Fungalcidal activity of Hst 5 is initiated by binding to cell surface proteins on Candida albicans, followed by intracellular transport to cytoplasmic effectors leading to cell death. As we identified heat shock 70 proteins (Ssa1p and/or Ssa2p) from C. albicans lysates that bind Hst 5, direct interactions between purified recombinant Ssa proteins and Hst 5 were tested by pull-down and yeast two-hybrid assays. Pull-down of both native complexes and those stabilized by cross-linking demonstrated higher affinity of Hst 5 for Ssa2p than for Ssa1p, in agreement with higher levels of interactions between Ssa2p and Hst 5 measured by yeast two-hybrid analyses. C. albicans ssa1Δ and ssa2Δ mutants were constructed to examine Hst 5 binding, translocation, and candidacidal activities. Both ssa1Δ and ssa2Δ mutants were indistinguishable from wild-type cells in growth and hyphal formation. However, C. albicans ssa2Δ mutants were highly resistant to the candidacidal activity of Hst 5, although the ssa1Δ mutant did not have any significant reduction in killing by Hst 5. Total cellular binding of 125I-Hst 5 in the ssa2Δ mutant was reduced to one-third that of wild-type strain. Intracellular transport of Hst 5 was significantly impaired in the ssa2Δ mutant strain, but only mildly so in the ssa1Δ mutant. Thus, C. albicans Ssa2p facilitates fungicidal activity of Hst 5 in binding and intracellular translocation, whereas Ssa1p appears to have a lesser functional role in Hst 5 toxicity.

Candida albicans is a significant fungal pathogen causing both superficial and disseminated infectious diseases in humans. C. albicans is an opportunistic pathogen, so that immunosuppressed patients or xerostomia individuals have a high incidence of oral candidiasis (1). Saliva is rich in proteins with antimicrobial activity; among these, histatins have potent fungistatic and fungicidal activity to yeast and filamentous forms of Candida (2). Thus, histatins are key components in the armamentarium of the innate host defense system. Among at least 50 observed histatin peptides found in saliva (3), histatin 5 (Hst 5)2 has the highest killing activity with C. albicans (2, 4).

Hst 5 must first penetrate the cell wall and cross the periplasmic space to access the cytoplasm where it exerts its toxic activity. The cell wall of C. albicans is a thick multilayered structure of chitin and mannoproteins that protects the cell and restricts passage of proteins. Our previous studies identified cell wall-binding sites for Hst 5 from intact cells (5), whose identity was consistent with either Ssa1p or Ssa2p members of the C. albicans HSP70 family (6). Hst 5-binding proteins of similar size were also observed in susceptible Saccharomyces cerevisiae strains, and S. cerevisiae cells with deletion of the SSA1 and SSA2 genes, while retaining SSA3 and SSA4 genes, had substantial reduction of Hst 5 binding and killing (6). This evidence supported the involvement of the Ssa protein family as functional binding partners involved in the uptake and fungicidal mechanism of Hst 5.

The cell wall of C. albicans is comprised of a multiple-layered glycoprotein-rich architecture and has been shown to be actively involved in many biological functions, especially the pathogenicity of this species (7). The major cell wall structure is composed of mannose-rich polysaccharides and glycoproteins interconnected by covalent bonds (8). In recent years, the development of sequential cell wall fractionation and analysis techniques, including two-dimensional PAGE and tandem mass spectrometry, have confirmed a heterogeneous population of noncovalently linked cell wall proteins (9, 10). Several proteins, previously thought to be associated exclusively in cytoplasmic compartments, have also been found in cell wall extracts. The lack of classical secretion signal sequence of these proteins implies that they may be sorted through alternative secretory pathways (11–13). Among them, the heat shock protein families (Hsp70, Hsp90, and Hsp104p) were found to be constitutively expressed at the cell surface of both C. albicans yeast and hyphal forms (6, 9–11). However, their functions at the cell surface have not been defined. It has been suggested that, in addition to their role in adaptation to temperature changes, these cell wall heat shock proteins may mediate direct interactions with the host environment (14–16).

Unlike S. cerevisiae, the Hsp70 Ssa family in C. albicans contains only two homologous family members, Ssa1p (656 amino acids) and Ssa2p (645 amino acids), which have 87.2% identity.

2 The abbreviations used are: Hst 5, histatin 5; BHst 5, biotin-labeled Hst 5; r, recombinant; HSP, heat shock protein; URA, uridine; DTSSP, 3, 3′-dithiobis-sulfosuccinimidylpropionate; RT, reverse transcription; TR, target region; ORF, open reading frame; DNP, 2,4-dinitrophenol.
of their protein sequences (6, 11, 17). In the cytoplasm, Ssa proteins are necessary for the translocation of newly synthesized polypeptides across the endoplasmic reticulum membrane by either co-translational or post-translational pathways (18, 19). They also participate as chaperones in protein folding and intracellular targeting to mitochondria and other cellular organelles (20, 21). All these processes are ATP-dependent and require the interplay between the ATPase domain and peptide-binding domains (22). Immuno-electron microscopy revealed that 70-kDa components, identified as Hsp70 proteins, are abundant at the yeast cell surface and extend through the cell wall and into plasma membrane in C. albicans (11). Such localization raises the possibility that Ssa proteins could serve to facilitate intracellular uptake of Hst 5, possibly by binding Hst 5 within the cell wall. However, it is not known whether both Ssa1 and Ssa2 proteins are part of the C. albicans cell wall structure or whether individual Ssa proteins have a role in facilitating translocation of Hst 5 to the cytosol. Hsp70 family members Ssa1 and Ssa2 are highly conserved genes in C. albicans, and expression levels of individual SSA genes have been reported (17, 23). In this study, direct interactions between C. albicans Ssa1 and Ssa2 proteins and Hst 5 are identified, and C. albicans SSA2 gene deletion mutants are found to be deficient in Hst 5 uptake and killing.

