Candidacidal Activity of Salivary Histatins

IDENTIFICATION OF A HISTATIN 5-BINDING PROTEIN ON Candida albicans*

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Candida albicans is the predominant species of yeast isolated from patients with oral candidiasis, which is frequently a symptom of human immunodeficiency virus infection and is a criterion for staging and progression of AIDS. Salivary histatins (Hsts) are potent in vitro antifungal agents and have great promise as therapeutic agents in humans with oral candidiasis. The molecular mechanisms by which Hsts kill yeast cells are not known. We report here, that unlike other antimicrobial proteins, Hsts do not display lytic activities to lipid membranes, measured by release and dequeenching of the fluorescent dye calcein. Analysis of the magnitude and time course of Hst-induced calcein release from C. albicans cells further showed that loss of cell integrity was a secondary effect following cell death, rather than the result of primary disruption of the yeast cell membrane. 125I-Hst 5 binding studies indicated that C. albicans expressed a class of saturable binding sites (Kd = 1 μM), numbering 8.6 × 105 sites/cell. Both Hst 3 and Hst 4 competed for these binding sites with similar affinities, which is consistent with the micromolar concentration of Hsts required for candidacidal activity. Specific 125I-
Hst 5 binding was not detected to C. albicans spheroplasts, which were 14-fold less susceptible to Hst 5 killing, compared with intact cells in candidacidal assays. In overlay experiments, 125I-Hst 5 bound to a 67-kDa protein detected in C. albicans whole cell lysates and crude membrane fractions, but not in the yeast cell wall fraction. Consistent with the overlay data, cross-linking of 125I-Hst 5 to C. albicans resulted in the appearance of a specific 73-kDa 125I-Hst 5-containing complex that was not detected in the cell wall. 125I-Hst 5 binding protein of similar size was also observed in susceptible S. cerevisiae strain T1#20. This is the first description of Hst 5 binding sites on C. albicans which mediate cell killing and identification of a 67-kDa yeast Hst 5 binding prote-

Oral candidiasis is a frequent superficial infection in humans associated with mechanical or traumatic factors or with an immunocompromised host (1). Candida albicans is the predominant species of yeast isolated from oral tissues of patients with oral candidiasis (2). Oral candidiasis is commonly associated with HIV* infection and is a criterion for the development and progression of AIDS (3). Relatively few antifungal drugs are available for clinical treatment of oral or systemic candidiasis. Increased use of these antifungal agents to treat candidiasis in late stage AIDS and cancer patients has resulted in a dramatic increase in emergence of candidal species with antifungal drug resistance, especially to azole-based drugs.

Growth or adhesion of pathogenic yeasts as well as bacteria in the oral cavity are regulated by glandular secretion of an array of salivary proteins (4, 5). Histatins (Hsts) are structurally related basic proteins of acinar cell origin, which possess in vitro candidacidal and candidastatic (6–8) activities and, to a lesser degree, bactericidal properties (9) and participate in nonimmune host defense of the oral cavity (10). Hsts have potential as therapeutic agents against oral candidiasis, being potent antifungal molecules with no toxicity to humans.

Histatin 1 (Hst 1), histatin 3 (Hst 3), and histatin 5 (Hst 5) are the major Hsts products found in saliva from human submandibular-sublingual and parotid glands (11, 12) and are present in healthy adults at concentrations of 50–425 μg/ml (13). Hst 1 and Hst 3 are the full-length precursor molecules, which are cleaved by specific proteolytic processing events during secretion to produce smaller histatins (14–16). In vitro, Hst 5 is the most potent candidacidal member of the family that kills pathogenic Candida species from 90% to 100% at physiological concentrations (15–30 μM) (10, 17). We have shown that Hst M, the middle portion of Hst 3 (residues 9–24), has equivalent candidacidal activity with the full-length molecule (17). Candidacidal activity of this fragment has been confirmed (8), and duplication of these residues (residues 13–24) as a tandem repeat within Hst 3 has produced a peptide with enhanced candidacidal activity at low concentrations (18).

