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Members of the Hsp70 Family of Proteins in the Cell Wall of *Saccharomyces cerevisiae*

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Western blot (immunoblot) analysis of cell wall and cytosolic extracts obtained from parental and *ssa1* and *ssa2* single- and double-mutant strains of *Saccharomyces cerevisiae* showed that the heat shock protein 70 (Hsp70) products of these genes, previously thought to be restricted to the cell interior, are also present in the cell wall. A cell wall location was further confirmed by indirect immunofluorescence with intact cells and biotinylation of extracellular Hsp70. Hsp70s have been implicated in translocation across the membrane and as molecular chaperones, and changes in the profile of cell wall proteins suggested that these proteins may have a similar role in the cell wall.

The heat shock response is one of the most evolutionarily conserved attributes of living organisms. Prokaryotic and eukaryotic responses to a sudden change of temperature and other adverse environmental conditions involve the increased production of a set of proteins collectively referred to as heat shock proteins. The production of these proteins presumably contributes to the protection and repair of cells under stress conditions (13). Several families of heat shock proteins have been identified, among them the Hsp70s, which have a molecular mass of approximately 70 kDa. The functions of Hsp70s are not restricted to heat shock protection; they also play a role in protein folding and the translocation of proteins across membranes (4, 6). Microbial Hsp70s are also major immunogens during different types of infection (9, 14). The yeast *Saccharomyces cerevisiae* has proven to be a good model system in which to study the physiological roles of heat shock proteins by using both genetic and biochemical approaches (4). In *S. cerevisiae*, at least 10 *HSP70*-like genes have been described and classified into five families from *SSA* to *SSE* (10, 17–19). Most of the proteins encoded by these genes are present at considerable levels under all growth conditions, although their synthesis is increased after exposure to stress. The *SSA* family includes four members, designated *SSA1* to *SSA4* (8), with DNA sequence similarity ranging from 80 to 97%. Cell viability requires moderate to high levels of at least one of the proteins encoded by this family (20). Under normal growth conditions both *SSA1* and *SSA2* genes are expressed at moderate levels, while *SSA3* and *SSA4* gene expression is undetectable. Heat shock results in increased expression of *SSA1* and strong induction of both *SSA3* and *SSA4* expression (8). Inactivation of either *SSA1* or *SSA2* does not result in an obvious phenotype. However, *ssa1 ssa2* double mutants grow more slowly than the parent strain at all temperatures tested and are unable to form colonies at 37°C (5). These strains are viable at temperatures lower than 37°C as a result of a high-level expression of the *SSA4* gene (20).

Until now, the gene products of the *SSA* subfamily of Hsp70s have been considered to be located exclusively in the cytosol of *S. cerevisiae* (4, 13). We have previously shown that a protein related to the Hsp70 family of proteins is present in the cell wall of *Candida albicans* (12). The sequence of the cDNA clone corresponding to the 3′ end of the gene was most similar to the *SSA1* and *SSA2* sequences of *S. cerevisiae* (12). In the present study we have investigated the presence of Hsp70s in the cell wall of *S. cerevisiae*. Evidence that members of the Ssa proteins are present in the cell wall of this budding yeast is presented.

