQLES.AX.LKN.:-:--:K-K:---D---:.----:.I-WLD..-.A.-EE:-:

ScSSA1     590 KLKELQDIANPIMSKLY.QAGGAPGGAAG.GAPGGFPGGAPPA........PEAEGPTVEEVD 642
ScSSA2     590 QLKELQEVANPIMSKLY.QAGGAPEGA....APGGFPGGAPPA........PEAEGPTVEEVD 639
ScSSA3     593 RQKELEGIANPIMTKFYGAGAGAGPGAG...ESGGFPGSMPNSGATG...GGEDTGPTVEEVD 6499
ScSSA4     593 RQKELEGVANPIMSKFYGAAGGAP.GAGPVPGAGAGPTGAPD.............NGPTVEEVD 642
CaHSP70    594 KRKELESVANPIISGAYGAAGGAPGGAGGFPGAGGFPGGAPGAGGPGGATGGESSGPTVEEVD 656
CaSSA1     141 QQKELESKANPIMTKAYQAGATPSGAAGA..APGGFPGGAAPE........PSNDGPTVEEVD 193
Consensus      --KEL:--ANPIM.K-Y..::G:--GA:-..--GGFP-GAP--G:.G---G..--GPTVEEVD
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FIG. 4. Nucleic acid and deduced protein sequences of *SSA2*. (A) Nucleic acid sequence of the cDNA clone. The sequence has been deposited in GenBank under the accession number U25718. (B) Deduced amino acid sequence of the longest open reading frame (2 to 584) of *C. albicans SSA2* (*CaSSA2*) of 193 amino acids with the corresponding C-terminal sequences for *S. cerevisiae* proteins for *SSA1* (*ScSSA1*), *SSA2* (*ScSSA2*), *SSA3* (*ScSSA3*), and *SSA4* (*ScSSA4*) and *C. albicans HSP70* (*CaHSP70*). Dots indicate gaps introduced during the alignment. On the consensus line, identity is indicated by the single-letter amino acid code, decreasing similarity is indicated by the colons and dots, and a lack of similarity and multiple differences are indicated by dashes. The numbering of amino acids is from the entire sequence of each protein, except the C-terminal deduced protein fragment of *C. albicans SSA2*.

TABLE 1. Nucleotide homology of 686-bp cDNA insert of clone R of *C. albicans* with the HSP70 gene family of *C. albicans* and *S. cerevisiae*[a]

Organism	Gene	Cellular location(s) of protein	% Homology with clone R insert	Overlap with clone R insert (bp)
C. albicans	*HSP70*	Cytosol	77	643
S. cerevisiae	*SSA1*	Cytosol, cell wall	72	675
	SSA2	Cytosol, cell wall	76	584
	SSA3	Cytosol	66	655
	SSA4	Cytosol	66	670
	SSB1	Cytosol	65	326
	SSC1	Mitochondria	67	303
	SSD1 (*KAR2*, *GRP78*, *BiP*)	Endoplasmic reticulum	65	517

[a] Homology obtained from the database as described in Materials and Methods. The location, expression, and function of *S. cerevisiae HSP70* genes have been recently reviewed (24, 47), and the presence of Ssa1p and Ssa2p in the cell wall has been demonstrated (46).

this distribution argued against an adventitious binding as the source of internal cell wall reactivity and supported a specific secretion and localization of the protein to the cell wall. Although the affinity-purified protein detected a single band in cell wall extracts, the possibility of a cross-reacting protein of the same size specified by a closely related gene of the *HSP70* family cannot be eliminated. The presence of an hsp in the cell wall of *C. albicans* is not unprecedented. Mathews et al. (55) found that a *C. albicans* 47-kDa antigen, a breakdown product of the candidal hsp90, was present in the cytoplasm and cell wall of both yeast and mycelial cells. The subcellular localization of the 70-kDa moiety noted above (Fig. 2) was similar to that observed for the 47-kDa moiety (55).

In addition to being highly conserved proteins, hsps are immunodominant antigens and major targets of host immune response during different types of infection (37, 38, 48). Immunity to conserved epitopes on hsps may provide a certain degree of protection against a variety of pathogens that is between innate immunity and acquired specific immune response (37). This is particularly true for members of the hsp70 family, which are among the most immunogenic proteins of pathogenic microorganisms. Several reports have implicated the presence of antibodies against hsps in the recovery from candidiasis both in humans and in animal models. The 47-kDa antigen, a breakdown product of the candidal hsp90, is thought to play a key role. Antibodies against this immunodominant antigen are protective in an animal model, and the presence of such antibodies has been implicated in the recovery in humans from candidiasis (53, 54). More recently, in an investigation of the humoral response in a murine model of candidiasis, a significant antibody response against a non-hsp 96-kDa antigen different from the hsp90 and against a 75-kDa hsp was detected (22). These authors concluded that resolution of candidiasis in their model was associated with the presence of antibodies against these two molecules. Furthermore, an 80-kDa cell wall-membrane protein in *Histoplasma capsulatum* with homology to hsp70 elicits cellular immune responses and mediates protective immunity (32).