**EXPERIMENTAL PROCEDURES**

**Strains and Materials—Escherichia coli DH5α competent cells (Invitrogen) were used as a host for plasmids and were grown in Luria-Bertani (LB) medium (Difco). Ampicillin (Sigma) was added at a final concentration of 100 µg ml⁻¹. The ura3” auxotrophic C. albicans CAF4-2 strain (24) was the parental strain for Hsp70 gene deletions. The strains generated by this study and their genotypes are listed in Table 1. Yeast nitrogen base (YNB; Qbiogene, Morgan Irvine, CA) without uracil or uridine and supplemented with 2% glucose was used for selection of the URA3” transformants. Yeast carbon base (YCB; Sigma) was used for induction of the URA3 marker Source

**TABLE 1 C. albicans strains used in this study**

| Strain     | Parent | Genotype | URA3 marker | Source |
|------------|--------|----------|-------------|--------|
| CAF4-2     | SCS314 | Δura3::imm434/Δura3::imm434 | ura3” | Ref. 24 |
| ssa1Δ      | CAF4-2 | Δura3::imm434/Δura3::imm434 Δssa1/ssa1::FRT, SSA2/ssa2::FRT | ura3” | This work |
| ssa2Δ      | CAF4-2 | Δura3::imm434/Δura3::imm434 Δssa2/ssa2::FRT | ura3” | This work |
| ssa1Δ/SSA1 | ssa1Δ | Δura3::imm434/Δura3::imm434 Δssa1/ssa1::FRT/RP10::SSA1ORF, SSA2/ssa2::FRT | URA3* | This work |
| ssa2Δ/SSA2 | ssa2Δ | Δura3::imm434/Δura3::imm434 Δssa1/ssa1::FRT Δssa2/ssa2::FRT/RP10::SSA2ORF | URA3* | This work |

**Construction and Analysis of a Yeast Two-hybrid System—**

Yeast two-hybrid screens were performed with DupLEX-ATM yeast two-hybrid system (OriGene Technologies, Inc.) to detect specific protein-protein interactions as we have described previously (6). Briefly, sequences encoding Hst 5 and the C. albicans SSA1 and SSA2 ORFs were amplified by PCR and in-frame ligated into the bait and target plasmids. The yeast host strain EGY48 was first transformed with plasmids harboring the bait fusion genes and the reporter plasmid pSH18-34. All baits were assayed for auto-induction and nuclear localization. The plasmids containing the target fusion genes were then transformed into strains already containing the bait and reporter plasmids. Determination of β-galactosidase activity in liquid culture was performed in duplicate assays in at least four independent experiments for each bait-target combination.

**Purification of Recombinant Ssa1p and Ssa2p—**

Recombinant C. albicans Ssa1 and Ssa2 proteins were expressed and purified using a S. cerevisiae expression system (Invitrogen). C. albicans SSA1 and SSA2 cDNA was obtained using PCR primers and inserted into the cloning site of the S. cerevisiae yeast expression vector pYES2/NT/C that contains polynucleotide tags at the 3’ and 5’ ends of Xpress-tagged and V5-tagged sites, in order to produce an Hα-Xpress–SSA–V5-H6c coding sequence under the control of a galactose-inducible promoter. The final constructs were transfected into competent INVSc-1 S. cerevisiae cells (Invitrogen). After growth for 9 h in 2 liters of induction media containing 2% galactose, cells were harvested and glass bead-disrupted in ice-cold lysis buffer, and clarified cell lysates were collected following centrifugation at 13,000 × g for 5 min. The clarified supernatant was loaded onto a Ni-Sepharose 6 Fast Flow column (Amersham Biosciences) that had been equilibrated in binding buffer A (20 mM NaH2PO4, 0.5 M NaCl, 30 mM imidazole, pH 7.4). After washing with 10 column volumes of buffer A, bound proteins were eluted with 10 column volumes of 300 mM imidazole in buffer A and desalted with PAGEprep advance kit (Pierce), and Ssa proteins were visualized by immunoblot analysis using Xpress or anti-HSP70 antibodies. Elution fractions judged to be at least 95% pure by SDS-PAGE and immunostaining were pooled and dialyzed against water for 7 days at 4 °C. Dialysis products were lyophilized and stored in −78 °C. The homogeneity and identity of recombinant Ssa proteins from purified fractions were confirmed by mass spectrometry analysis (Yale Cancer Center Mass Spectrometry Resource & W. M. Keck Foundation Biotechnology Resource Laboratory).

**Pulldown Assay of Ssa-BHst 5 Complexes—**

BHst 5 (46 µg) was combined with purified recombinant Ssa1p or Ssa2p (15 µg) in binding buffer B (0.1 M phosphate, 0.15 M NaCl, pH 7.2), containing 0.8 mM ATP and 6 mM Mg2⁺, and incubated for 2 h at 4 °C to allow complex formation. For cross-linking experiments, 5 mM 3,3’-dithiobis-sulfsuccinimidyldipropionate (DTSSP) (Pierce) was added to the incubation mixture to stabilize Ssa-BHst 5 complexes. The reaction mixture was combined with immobilized streptavidin-agarose beads (50 µl) (Pierce) that had been pre-equilibrated in binding buffer B, placed in a spin column, and then incubated with gentle stirring.
for 1 h at 4 °C. Unbound proteins were removed by washing the beads four times with 500 μl of buffer B. Beads were incubated with 300 μl of SDS-PAGE sample buffer (2% SDS, 62.5 mM Tris, 10% glycerol, 2.5% 2-mercaptoethanol, pH 6.8) and placed in a water bath at 85 °C for 8 min to remove protein complexes and then collected from the spin column by centrifugation (1,200 × g, 1 min). Control reactions were performed with either rSsa1 or rSsa2 protein and streptavidin-agarose beads alone in identical pulldown assay conditions. Pulldown proteins were subjected to 7.5% SDS-PAGE, and rSsa proteins were detected by Western blotting with anti-Hsp70/Hsc70 monoclonal antibody or by Coomassie staining.

**DNA Manipulations**—Standard conditions for molecular cloning, DNA isolation, and transformation were carried out as described previously (25). All oligonucleotides used in strain constructions were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Restriction endonucleases and GeneRuler DNA markers were purchased from Fermentas (Hanover, MD). Fast-Link DNA ligation kit was from Epicenter Technologies (Madison, WI). Plasmid DNA was prepared by QIAprep spin miniprep kit from Qiagen (Valencia, CA). Genomic DNA of C. albicans strain was isolated by PUREGENE DNA isolation kit from Gentra System (Minneapolis, MN).