A parental strain, T211 (**a** *GAL2 his3-11,15 leu2-3,112 lys1 lys2 trp1-Δ1 ura3-52*), and a double-mutant strain containing disruptions in *SSA1* and *SSA2*, T212 (**a** *GAL2 his3-11,15 leu2-3,112 lys1 lys2 trp1-Δ1 ura3-52 ssa1*::*HIS3 ssa2*::*LEU2*), were obtained from E. A. Craig. The strains were grown at 23°C in a medium containing 2% yeast extract, 1% peptone, and 2% glucose. Protein and glycoprotein components of the walls from both strains were released from intact cells by treatment with β-mercaptoethanol (βME) basically as previously described by Casanova et al. (1, 2). Briefly, the cells were resuspended in ammonium carbonate buffer containing 1% (vol/vol) βME and incubated for 30 min at 37°C in a rotary incubator. After treatment, the cells were sedimented, and the supernatant fluid was recovered, dialyzed against distilled water, and lyophilized (βME extract). It has previously been shown that this extraction procedure leads to solubilization of genuine cell wall components (2). Extracted cells from both the parent strain and the double mutant were washed with ice-cold lysis buffer (10 mM phosphate [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, 2 mM $MgCl_2$) and broken by vortexing with glass beads in the presence of a small volume of lysis buffer. Unbroken cells and particulate matter were removed by centrifugation, and the supernatant (cytosolic extract) was collected. The protein content of each sample was estimated by measuring A_{280}. The different extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 4 to 15% acrylamide gradient minigels (Bio-Rad, Hercules, Calif.) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) by using a semidry electroblotter (Hoefer Scientific Instruments, San Francisco, Calif.) at 0.8 mA/cm^2 for 1 h. Separated components were analyzed by immunoblotting with rabbit polyclonal antibody (PAb) 343, generated against the C-terminal peptide (APPA PEAEGPTVEEVD) of Ssa1p, and PAb 535, generated against a common epitope of Hsp70s (VGIDLGTTYSC). Analysis with PAb 343 revealed the presence of a reactive band with an apparent electrophoretic mobility of approximately 70 kDa in

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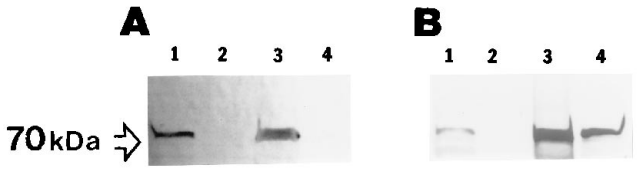

FIG. 1. Immunoblot analysis of cell wall and cytosolic extracts with antibodies recognizing Hsp70s. Protein extracts (10 μg of protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes as described in the text. Immunoblotting was performed with PAb 343 (1:300 dilution) against the carboxy-terminal domain of Ssa1p (A) and with PAb 535 (1:300 dilution) against a common epitope of Hsp70s (B) of βME cell wall extracts (lanes 1 and 2) and cytosolic extracts (lanes 3 and 4) from an *S. cerevisiae* parent strain, T211 (lanes 1 and 3), and an *ssa1 ssa2* double-mutant strain, T212 (lanes 2 and 4). Electrophoretic mobility was determined by comparison with protein standards run in parallel.

the cell wall and cytosolic extracts from the parental strain (Fig. 1A, lanes 1 and 3, respectively). As expected, no reactivity was detected in either extract from the *ssa1 ssa2* double mutant (Fig. 1A, lanes 2 and 4). When PAb 535, generated against the conserved epitope of Hsp70s, was used as a probe in the immunoblots (Fig. 1B), reactive bands were detected, as expected, in the cytosolic extracts from both the parent and the double-mutant strain (lanes 3 and 4, respectively). However, the same antibody preparation recognized a moiety only in the cell wall extracts from the parent strain (Fig. 1B, lane 1), not in those from the double mutant (Fig. 1B, lane 2). Together, these results suggested the presence of either Ssa1 or Ssa2 protein, or both, in the cell wall of *S. cerevisiae*, whereas other members of the family (and, more specifically, Ssa4p, which is known to be overexpressed in the double mutant) are restricted to a cytosolic location. The absence of the abundant cytosolic Ssa4p in the cell wall extracts from the double-mutant strain ruled out the possibility of cytosolic contamination of these cell wall extracts.

To investigate further the presence of Ssa1 and/or Ssa2 proteins in the cell wall of *S. cerevisiae*, cell wall and cytosolic extracts were obtained from *ssa1* and *ssa2* single-mutant strains JN114 (**a** *GAL2 his3-11,15 leu2-3,112 trp1-Δ1 ura3-52 lys2? ssa1-3*) and JN115 (**a** *GAL2 his3-11,15 leu2-3,112 trp1-Δ1 ura3-52 lys2? ssa2-2*), respectively. The extracts were separated by SDS-PAGE and transferred to nitrocellulose papers as described above. The nitrocellulose membranes containing the separated materials were incubated in the presence of PAb 1173, generated against denatured Ssa1p C1ΔB. As seen in Fig. 2, cell wall extracts (lanes 1 and 2) and cytosolic extracts (lanes 3 and 4), from both *ssa1* (lanes 1 and 3) and *ssa2* (lanes 2 and 4) single mutants contained reactive moieties with an apparent molecular mass of approximately 70 kDa, indicating