Histatins should be added to the described families of natural antibiotics produced by host tissues in contact with indigenous microorganisms (19). These antimicrobial proteins are produced by a variety of tissues and organisms as a means of nonimmune host defense. Although Hsts have similar size and net positive charge as other naturally occurring antimicrobial

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1 The abbreviations used are: HIV, human immunodeficiency virus; Hst, histatin; Fmoc, N-(9-fluorenylmethoxycarbonyl); Bac, bacitracin; Tricine, N-tris(hydroxymethyl)methylglycine; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TES, Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; HRP, histidine-rich polypeptide; ePC, egg phosphatidylcholine; PI, phosphatidylserine; DSS, disuccinimidyl suberate; BS, bistrifluoroacetimidyl suberate.

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peptides, they possess structural features unique from other described low molecular weight cationic proteins. The high histidine content, lack of disulfide bonds, and weak amphipathic character of the α-helical structures distinguish Hsts from the known natural antimicrobial proteins such as defensins, bactenecins, and cecropins (19).

The mode of action of a number of cationic host defense polypeptides is related to increased membrane permeability and disruption of microbial cell structure. Studies with artificial lipid bilayers have demonstrated a range of effects including channel formation by defensins (20–22) and disruption of lipid packing by bactenecins (23), dermaseptin (24), nisin (25), cecropin (26), and tachypleisin (27), which results in permeabilization of the membrane. Lytic properties have also been described for the polyene antimycotics, which are currently used in drugs in treatment of candidiasis. Polyene antimycotics form pores in the plasma membrane by complex formation with ergosterol of the plasma membrane (28, 29), resulting in loss of potassium and leakage of other cell products. These drugs have additional secondary effects on inhibition of plasma membrane ATPase leading to proton efflux as well as reduction of activity of many biosynthetic enzymes (29). The synthetic azole derivatives are the other major class of antifungal drugs. The azole-based drugs act on yeast mitochondria and through a complex multistep pathway inhibit the biosynthesis of ergosterol, the major sterol of yeast plasma membranes (30, 31).

The mechanisms by which salivary Hsts exert candidacidal activity are not well understood. Electron micrographs of C. albicans cells following Hst treatment revealed damage at the cell membrane, as well as the presence of cytoplasmic vacuoles, suggesting loss of intracellular materials (32). In addition, C. albicans cells were found to release potassium in the presence of a fraction of histidine-rich polypeptides purified from human parotid saliva, indicating a loss of yeast membrane integrity (33). Further, Hsts were effective in killing azole-resistant Candida species, thus implicating a different mechanism of Hst action from that of the azole-based antifungal drugs (34, 35). Studies using Hst 5 variants with one or two amino acid substitutions showed that His18, Lys13, Arg22, and the dipeptide sequence Phe14/His15 may be important for optimal activity (36). Moreover, Hst 5 variants with reduced killing ability exhibited similar helical content to Hst 5, suggesting that the α-helical conformation alone is not solely responsible for optimal candidacidal activity (36).

Although earlier potassium release results suggested that Hsts’ biological effects may be related to direct peptide-membrane interactions, recent structural studies of the active fragment of Hst (37) and two-dimensional NMR studies of Hst 5 (38) revealed that the weak amphipathic character of the helical structure precludes spontaneous insertion into the membrane. The size of the active fragment (16 residues) also argues against its ability to function as a channel-forming peptide. Moreover, the yeast cell wall is rich in mannoproteins and glucans containing anionic sites, which may form electrostatic complexes with Hsts and prevent interaction with the cell membrane.

Hsts’ potent antifungal activity, lack of toxicity to humans, and ability to kill azole-resistant yeast strains underscore the importance of detailed understanding of their mechanism of action. In this study, we examined whether Hsts can induce direct physicochemical damage to yeast cell membranes (cathodic peptide model) or whether binding to a specific yeast component is required for Hst killing by performing biological and fluorescent dye release assays, Hst binding, and cross-linking studies on C. albicans intact cells and spheroplasts.