**Construction of pSSA1/2-URA Plasmids**—The URA3-flipper technique (26) was used to replace the first allele of each SSA1 or SSA2 gene. The pSFCU2 plasmid (kindly provided by Dr. J. Morschhauser) containing two FRT sites, a SAP2 promoter-driven FLIP gene and a URA3 marker, was used as the starting vector. The 5′-flanking sequence, including an ~550-bp length of the promoter region from either SSA1 or SSA2 gene, was subcloned and ligated with the KpnI- and XhoI-digested plasmid. The constructed plasmids containing 5′-flanking region of SSA4 genes were confirmed by PCR. Fragments including ~600 bp of 3′-flanking sequence, spanning the untranslated region of SSA1 or SSA2 genes, was then digested by BglII and SacI and ligated into the pSSA-5FR-URA plasmid. Insertion of SSA4 genes into the vector was confirmed by restriction digestion and PCR. The final gene disruption constructs were named pSSA1-URA and pSSA2-URA.

**Construction of pSSA1/2-URA Plasmids**—The pDBU3 plasmid (~5.1 kb), containing 2.37-kb length of the URA3 opening reading frame, was used as a starting vector. In addition to the 5′- and 3′-target sequences of the SSA1/2 gene (5′ TR and 3′ TR, ~350 bp each), a second 3′-target region (3′ TR-2, ~300 bp) amplified from the sequence immediately downstream of the first 3′-TR was added. The PCR product of 3′ TR-2 was introduced into the vector between the 5′ TR and URA3 marker. This additional flanking region served as the recognition site for fluoroorotic acid-induced intra-chromosomal recombination. Therefore, the final gene disruption constructs, pSSA1-URA and pSSA2-URA, were used to target the second allele of SSA1/2 gene.

**Disruption of SSA1 and SSA2 Genes in CAF4-2 Strain**—Because of the diploid genome of C. albicans, a combination of two genetic strategies was employed to sequentially remove both alleles of SSA genes. To achieve stable transformation, the pSSA-URA plasmids were linearized by overnight digestion with EamI1105I and then transformed into CAF4-2 strain by the lithium acetate/polyethylene glycol method (28). For each transformation, 50 μl of competent cells were mixed with 5 μl of 10 mg ml−1 denatured sheared salmon sperm carrier DNA (Clontech) and 5 μl of the linearized SSA disruption cassette. Transformed cells were spread onto uracil-deficient agar plates. Candida genomic DNA was isolated, and integration of gene disruption cassette was confirmed by PCR with control primers. To recycle the URA3-flipper cassette, selected colonies were further inoculated into 2 ml of YCB plus bovine serum albumin (4 mg ml−1) and uridine (100 μg ml−1) medium to enable the induction of SAP2 promoter-regulated FLP recombinase. A culture diluted overnight was directly plated onto YNB agar plates containing uridine (50 μg ml−1), and transformants were analyzed to screen for heterozygous mutants of SSA1/2 genes.

Because the same gene disruption cassette has poor efficiency for targeting the second allele, fluoroorotic acid-induced recombination was utilized as described previously (27) to disrupt the remaining wild-type copy of SSA1/2 genes. The pSSA-URA plasmids were linearized by Eco31I digestion and transformed into the heterozygous mutants (SSA1/ssa1::FRT or SSA2/ssa2::FRT). Successful replacement of the second allele SSA genes was confirmed by PCR. Selected colonies were spread onto YNB agar plates containing fluoroorotic acid (1 mg ml−1) and uridine (50 μg ml−1) to induce intra-chromosomal recombination and eliminate colonies containing the URA3 gene. Resulting homozygous mutants with either SSA1 or SSA2 gene deletions were obtained. For additional deletion of SSA genes, these mutants were transformed with another series of cassettes. Deletion of one allele of the second SSA1 or SSA2 gene was obtained, and the strains were designated as SSA1Δ and SSA2Δ (Table 1). However, no transformants with complete deletion of both SSA1 and SSA2 genes could be obtained, showing that at least one copy of either SSA1 or SSA2 gene is essential for viability.

**Restoration of SSA1 or SSA2 Genes in Deletion Mutant Strains**—To confirm gene-specific effects, two complementation strains were constructed by introducing a wild-type allele of SSA1 or SSA2 genes into ssa1Δ or ssa2Δ strains, respectively, at the RP10 locus (27). The ORF of either SSA1 or SSA2 genes (~3.5 kb), including ~1-kb promoter region and ~550-bp untranslated region, were cloned and ligated into the digested vector pDBU3.R. The resulting plasmids (pRP10-SSA1ORF and pRP10-SSA2ORF) were linearized with NcoI enzyme and transformed into the ssa1Δ and ssa2Δ mutants, respectively. The correct integration of these cassettes into the RP10 locus was verified by PCR. The genotypes of these complementation strains, designated as ssa1Δ/SSA1 and ssa2Δ/SSA2, are shown in Table 1.

**Southern Blot to Confirm SSA1/2 Gene Deletions**—Southern blotting was performed in wild-type and sequential deletion mutants using the HybQUEST DNP complete system (Mirus, Gene Transfer, Madison, WI) according to the manufacturer’s protocol. DNA probes (~700 bp) of SSA1/2 genes were cloned (bgSSA1–5′, GGAAGATCTATGAGGAAGAATAGGTAGTATTTAC; SSA1-r, TCTGATGAAATCATCTCAATATTTTAC; SSA2(C)-2, TGTAGATGAATTGATTA-
GTGGTG; and xhSSA2–3’, AACCTCGAGCATGATTTAATTATTAGTTGGATTATC) and labeled with HybQUEST DNP from kits. Membranes containing digested genomic DNA were pre-hybridized with 10 mg ml−1 denatured sheared salmon DNA to block nonspecific binding. Denatured DNP-labeled SSA probes (50 μl) next were added into 3.5 ml of pre-warmed hybridization solution and incubated at 42 °C overnight. The membrane was blotted following stringency washes in (2×, 0.5×, and 0.1×) SSC buffer with 0.1% SDS. Hybridized bands were visualized by exposure of the membrane to Lumi-PhosPlus chemiluminescent substrate and developed on film.