More recently, La Valle and colleagues reported that sera from three normal healthy individuals contained antibodies against *C. albicans* hsp70, the product of *C. albicans SSA1* (HSP70), and suggested that the presence of such antibodies could contribute to protection against infection (41). These authors pointed out that the variability in this generally conserved protein was predominantly in the C-terminal region of the protein, the region of the protein that is usually immunodominant (38). We observed reactivity of both normal and patient sera with the C-terminal portion of *C. albicans SSA2* in this study. In this gene also, differences from conserved sequences compared with either the *S. cerevisiae* deduced amino acid sequence or the *C. albicans SSA1* (*HSP70*) deduced sequence reside in the C terminus (Fig. 4). Due to the high levels of homology between the different hsp70s as well as the variability at the C terminus, this observation could result from the combination of antibodies in the sera against this as well as other *C. albicans* hsp70s or even hsp70 from other organisms.

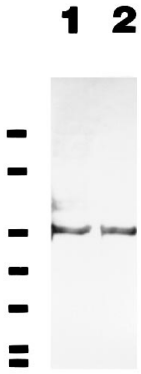

FIG. 5. Western blot analysis with antibody to the common epitope of Ssa proteins. βME cell wall extracts (lane 1) and cytosolic extracts (lane 2) from *C. albicans* blastoconidia were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis was performed with PAb 1173 to common epitopes of Ssa proteins of *S. cerevisiae* as described in Materials and Methods. Electrophoretic mobilities of protein standards (7, 18, 31, 42, 72, 130, and 217 kDa from bottom to top) run in parallel are indicated on the left.

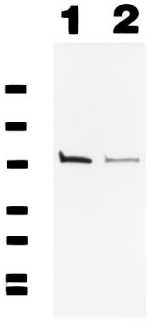

FIG. 6. Western blot analysis of biotinylated proteins. Unfractionated cell wall extract (lane 1) or biotinylated proteins purified from the extract by streptavidin affinity chromatography (lane 2) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Monovalent affinity-purified antibody recognizing the cloned Hsp70 fusion protein was used for the Western blot analysis. Electrophoretic mobilities of protein standards (7, 18, 31, 42, 72, 130, and 217 kDa from bottom to top) run in parallel are indicated on the left.

The presence of an hsp70 in the cell wall and at the surface makes the antigen present on the intact cell and may facilitate any potential protective role of antibody.

Northern blot analysis confirmed the expression of the gene in cells independent of morphology, growth medium, and temperature (Fig. 3). The transcript amount detected in growing cells was similar. There was no apparent change in expression in germ tubes growing at 37°C 6 h after induction. However, in nongrowing cultures the transcript amount appeared to be reduced. While some hsp70-encoding genes of *S. cerevisiae* respond to stress with an increased expression, others show constitutive or high basal levels of expression (47). The *S. cerevisiae SSA1* and *SSA2* genes are constitutively expressed, although *SSA1* expression is elevated by a heat stimulus while *SSA3* and *SSA4* are minimally expressed in the absence of heat shock (23, 47). *C. albicans SSA1* (*HSP70*) expression increased following an increase in growth temperature (28, 41).

In conclusion, in this report we present evidence for the presence in the cell wall of *C. albicans* of a protein with a high degree of homology to the hsp70 family of proteins. This protein is a genuine cell wall component and is constitutively expressed by *C. albicans* cells in all conditions tested. Immunodominant hsp70s appear to serve a chaperone function, as apparently do hsp90s (24, 29, 47). We have recently shown in *S. cerevisiae* that Ssa1p and Ssa2p are present in the cell wall (46). In an *ssa1 ssa2* strain, the 70-kDa moiety is absent and is not replaced by Ssa4p, which is upregulated in this strain. In addition, at least one other major band was missing from the profile of proteins in the cell wall extract of the mutant strain compared with that of the parental strain. This finding suggested that hsp70 in the cell wall has functions similar to those described in the cytoplasm.

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