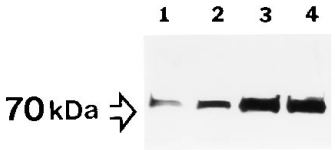

FIG. 2. Immunoblot analysis of cell wall and cytosolic extracts from strains defective in either *SSA1* or *SSA2*. Protein extracts (10 μg of protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes as described in the text. Immunoblot analysis was performed with PAb 1173 (1:500 dilution), which recognizes a common epitope of Ssa proteins of βME cell wall extracts (lanes 1 and 2) and cytosolic extracts (lanes 3 and 4) from an *S. cerevisiae ssa1* single-mutant strain, JN114 (lanes 1 and 3), and an *ssa2* single-mutant strain, JN115 (lanes 2 and 4). Electrophoretic mobility was determined by comparison with protein standards run in parallel.

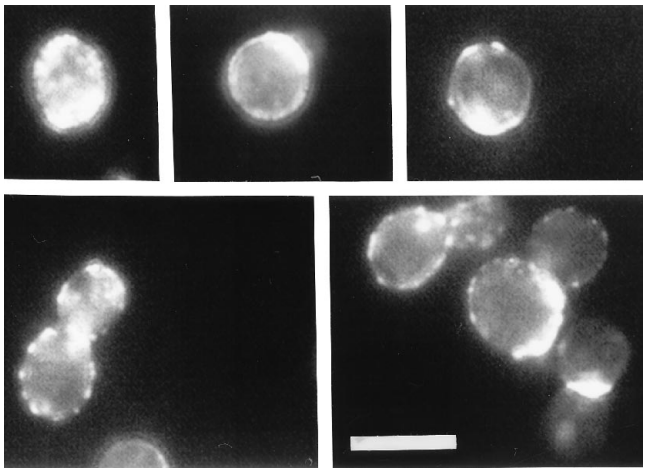

FIG. 3. Surface localization of Hsp70. PAb 343 was used in an indirect immunofluorescence assay with cells of the parental strain, T211. All cells were fluorescent, and images were obtained with a cooled charge-coupled device camera. The digital image was converted to a tagged image file format, processed for brightness and contrast, scaled (imgworks; Silicon Graphics Inc., Mountain View, Calif.), and printed (Codonics printer; Codonics Inc., Middleburg, Ohio). Bar, 5 μm.

the presence of both Ssa1 and Ssa2 proteins in the cell wall of *S. cerevisiae*. The experimental design cannot distinguish whether one or both proteins are present in the parental strain.

We used two additional methods to demonstrate that Hsp70 was a bona fide extracellular component. Indirect immunofluorescence was used to examine surface localization. Yeast cells of the parental strain, T211, were incubated sequentially with a 1:10 dilution of PAb 343 and a fluorescein-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim, Indianapolis, Ind.) as the secondary antibody as previously described (12). Microscopic images were obtained with a cooled charge-coupled device camera (12). Essentially, all cells of the parental strain were fluorescent (Fig. 3). No fluorescence was observed with parental cells incubated in the absence of PAb 343 or with cells of the *ssa1 ssa2* double-mutant strain incubated with or without PAb 343. These observations further demonstrated the specificity of the reaction and the presence of an Hsp70 on the cell surface of the parental strain. In a second experiment, sulfo-*N*-hydroxysuccinimide-biotin (Pierce, Rockford, Ill.), which cannot permeate the cell membrane (3), was used to biotinylate cell wall materials from the parental strain, T211. After biotinylation, cell surface components were extracted with βME as described above. Biotinylated proteins in a portion of the extract were affinity purified by chromatography on immobilized streptavidin (Pierce) (12). PAb 343 was used for a Western blot (immunoblot) analysis (Fig. 4). PAb 343 reacted with a 70-kDa moiety in both the βME extract (lane 1) and a purified biotinylated protein fraction (lane 2).