Total RNA Isolation, cDNA Synthesis, and Real Time RT-PCR—Transcriptional levels of each gene were analyzed following growth at room temperature and after heat shock conditions at 37 °C for 1 h. Total RNA isolation was performed using the RNaseq mini kit from Qiagen. Samples were both in-column-treated with DNase I, and off-column-treated using the TURBO-DNA-Free set from Ambion. The absence of genomic DNA contamination was confirmed by PCR amplification, and quencher dye for the probes were 6-carboxyfluorescein (FAM) labeled probes (50 nM of the forward and reverse primers. Fluorescent data were collected and analyzed with iCycler iQ software. The reporter dye value was obtained by calculating the difference Ct values of the target gene (SSA1 or SSA2) and the normalizer (EFB1). The ΔCt of CAF4-2 at room temperature was used as a reference (base line). The ΔΔCt values were then calculated as the difference between the ΔCt of each sample and the base line and were transformed to absolute values (2−ΔΔCt) to calculate comparative expression levels.

Candidacidal Assays of Histatin 5—Antifungal activities of Hst 5 with C. albicans strains were examined by microdilution plate assays (6). Single colonies of each strain were inoculated into 10 ml of YPD medium with uridine (50 μg/ml) and grown overnight at room temperature until A600 values reached 1.6–1.8. Cells were washed twice with 10 mM phosphate buffer, pH 7.4, and then cells (106) were mixed with different concentrations of Hst 5 and incubated at room temperature with constant shaking for 1 h. For heat shock conditions, cells were transferred to pre-warmed (37 °C) buffer and incubated at 37 °C for 30 min prior to addition of 125I-Hst 5 and then incubated at 37 °C for another 30 min to allow uptake of 125I-Hst 5. Following two rinses with binding buffer to remove nonspecifically bound protein, total cellular uptake of 125I-Hst 5 was measured in a γ-counter. Data from at least three independent experiments were used to obtain the mean total uptake of 125I-Hst 5 for each cell type and condition.

Western Blot of Cell Wall and Cytoplasmic SSA1/2 Proteins and Transported Hst 5—Expression levels of SSA1/2 proteins in C. albicans cell wall and cytoplasm were assessed as described previously (6). Briefly, each strain was grown under the same conditions as for RNA isolation. Cells were washed twice with 10 mM sodium phosphate buffer, pH 7.4, and cell wall components were released by incubation of cell suspension in the ammonium carbonate buffer (1.89 g/liter, pH 8.29) containing 1%/v/v β-mercaptoethanol for 30 min, 150 rpm at 37 °C. The supernatant, containing β-mercaptoethanol cell wall extracts, was collected following centrifugation (2,300 × g) for 3 min. Proteins were immediately concentrated using Centricon YM-30 filters (Millipore Corp., Bedford, MA) at 4 °C. The remaining cell pellets were suspended in ice-cold lysis buffer, and cytosolic proteins were extracted by breaking the cells in a Lysing Matrix C tube (QBiogene). After measuring protein concentrations, 7.5 μg of total proteins from each sample were loaded onto SDS-polyacrylamide gels. Each Hst 5 protein was transferred to a PVDF membrane (Millipore Corp.) and probed with anti-Hst 5 (numbers of colonies from suspensions with Hst 5/numbers of colonies from control suspensions) × 100.

For time course assays of cytosolic levels of Hst 5, cells (1 × 108) were suspended in 1 ml of phosphate buffer, and BHst 5 was added to a final concentration of 31.25 μM. The cell mixtures were incubated with constant shaking for 0, 15, 30, 45, 60, 75, and 90 min. The reaction was stopped at each time point, and the cell pellet was subjected to cell wall and cytoplasmic extraction as described above. Equal amounts of protein from C. albicans Ssa2p Facilitates Histatin 5 Toxicity

ΔCt value was obtained by calculating the difference between Ct values of each gene (SSA1 or SSA2) and the normalizer (EFB1). The ΔCt of CAF4-2 at room temperature was used as a reference (base line). The ΔΔCt values were then calculated as the difference between the ΔCt of each sample and the base line and were transformed to absolute values (2−ΔΔCt) to calculate comparative expression levels.
TABLE 2
Quantification of protein-protein interactions between C. albicans Ssa1p, Ssa2p, and Hst 5

| Bait-target | Hst 5 | Ssa1p | Ssa2p |
|-------------|-------|-------|-------|
| Hst 5       | 8.27 ± 0.16 | 1.88 ± 0.20 | 7.67 ± 0.48 |
| Ssa1p       | 6.76 ± 0.38 | 0.55 ± 0.03 | 4.44 ± 0.47 |
| Ssa2p       | 9.03 ± 1.52 | 1.89 ± 0.06 | 3.64 ± 0.11 |

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each extract were subjected to 12.5% SDS-PAGE, and BHst 5 (~3 kDa) was detected using streptavidin conjugated with horseradish peroxidase (Pierce). Quantitative analysis of cytosolic BHst 5 and Ssa proteins was performed with a Bio-Rad GS-700 Imaging Densitometer (Arcus II, Agfa) and Quantity One software (version 4.2). Levels of cytoplasmic BHst 5 from at least three independent experiments were averaged, and the means were curve fitted using Prism 4.03 software.

Statistical Analyses—Differences between experimental groups were evaluated for significance by unpaired t test or one-way analysis of variance by using Prism 4.03 software.

RESULTS

Protein-Protein Interactions Determined by Yeast Two-hybrid System Analysis—Previously, we examined Hst 5-Ssa1 protein interactions by yeast two-hybrid analysis (6) and found moderate interaction between these proteins when Hst 5 was used as the bait protein, but less interaction when Hst 5 was used as a target. Therefore, we extended these experiments to include C. albicans Ssa2 proteins in parallel with Ssa1p in order to directly compare levels of interactions. To exclude the possibility of auto-activation induced by bait fusion proteins alone, the LexA-bait fusion constructs containing the SSA1, SSA2, or Hst 5 were transformed into the EGY48 strain, and no detectable β-galactosidase activities were observed. Consistent with previous results, we found moderate levels of activation between Hst 5 and Ssa1p when used as bait and target, respectively, but little activation with the reverse combination (Table 2). However, EGY48 strains co-expressing SSA2 and Hst 5 exhibited significant β-galactosidase activity in both bait and target combinations, indicating strong interactions between C. albicans heat shock protein Ssa2p and Hst 5. Some interaction of Ssa2p with itself was observed, although it was not remarkably as strong as that of Hst 5-Ssa1p interactions. The combination of SSA2 and SSA1 as the bait and target exhibited over twice the β-galactosidase activity of reverse combination. Thus, although Ssa1 and Ssa2 proteins show some interactions with each other, by far the strongest interaction among the combinations tested was between Ssa2p and Hst 5.