**EXPERIMENTAL PROCEDURES**

**Materials—** C. albicans strain DS1 was isolated from the palate of a denture stomatitis patient (39) and Saccharomyces cerevisiae strains T670 and BJ2169 were obtained from Dr. J. Brunn (Department of Biological Sciences, SUNY, Buffalo, NY). Sabouraud dextrose agar and YPD media were from Difco. Wang resins and Fmoc amino acid derivatives were purchased from Calbiochem (Novabiochem Co., La Jolla, CA) and Sigma. Na235I was purchased from Amersham Pharmacia Biotech. Zymolyase-20T was from ICN Biomedicals (Costa Mesa, CA). Calcein-AM and calcein were obtained from Molecular Probes (Eugene, OR). Egg yolk phosphatidylcholine (ePC) and phosphatidylserine (PS) were obtained from Avanti Polar Lipids (Birmingham, AL); cholesterol and ergosterol were purchased from Sigma. Sephadex G-25 and Sephadex G-10 were from Amersham Pharmacia Biotech; discucinimidyl suberate (DSS), bis(sulfosuccinimidyl)suberate (BS), and BCA protein reagent were purchased from Pierce, and the electrophotoreic reagents were from Bio-Rad.

**Peptide Synthesis and Purification—** Full-length Hst 3 (DSHARKH-HGYKKRF HEKHHHSHGRYNNLYLD N) containing N-terminal (N = DSHARKH), middle active (M = GYKKRFHEKHHSHGRY), and C-terminal (C = RSNNYLYDN) regions and Hst 5 (DSHARKHGYKKRF- HEKHHHSHGRYNNLYDN) containing N and M regions were synthesized using a Beckman System 990 synthesizer, standard solid phase synthesis protocols, and Fmoc chemistry (Table I). Peptides were cleaved from the resin and depurified under anhydrous conditions using trifluoroacetic acid, 13% triethylsilane, and 6% phenol. Hst 4 (RKFHEKHHSHGRYNNLYDN) and Hst 4α (GYRKHHFHEHSSGRGYSNLNLNDN) containing M and C regions, region M (GYRKHHFHEHSSGRGYSNLYDN), and N regions (C = RSNNYLYDN) were synthesized using T-butoxycarbonyl chemistry as described previously (17, 37).

**Purification of Hsts** was performed by reversed phase high-performance liquid chromatography using a Rainin Dynamax-60A C 18 column and a gradient of acetonitrile and water (both containing 0.1% trifluoroacetic acid) as the solvent system. Purity of Hsts was assessed by amino acid analysis, mass spectroscopy and confirmed by amino acid analysis using an Applied Biosystems 492 on-line amino acid analyzer (Applied Biosystems, Foster City, CA). Hsts were purified using a Beckman System 990 synthesizer, standard solid phase synthesis protocols, and Fmoc chemistry (Table I). Peptides were cleaved from the resin and depurified under anhydrous conditions using trifluoroacetic acid, 13% triethylsilane, and 6% phenol. Hst 4 (RKFHEKHHHSHGRYNNLYDN) and Hst 4α (GYRKHHFHEHSSGRGYSNLNLNDN) containing M and C regions, region M (GYRKHHFHEHSSGRGYSNLYDN), and N regions (C = RSNNYLYDN) were synthesized using T-butoxycarbonyl chemistry as described previously (17, 37).

**Candidacidal Assay—** C. albicans was maintained on Sabouraud dextrose agar plates as described previously (17), and S. cerevisiae was maintained on YPD agar plates. C. albicans was inoculated into 10 ml of sucrose-salts-biotin yeast synthetic medium (SSB) (17, 41), and S. cerevisiae was inoculated into 10 ml of YPD medium. Cells were grown to stationary phase for 48 h at 25 °C with rotary agitation at 200 rpm. Blastocindial cell morphology and cell numbers were determined by phase contrast microscopy using a cell counting chamber.