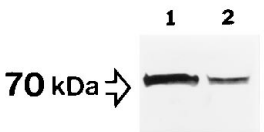

FIG. 4. Western blot analysis of biotinylated proteins. Unfractionated cell wall extract (lane 1) or biotinylated proteins purified from the extract by streptavidin affinity chromatography (lane 2) were separated by SDS-PAGE and transferred to nitrocellulose membranes. PAb 343 generated against the C-terminal peptide of Ssa1p (1:300 dilution) was used for the Western blot analysis.

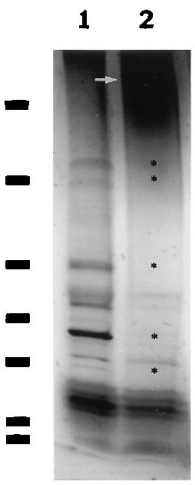

FIG. 5. Analysis of the protein composition of cell wall extracts from the parent strain and the *ssa1 ssa2* double-mutant strain. βME extracts (10 μg of protein) from the parent strain, T211 (lane 1), and the double-mutant strain, T212 (lane 2), were separated by SDS-PAGE and then silver stained as described in the text. Asterisks indicate proteins present in the cell wall extract from the parent strain but absent in that from the double-mutant strain. The white arrow indicates a high-molecular-weight polydisperse material which appeared to be more abundant in the cell wall extract from the double-mutant strain. The electrophoretic mobilities of protein standards run in parallel (from bottom to top, 7, 18, 31, 42, 72, 130, and 217 kDa) are indicated.

These observations confirmed that Hsp70 was present outside the cell membrane prior to extraction and that it is a bona fide cell wall protein.

Fungal cell wall protein composition is complex. Since Hsp70s have been implicated in translocation across the membrane and as molecular chaperones, their presence in the cell wall may indicate a role for them in the overall process of the biosynthesis, secretion, and assembly of cell wall components and in the cell wall structure. Thus, the absence of Hsp70 cell wall proteins may result in changes in the protein composition of the cell wall. To investigate this hypothesis, cell wall extracts from the parent strain and the *ssa1 ssa2* double-mutant strain were separated by SDS-PAGE. Silver staining analysis of the gel revealed changes in the polypeptidic patterns associated with the cell wall extract from the *ssa1 ssa2* double-mutant strain compared with the parent strain (Fig. 5). Some proteins were missing, including a protein with an apparent molecular mass of approximately 70 kDa. Also, increased quantities of a high-molecular-weight polydisperse material (shown previously to be highly glycosylated) were detected in the extract from the double-mutant strain.

In summary, the previous observation of an Hsp70 protein in the cell wall of *C. albicans* prompted us to investigate the presence of members of this family of proteins in the cell wall of *S. cerevisiae*. Results presented in this report suggest that, in addition to their well-known and well-studied cytosolic location, both Ssa1 and Ssa2 proteins are located in the cell wall of *S. cerevisiae*, whereas other members of the family are restricted to the cytosol. The absence of a conventional N-terminal signal sequence, found on most secretory proteins, for these Hsp70s suggests an alternative mechanism of secretion to the classical endoplasmic reticulum-Golgi secretory pathway (10). The change in the cell wall protein profile of the deficient strain supports a role for Hsp70s in cell wall biosynthesis either at the immediate level of the plasma membrane and the cell wall or at an earlier step in synthesis and secretion, or both. The presence of heat shock proteins in fungal cell walls is not unprecedented. In *C. albicans*, a 47-kDa protein which is a breakdown product of Hsp90 has been identified in the cell wall, where it is an immunodominant antigen (15, 16). An 80-kDa cell wall antigen with homology to Hsp70s has been reported for *Histoplasma capsulatum* (7). Also, since Hsp70s have been implicated in translocation across the membrane and as molecular chaperones, their presence in the cell wall and changes in cell wall composition in the double-mutant strain seem to indicate a role for these proteins in processes associated with overall fungal cell wall biosynthesis.

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