Assessment of Direct Interactions between Hst 5 and Ssa Proteins—Because we had observed binding by far Western assay of Hst 5 with a protein obtained from C. albicans cell extracts whose identity was consistent with either Ssa1 or Ssa2 protein, binding assays using individual rSsa1 and rSsa2 proteins with Hst 5 were carried out. Purified recombinant Ssa1 and Ssa2 proteins were tested for direct interactions with Hst 5 by in vitro pulldown assays in order to assess their relative strengths of interaction, and we compared them with those observed in the yeast two-hybrid system. When Ssa1 protein was incubated with BHst 5, only very weak interaction was found, as evidenced by the very minor amount of protein detected by pulldown with SA beads following incubation with BHst 5 (Fig. 1, upper panel, 3rd lane). Because HSP70 proteins are molecular chaperones characterized by transient binding with substrates, the chemical cross-linker DTSSP was used to stabilize any weakly bound protein complexes. Following chemical cross-linking, small amounts of Ssa1 protein were detectable in pulldown complexes (Fig. 1, upper panel, 4th lane), showing that Hst 5-Ssa1 protein association does occur; however, these complexes may be weak or are transient. In contrast, Ssa2 protein was readily observed upon pulldown of BHst 5-Ssa2 complexes with SA beads without use of a chemical cross-linker (Fig. 1, bottom panel, lane 3), and addition of DTSSP revealed substantial amounts of Ssa2p-BHst 5 complexes upon pulldown (Fig. 1, bottom panel, lane 4). The specificity of these observed interactions was verified, because no interaction between either Ssa protein or SA beads alone was detected (Fig. 1, 2nd lane). Using equal amounts of input Ssa1 and Ssa2 proteins, nearly 10-fold more Ssa2p-BHst 5 was detected in cross-linked pulldown assay than Ssa1p-BHst 5 complexes (Fig. 1, bottom panel, far right lane). Thus, both native and chemically cross-linked assays demonstrated the higher affinity of Hst 5 for Ssa2p than for Ssa1p. These results are in agreement with the higher levels of interactions between Ssa2 and Hst 5 measured by yeast two-hybrid analyses (Table 2). However, both Ssa1 and Ssa2 protein associations with BHst...
C. albicans $\text{Ssa2p}$ Facilitates Histatin 5 Toxicity

5 appear to be transient as demonstrated by the large increase in pulldown protein following complex stabilization by cross-linking. Because both pulldown and two-hybrid analyses found pulldown protein following complex stabilization by cross-

C. albicans $\text{Ssa2p}$ Facilitates Histatin 5 Toxicity

mRNA Levels of $\text{SSA1}$ and $\text{SSA2}$ Genes in C. albicans Wild-
type and Mutant Strains—S. cerevisiae contains four $\text{SSA}$ genes, whose expression is induced by heat shock or other stressors (35). In wild-type C. albicans, both $\text{Ssa1}$ and $\text{Ssa2}$ proteins are constitutively expressed, and total cellular levels are further increased upon temperature shift to 37 °C (6, 17). However, quantification of relative levels of $\text{Ssa1}$ and $\text{Ssa2}$ proteins and their cellular localization in mutant strains lacking functional copies of either $\text{SSA}$ gene have not been investigated. To determine relative expression levels of each family member, quantitative real time RT-PCR was performed to measure the transcript levels of $\text{SSA1}$ and $\text{SSA2}$ genes in wild-type and null mutants. Alignment of $\text{SSA1}$ and $\text{SSA2}$ cDNA sequences showed 85.5% identity, with the highest homology at the N terminus. Therefore, C-terminal variable regions (the last 150 bp of coding sequence) were employed for design of primer pairs and probes to differentiate between these related genes. The housekeeping gene $\text{EFB1}$ was examined in parallel as the internal control because it was not affected by temperature or by $\text{SSA}$ gene mutation. The total basal transcriptional level of $\text{SSA1}$ in wild-type cells was nearly 100-fold higher than $\text{SSA2}$ transcripts, showing that the $\text{SSA1}$ gene is constitutively expressed at high levels relative to $\text{SSA2}$ during normal growth in C. albicans. For purposes of analyses, the transcriptional level of each gene was expressed relative to basal $\text{SSA1}$ or $\text{SSA2}$ transcripts at room temperature.

In the wild-type CAF4-2 strain, heat shock induction at 37 °C for 1 h resulted in 2.66 ± 0.34-fold up-regulation of $\text{SSA1}$ transcript (Fig. 2A) and 1.78 ± 0.46-fold increase in transcript of the $\text{SSA2}$ gene (Fig. 2B). The $\text{ssa2}$ mutant, having only one remaining $\text{SSA1}$ allele, had basal transcripts of $\text{SSA1}$ of 0.59 ± 0.23 relative to wild-type strain. Heat shock conditions induced a 2-fold increase in $\text{SSA1}$ transcripts (1.26 ± 0.44), returning levels to that of the wild-type strain. As expected, $\text{SSA2}$ gene transcripts in this strain were not detectable, confirming the specificity of primers and probes used in real time RT-PCR experiments. In contrast, basal levels of $\text{SSA2}$ transcripts in the $\text{ssa1}$ mutant, having only one remaining $\text{SSA2}$ allele, were increased to 1.63 ± 0.53 that of the wild-type strain, which likely reflects a compensatory response to loss of the highly expressed $\text{SSA1}$ gene. Heat shock treatment of $\text{ssa1}$ further increased $\text{SSA2}$ transcription by 6.02 ± 1.76-fold compared with the wild-type strain. No $\text{SSA1}$ transcripts were detected in the $\text{ssa1}$ mutant, confirming knock-out of the $\text{SSA1}$ gene.