**Conformation of Yeast Cells to Spheroplasts—** C. albicans cells were grown for 48 h at 25 °C in SSB medium and converted to spheroplasts with Zymolyase 20T (ICN Biomedicals, Inc.) as described (42). Briefly, C. albicans cells from 10 ml cultures were harvested by centrifugation at 600 × g, washed twice with TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA), and resuspended in 900 μl of 0.5% (w/v) weight cells spheroplasting buffer (10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM sorbitol as an osmotic protector). After the addition of 2-mercaptoethanol to a final concentration of 30 mM and 150 units Zymolyase-20T/g wet weight yeast cells, cells were incubated for 1–1.5 h at 30 °C with gentle shaking. Spheroplast formation was monitored microscopically by lysis of osmotically sensitive cells in 5% SDS; under these conditions, more than 90% of the cells were converted to spheroplasts. Spheroplasts were washed twice and resuspended in buffer containing 1 mM sorbitol for further use. In some cases, spheroplasts were solubilized in Laemmli sample buffer and subjected to SDS-PAGE.

**Candidacidal Assay—** Antifungal activity of Hsts was examined by microdilution plate assay as described previously (17) with the following modifications. Fungicidal assays were performed on stationary C. albicans cells in the presence or absence of Hst 5, Hst 3, Hst 4, Hst M, Hst 4α, or Hst C (0.98–125 μM) or 500 μM amylase, cytochrome c, and insulin (100 μg/ml). Briefly, C. albicans cells were washed twice with 10 mM sodium phosphate buffer (Na2HPO4/NaH2PO4), pH 7.4, and resuspended at 1 × 107 cells/ml. Cell suspensions (20 μl) were mixed with 20 μl of 10 mM phosphate buffer, pH 7.4, containing the indicated proteins and incubated for various times at 37 °C with shaking. Control cultures were incubated with 20 μl of 10 mM phosphate buffer alone. The reaction was stopped by addition of 360 μl of yeast nitrogen base (YNB); 40 μl of the suspension (300 cells) were spread onto Sabouraud
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dextrose agar plates and incubated for 48 h at 37°C. In some experiments, candidicial assays were performed in 10 mM phosphate buffer, pH 7.4, with 1 mM sorbitol. In preliminary experiments, Hst 5 candidicial activity was tested in 10 mM phosphate buffer containing either 1 mM sorbitol, 2 mg/ml BSA, 0.5 mM KCl, 0.5 mM NaCl, or in 40 mM HEPES. Histatin 5 candididal activity was also detected in 10 mM phosphate buffer containing 10 mM sorbitol or 2 mg/ml BSA; however, it was completely inhibited in buffers containing high salt concentration or in HEPES buffer and therefore these reagents were not used throughout the experiments. Candidicial assays were performed in duplicate or triplicate. Loss of viability was calculated as [1− (colonies from suspension with peptide/colonies from suspension with saline)] × 100. The lowest concentration required to kill 50% of colony-forming unit yeast cells (LD50) was calculated from the dose-response curves. Statistical significance of the results was calculated using Student's t test.

Cell Permeability Assay—Cell permeability was monitored by release of the intracellular dye calcein. Following cellular uptake of calcein, calcein-AM and subsequently converted to spheroplasts as described above and measurements were performed in 10 mM phosphate buffer containing 1 mM sorbitol. Hst 5 (50 or 200 μg) was then added to calcein-loaded cells, and the fluorescence intensity of the induced calcein release (Iint) was recorded every 5 min at excitation and emission wavelengths of 485 and 530 nm, respectively, in a Hitachi F-2000 fluorescence spectrophotometer. Experiments were run for 60 or 90 min. The optimal number of cells, incubation time, and calcein-AM concentration were determined by preliminary experiments to reduce potential artifacts from overloading, dye leakage, and toxic effects. Fluorescence intensity corresponding to 100% of potentially available calcein (Ical) was determined by boiling the cells for 10 min. To confirm that all intracellular calcein was counted, calcein-loaded whole cells were converted to spheroplasts, lysed in boiling water, and total fluorescence measured. Total fluorescence released by lysis of osmotically sensitive cells was very similar to that released following boiling of whole cells. Total fluorescent intensity of the cell population following boiling was assumed to be equivalent to total potentially available intracellular calcein (I0). The fluorescence intensity of calcein released from loaded cells before the addition of peptides was measured as background (I0bg). Only cell preparations that showed less than 0.1% changes in the background over 10 min were used for further experiments. Calcein efflux was calculated as percent fluorescent release = (Iint - I0bg)/(I0 - I0bg)) × 100.