Reconstitution of the $\text{SSA1}$ gene in the $\text{ssa1}$ mutant background resulted in nearly complete recovery of $\text{SSA1}$ transcript levels (0.72 ± 0.16-fold at room temperature and 1.61 ± 0.08-fold at 37 °C heat shock) (data not shown). Interestingly, complementation of the $\text{SSA1}$ gene also returned $\text{SSA2}$ transcription to that of the wild-type strain at both room temperature and heat shock conditions (1.00 ± 0.28 and 1.67 ± 0.77-fold, respectively) despite $\text{SSA2}$ hemizygous gene status. Re-introduction of the $\text{SSA2}$ gene in $\text{ssa2}$ mutant background restored $\text{SSA2}$ transcripts to nearly that of wild-type levels (0.68 ± 0.06 at room temperature and 1.35 ± 0.69 after heat shock) but did not alter $\text{SSA1}$ mRNA levels (data not shown). These complementation experiments confirm that expression
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FIGURE 2. Deletion of the C. albicans SSA1 gene induces compensatory expression of the remaining SSA2 allele. Transcriptional profiles of SSA1 and SSA2 genes were characterized by quantitative real time RT-PCR from at least three independent experiments. Quantitative mRNA levels of SSA1 (A) and SSA2 (B) were expressed relative to the baseline of wild-type cells grown at room temperature (RT). Wild-type (WT) cells (black bars) had over a 2-fold increase of transcription of SSA1 and 1.5-fold increase in SSA2 genes following heat shock (HS). Transcripts of SSA1 in the ssa2Δ mutant strain (gray bars) were equivalent to wild-type levels at room temperature, but transcriptional response to heat shock was attenuated. In contrast, transcripts of SSA2 in the ssa1Δ mutant strain (white bars) were slightly increased at room temperature and elevated by 6-fold following heat shock. No mRNA was detected from SSA1 or SSA2 genes in each respective null construct.

FIGURE 3. C. albicans cell wall-localized Ssa proteins are increased following heat shock. Cell wall (left) and cytoplasmic proteins (right) were extracted from C. albicans strains at either room temperature (25 °C) or following heat shock for 1 h at 37 °C. Equal amounts of protein were loaded in each lane, and Ssa1 and Ssa2 proteins were resolved by 7% SDS-PAGE and detected by Western blot hybridization with anti-Hsp70/Hsc70 monoclonal antibody. C. albicans Ssa2 protein has a larger apparent molecular mass (upper band) than Ssa1 protein (lower band). Lanes 1 and 2, wild-type strain CAF4-2; lanes 3 and 4, ssa1Δ mutant; lanes 5 and 6, ssa2Δ mutant. Proteins extracted following growth at room temperature are indicated with a minus sign, whereas proteins extracted following heat shock are indicated with a plus sign. The apparent molecular mass is indicated at the right.

of C. albicans SSA1 and SSA2 genes is interdependent, as has been shown for the SSA family in S. cerevisiae (35).

Ssa1p Is the Major Cell Surface-localized HSP70 Protein in C. albicans—Levels of cytoplasmic and cell wall Ssa1 and Ssa2 proteins were quantified by Western blot in wild-type and mutant strains both at room temperature and following heat shock (Fig. 3). Two proteins (70 and 75 kDa) were detected with monocolonal anti-Hsp70 in wild-type cells (Fig. 3, lanes 1 and 2). The 70-kDa band was identified as Ssa1p, because it contained significantly more protein than the 75-kDa band and corresponded with the ssa1Δ mutant (Fig. 3, lanes 3 and 4) and ssa2Δ mutant (Fig. 3, lanes 5 and 6), which express only Ssa2 or Ssa1 proteins, respectively. Because the predicted size of Ssa1p is greater than the Ssa2p, the higher apparent molecular mass of Ssa2p found by SDS-PAGE suggested the possibility of additional post-translational modifications. Inspection of the primary sequence of the variable C terminus of Ssa2p revealed eight Ser/Thr substitutions as potential O-glycosylation sites, and one potential N-glycosylation site (NQT) not present in Ssa1p. Four of the Ser/Thr substitutions were closely spaced and were clustered with proline residue substitutions, further suggesting a strong propensity for O-glycosylation at these sites (36). Therefore, it is possible that the larger apparent molecular mass of Ssa2p is a result of additional glycosylation in the C-terminal region compared with Ssa1p.

As expected from higher relative transcriptional levels of SSA1, cell wall levels of Ssa1p were 4–5-fold more abundant than Ssa2p in wild-type cells at room temperature (Fig. 3, lane 1). Clearly, Ssa1p is the predominant HSP70 family member localized at the cell wall of C. albicans. Heat shock elevated both cell surface Ssa1 and Ssa2 proteins in wild-type cells (Fig. 4), resulting in 1.6 ± 0.4-fold increase in cell surface Ssa1p and 3.7 ± 1.6-fold augmentation in cell wall Ssa2p levels (Fig. 4A). In contrast, cytoplasmic levels of Ssa1p were unchanged upon heat shock in wild-type cells, whereas Ssa2p was only slightly increased (Fig. 3 and Fig. 4B).

Because deletion of one C. albicans SSA gene resulted in compensatory increase in expression of the remaining gene (Fig. 2), we examined SSA protein levels in the cytosol and cell wall of each mutant. Cell wall levels of Ssa1p in the ssa2Δ mutant were elevated further by 1.7 ± 0.2-fold at room temperature and by 2.2 ± 0.3-fold following heat shock compared with the wild-type strain (Fig. 4A). However, cytosolic levels of Ssa1p were unchanged in the ssa2Δ mutant at either temperature (Fig. 4B). The ssa1Δ mutant showed a similar, but less pronounced, elevation in cell wall Ssa2p that was increased by 1.6 ± 0.1- and 4.2 ± 2.0-fold at room temperature and after heat shock, respectively. As for Ssa1p in the ssa2Δ strain, total cytoplasmic levels of Ssa2p were unchanged following heat shock treatment in the ssa1Δ (Fig. 4B). Thus, each mutant retained normal cytosolic levels of its remaining Ssa protein but had elevated cell wall levels of the respective remaining SSA protein compared with the wild type.