Preparation of Phospholipid Vesicles—The reverse-phase evaporation method of vesicle formation (43, 44) was used to prepare small unilamellar vesicles using ePC or PS in liposome buffer (150 mM NaCl, 10 mM TES, pH 7.5) and 25 mM calcein. Lipozyme, containing sterols, cholesterol or ergosterol were recrystallized twice from ethanol and were incorporated into ePC and PS preparations at a lipid/sterol ratio of 2:1. Liposomes were extruded through 0.1-μm pore polycarbonate membranes using a high pressure-extruder to produce uniform-sized small unilamellar vesicles. Liposomes were then separated from unencapsulated calcein by gel chromatography with a Sephadex G-25 column by eluting with liposome buffer. The phospholipid concentration of collected fractions containing liposomes was determined by phosphorus assay (45). The lipid concentration of each liposome preparation was 1–2 mM. Liposome preparations were adjusted to 0.1 mM phospholipid in liposome buffer, and background fluorescence monitored for 30 min to ensure stability of each preparation. Hst 5, Hst M, and Bac 5 were added to the liposome preparations at varying lipid to peptide concentration ratios and fluorescence of the released dye was recorded as described above. Liposomes were then lysed with 0.5% Triton X-100 to establish total fluorescent content of each preparation. Percent fluorescent release was calculated as ([I0 - I0bg]/[I0 - I0bg]) × 100.

Iodination of Histatin 5—Synthetic histatin 5 (25 μg) was dissolved in 10 mM sodium phosphate buffer, pH 7.8, and radioiodinated using a chloramine T method as described previously (46). Free iodine was separated from 131I-Hst 5 on Sephadex G-10 column that had been pre-equilibrated with 1 mg/ml BSA. The specific activity of the radioiodinated Hst 5 was typically around 20 cpm/fmol. The purity of radio-labeled Hst 5 was assessed by 15% SDS-PAGE using a Tris-Tricine electrode buffer and appeared as a single band. Based on the specific activity of 131I-Hst 5, 1000 cpm (approximately 200 pg) of 131I-Hst 5 was added to 10 mM phosphate buffer, pH 7.4 (~20 cpm/fmol), the iodotyrosines were estimated to be less than 2% of the tyrosine residues in the final product. Nonradioactive iodination of Hst 5 was performed parallel to the radioactive labeling, except that 900 ng of Na2125I was used in place of Na2131I. Fractions collected after Sephadex G10 chromatography were concentrated by lyophilization and dissolved in 10 mM phosphate buffer, pH 7.4. Protein concentration was determined by amino acid analysis and assuming a molecular weight of 5300. Hst 5 was incubated in 10 mM phosphate buffer, pH 7.4, or 10 mM phosphate buffer with 2 mg/ml BSA was further tested for biological activity on candidicial bioassay.

Binding Studies—125I-Hst 5 binding was performed on C. albicans cells that were grown for 48 h in SSB medium. Cells were washed twice with 10 mM sodium phosphate buffer, pH 7.4, and resuspended at 4 × 107 cells/ml in binding buffer (10 mM phosphate buffer, pH 7.4, 2 mg/ml BSA). Liposome preparations containing 100 μg of liposomes and 1 μg of labeled peptide (131I-Hst 5) were added to 5 × 107 cells/ml of C. albicans. Three minutes after the addition of liposomes, the cell suspension was lysed with 0.5% Triton X-100 to release protein antigens from the cell membrane. Lysates were centrifuged at 15,000 rpm for 30 min, and the supernatants were used to determine the extent of binding of labeled peptide to C. albicans. Competition binding studies were carried out in binding buffer containing various concentrations (100 nM to 500 μM) of Hst 5, Hst 3, or Hst 4; plus 500 μM amounts of cytochrome c, insulin-β-chain, salivary amylase, or MUC7-derived peptides. Optimal cell number, incubation time, and temperature were determined by preliminary experiments. Cells were assayed for each experimental point in triplicate. The competition curves were fitted to the exponential points using the equation for one binding site using curve fit program LIGAND (Munson, NIH and Kaleidagraph (Synergy System, Reading, PA).