C. albicans Ssa2Δ Mutants Are Resistant to Hst 5 Killing—Our previous results using S. cerevisiae showed that fungicidal activities of Hst 5 were substantially decreased in the Δssa1Δssa2 double mutant (6), suggesting the functional relevance of Ssa proteins for Hst 5 activity. Based upon these findings, we assessed the sensitivity of C. albicans ssa1Δ and ssa2Δ mutants to Hst 5 in order to determine the role of these proteins in Hst 5-mediated toxicity and to compare functional differences between these two family members.
Assays using Hst 5 were carried out to correlate killing with the expression levels of Ssa1p and Ssa2p. At room temperature, wild-type cells (Fig. 5A, solid squares) had typical dose-dependent sensitivity to Hst 5 in which killing rose from 34 to 71% following incubation with increasing doses from 3.8 to 31.25 μM of Hst 5. Compared with the wild-type strain, the ssa1Δ mutant (Fig. 5A, open circles) had no statistically significant difference in sensitivity to Hst 5 (p > 0.05), showing that loss of Ssa1p did not affect Hst 5 killing. In contrast, the sensitivity of the ssa2Δ mutant to Hst 5 was significantly (p < 0.01) decreased (Fig. 5A, solid triangles) so that only 23% of cells were killed at 31.25 μM dosage of Hst 5. These results show that Ssa2p contributes substantially to Hst 5 killing of cells under nonheat shock conditions, whereas Ssa1p, even though overexpressed in the ssa2Δ mutant strain, has a lesser role in Hst 5 fungicidal activity.

To further examine the role of Ssa2p in Hst 5 killing, cells were subjected to heat shock conditions prior to treatment with Hst 5. Wild-type cells (Fig. 5B, solid squares) displayed increased sensitivity to Hst 5 following heat shock treatment compared with cells incubated at room temperature (Fig. 5A), as expected from increased levels of cell wall localized Ssa proteins. Killing of wild-type cells with 31.25 μM Hst 5 was increased to 89%. However, the candididal effect of Hst 5 with ssa1Δ mutant strain (Fig. 5B, open circles) was not significantly different (p > 0.05) from wild-type cells following heat shock, in good agreement with similar cell wall expression levels of Ssa2p upon heat shock in these two strains (Fig. 4A). In contrast, the ssa2Δ mutant strain (Fig. 5, solid triangles), despite having higher cell wall levels of Ssa1p than heat-shocked wild-type cells, had significantly reduced susceptibility to Hst 5 compared with wild-type cells. However, heat shock conditions elevated the overall sensitivity of ssa2Δ cells to Hst 5, suggesting that other receptors for Hst 5 may be induced under these conditions, perhaps additional cell wall-localized heat shock proteins.

**Total Cell-associated 125I-Hst 5 Is Decreased in C. albicans Ssa2Δ Mutants—Because in vitro experiments showed direct physical interactions between both Ssa proteins and Hst 5 (Fig. 1), these proteins may also have an important role in mediating binding of Hst 5 with the cell in vivo. Therefore, we assessed the levels of total cell-associated 125I-Hst 5 in C. albicans wild-type and mutant strains following 30 min of incubation of 3 μM 125I-Hst 5 peptide. Wild-type cells (Fig. 6, black bars) had an average total cellular uptake of 6.35 pmol of 125I-Hst 5/10^6 cells at room temperature, and this value was used as a base line for comparison with other strains. Preincubation of wild-type cells under heat shock conditions increased total cellular uptake of 125I-Hst 5 by 1.6 ± 0.1-fold, mirroring the increase in cell wall Ssa proteins induced by heat shock (Fig. 3). In contrast, the ssa2Δ strain (Fig. 6, gray bars) was significantly (p < 0.05) reduced to 0.3 ± 0.1 that of wild-type cells at room temperature and 0.6 ± 0.3 upon heat shock. Uptake of 125I-Hst 5 by the ssa1Δ strain (Fig. 6, white bars) at room temperature was slightly reduced to 0.6 ± 0.2 of wild-type cells, but this difference was not statistically significant, whereas total cellular uptake of Hst 5 by ssa1Δ cells was equivalent (1.5 ± 0.5) to wild-type cells following heat shock treatment. Replacement of SSA1 or SSA2 genes in their respective deletion mutant resulted in restoration of wild-type levels of 125I-Hst 5 uptake (no statistically significant difference compared with wild-type cells) in both ssa1Δ/SSA1 (Fig. 6, cross-hatched bars) and ssa2Δ/SSA2 (square-hatched bars) strains. These results further showed a more critical requirement for Ssa2 proteins for total cellular uptake of Hst 5.

**C. albicans ssaΔ Mutants Are Impaired in Intracellular Translocation Hst 5—Intracellular transport of Hst 5 is essential for its killing activity; however, the relationships between...
levels of total cell binding and transport are not known. Therefore, we carried out experiments to assess intracellular transport of Hst 5 in wild-type and mutant cells having differing levels of cell wall Ssa proteins. Quantification of cytoplasmic levels of internalized BHst 5 was obtained from cell lysates following β-mercaptoethanol extraction of cell wall proteins to strip extracellularly bound Hst 5. Cell lysates were blotted and probed for BHst 5 at 15-min intervals following Hst 5 incubation (Fig. 7A), and cytoplasmic levels of BHst 5 were quantified by densitometry (Fig. 7B). Significant amounts of intracellular (cytoplasmic) BHst 5 were detected in the wild-type strain at 15 min, showing that intracellular transport of the protein occurs rapidly following introduction with Hst 5 (Fig. 7B, solid squares). Wild-type cells continued to accumulate cytosolic Hst 5 at nearly a linear rate over the 90-min observation time. The ssa1Δ strain accumulated BHst 5 at a similar initial rate for the first 30 min compared with the wild-type strain (Fig. 7B, open circles), in agreement with total cellular levels of 125I-Hst 5 observed at 30 min (Fig. 6). However, the level of cytosolic BHst 5 did not increase after 45 min, so that by 90 min, total cytoplasmic accumulation of BHst 5 in the ssa1Δ strain was decreased by 50% of wild-type levels ($p < 0.05$). Thus, although the ssa1Δ strain has wild-type levels of Ssa2p in the cell wall, these cells can only sustain wild-type levels of Hst 5 uptake for 30 min, showing that other factors are required for continued uptake, perhaps an adequate ATP supply.

In contrast, the 30-min cytosolic accumulation of BHst 5 in the ssa2Δ strain (Fig. 7B, closed triangles) was reduced to less than one-third of wild-type levels, again in close agreement...
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with reduction in total cellular levels of \(^{125}\text{I}-\)Hst 5 at 30 min (Fig. 6) and further showing that the majority of Hst 5 is translocated to the cytosol by 30 min. By 90 min, the cytosolic accumulation of BHst 5 in the ssa2Δ strain was decreased to only 10% of wild-type levels of Hst 5 (p < 0.01), showing substantial reduction in translocation of Hst 5 in this mutant, despite having greater levels of cell wall Ssa1p compared with wild-type cells (Fig. 4A). Thus, Ssa2p appears to have an important role in facilitating intracellular transport of Hst 5 in C. albicans.

**DISCUSSION**

The presence of Ssa1 and Ssa2 proteins as bona fide constituents of the C. albicans cell envelope has raised questions regarding their function in this cellular compartment. To assess these two highly related proteins in C. albicans in respect to their potential function with Hst 5, we constructed isogenic SSA deletion mutants. It is important to note that neither ssa1Δ nor ssa2Δ mutant exhibited any differences in growth rates or in ability to form hyphae that would be indicative of cell wall defects. Thus, either Ssa proteins do not have a major functional role in formation or maintenance of the cell wall or the remaining Ssa protein is able to fully replace the deleted Ssa protein in respect to cell wall functions in C. albicans. The latter possibility is supported by the functional overlap of Ssa proteins in S. cerevisiae (29), and by our data that show a compensatory increase in cell wall levels of the remaining Ssa protein in each deletion mutant (Fig. 3).

Deletion of the SSA2 gene resulted in a significant reduction in total cell binding and intracellular translocation of Hst 5, despite elevated cell wall levels of Ssa1 protein that are normally 5-fold more abundant than Ssa2p in wild-type cells. This reduction in Hst 5 uptake was mirrored by reduced sensitivity to Hst 5 when compared with the wild-type strain, as expected because Hst 5 transport to the intracellular compartment is required for toxicity. In contrast, deletion of the SSA1 gene, which removed the predominant Ssa1 protein from the cell wall, had much less effect on Hst 5 binding and uptake. These results argue for the specificity of observed effects between Hst 5 and Ssa2p, because any secondary effects as a result of deletion of Ssa proteins would be expected to be more significant upon loss of the major Ssa1p in the cell wall. These results were also supported by pulldown assays that showed a higher relative level of binding of Ssa2p with Hst 5, as compared with Ssa1p (Fig. 1), as well as higher interaction scores between Ssa2p and Hst 5 identified in yeast two-hybrid assays (Table 2). Collectively, these data suggest that although both Ssa proteins are capable of interaction with Hst 5, Ssa2 protein appears to have a higher propensity for association with Hst 5, as well as a more significant functional role within the cell wall in respect to toxicity of Hst 5.

Heat shock proteins are abundant molecular chaperones of eukaryotic cells that bind client proteins in association with other co-chaperones. Their binding role in the cytosol induces protein folding and structural changes that allow the client protein to attain full activity. Their function as noncovalently bound, freely extractable cell wall components is presumed to be similar to characterized cytosolic functions. In C. albicans, at least three other heat shock proteins, including Hsp60p, Hsp90p, and Hsp104p, have been identified as cell surface proteins (9, 10), suggesting that elements to form co-chaperone complexes are also present in the cell wall. ATP is required for cyclic binding and release of client proteins by these molecular chaperone complexes. ATP in the yeast cell wall and cell envelope may be readily available from cellular sources. In this regard, we found that Hst 5 treatment of C. albicans induces cellular efflux of ATP that mediates cytotoxicity, because elimination of ATP from extracellular medium by addition of the phosphatase enzyme apyrase significantly protected cells from Hst 5-induced killing (37). Thus, the cell itself under certain conditions may release ATP that may be used for HSP-client binding interactions in the cell wall.

A striking feature of the role of Ssa2 protein, as illustrated by deletion constructs, is the close relationship between bound total Hst 5 and its subsequent cytosolic translocation. Protein transport processes have been well studied because they are essential for cell survival and growth. The import of protein toxins such as E. coli colicins is one such process that is accomplished by toxin binding to specific receptors on susceptible target cells, before being translocated across the cell membrane by transporter complexes (38–40). Colicins are opportunistic cargo for receptors in the bacterial outer membrane whose normal physiological function is to bind and transport metabolites such as metals, sugars, and vitamins. Translocation is mediated by two receptors, one of which is mainly used for initial binding and sequestering of colicin, and facilitates entry into a second neighboring receptor-translocator complex. These features very closely resemble the observed process of Hst 5 binding and translocation found in the present studies. Although heat shock proteins have not yet been characterized to be involved in intracellular transport of client proteins, such interactions would be consistent with their chaperone functions. In this regard, recent studies analyzing Hsp90 interactors identified 28 membrane transport proteins, including six plasma membrane permeases as being novel interacting partners (41). Thus, it is possible that Ssa2 protein initially binds Hst 5 as it comes into contact with the cell wall and then transfers Hst 5 to another membrane-associated translocator complex, which subsequently imports Hst 5 into the cytoplasm. A possible candidate for this function is the yeast nonspecific cation transporter, NSC (42).

The colicin model suggests that C. albicans Ssa proteins located in the cell wall may function as receptors to bind and transport extracellular metabolites. Our findings that heat shock significantly enhanced the cell wall distribution of Ssa proteins support the notion that these proteins are rapidly recruited in order to adapt cells to survive temperature stress by increasing uptake of essential compounds. However, cell surface-localized heat shock proteins play other roles, including modulation of the immune response (5, 43, 44) and regulation of recruitment of natural killer cells and phagocytic cells (45, 46). In this regard, extracellular Hsp70 initiated uptake of granulocyte B in tumor cells (47), whereas Hsp60 of Histoplasma capsulatum was identified as a cell surface ligand for the complement receptor CR3 (CD11b/CD18) on human macrophages (48).

More definitive elucidation of the mechanism of action of Ssa2p and its precise role in translocation of Hst 5, and perhaps
other toxic peptides, needs further investigation. Nevertheless, the present data demonstrate that Ssa2p, and to a lesser extent Ssa1p, play crucial roles in binding and coordinate intracellular transport of Hst 5 required for its toxicity. The wide range of functions of cell wall-associated Hsp family members suggests that they may carry out multiple functions in yeast. One intriguing possibility is that Ssa proteins not only function in nutrient capture and transport but may also have a further role in *C. albicans* adhesion and development of oropharyngeal candidiasis.

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