Cell Fractionation—C. albicans cells were grown for 48 h in SSB, harvested by centrifugation, and washed twice with 10 mM phosphate buffer, pH 7.4. Cells (0.7–1.0 g wet weight) were resuspended in 15 ml of cold homogenization buffer (10 mM phosphate buffer, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 g/ml benzamidine) and added to a 50-ml Bead Beater Chamber (Biospec, Bartlesville, OK) containing 15 ml w/v of prechilled 0.5-mm glass beads. Homogenization was carried out in five 1-min bursts at 4°C. The beads were rinsed twice with 5 ml of homogenization buffer, and the lysate was centrifuged for 5 min at 1000 × g at 4°C to remove unbroken cells and cell debris. Crude membrane fraction was separated from the soluble proteins by centrifugation at 170,000 × g (1 h) at 4°C in a Beckman 50 Ti rotor (150,000 × g, 30,000 × g, and 10,000 × g for 5 days on X-Omat film (Eastman Kodak Co.).

Cross-linking Studies—Washed C. albicans cells were resuspended in binding buffer at 1.8 × 1010 cells/ml and 150 μl (3 × 105 cells) were incubated for 20 min at room temperature with 100 nM 125I-Hst 5 in a final incubation volume of 200 μl. Where indicated, a 1000-fold excess of Hst 5 was added as an unlabeled competitor. Cells were then placed on ice and subsequently incubated for 20 min with 0.9 mM non-cleavable membrane-permeable DSS or membrane-impermeable BS cross-linking reagents (47), followed by three washes with binding buffer. Yeast pellet was prepared from the 125I-Hst 5 bound to C. albicans by enzymatic digestion of the cell wall and subsequent lysis of the osmotically sensitive wall-free cells. Cross-linked C. albicans cells were resuspended in 200 μl of spheroplasting buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM sorbitol, 480 μg of Zymolase-20T, 30 mM 2-mercaptoethanol) and incubated for 1 h at 30°C with gentle shaking. Wall-free cells were separated from the released cell wall material by centrifugation, solubilized in Laemmli sample buffer, boiled, and subjected to SDS-
Candidacidal assays were performed on *C. albicans* cells in the presence of Hst 5, Hst 3, Hst M, Hst 4, and Hst C (0.98–125 μM) and on spheroplasts in the presence of Hst 5 (0.98–250 μM), as described under "Experimental Procedures." Loss of viability is expressed as 1 – (colonies after peptide addition/colonies after incubation in buffer only) × 100. Results represent means ± S.D. of duplicates from 11 (H5, whole cells), 3 (Hst 5, spheroplasts), and 2 (Hst M, Hst 3, Hst 4, Hst C) experiments. Statistical significance was calculated using Student’s *t* test for data from Hst 5-treated whole cells compared to loss of cell viability induced by Hst 4 (*, *p* < 0.05), Hst 3 (**, *p* < 0.005), and Hst 5 induced loss of spheroplast viability (***, *p* < 0.0005). LD₅₀ peptide concentration (μM) required to induce half-maximal loss of viability.

| Histatin | C. albicans | LD₅₀ μM |
|----------|-------------|---------|
| Whole cells | 1.8 ± 0.5 |
| Spheroplasts | 25.3 ± 3.1*** |
| Whole cells | 9.2 ± 0.4*** |
| Whole cells | 2.0 ± 0.2 |
| Whole cells | 4.7 ± 0.1* |
| Whole cells | 0 ± 0 |

**RESULTS**

Histatin-induced Loss of Yeast Cell Viability—The antifungal activity of the major members of the Hst family has been characterized and active concentrations for candidacidal activity determined (6, 11, 17). We have confirmed and extended these results by testing the concentration dependence of synthetic Hsts representing various regions of the major Hsts (Table I) on *C. albicans* whole cells and spheroplasts and on the related yeast *S. cerevisiae* (Fig. 1). Candidacidal activity of Hst 5 was compared with the activities of Hst 3, Hst M, Hst 4, Hst 4a, and Hst C. Hst 5 exhibited similar candidacidal activity as Hst M and was 2.5- and 5-fold more potent than Hst 4 and Hst 3 (LD₅₀ = 1.8 μM versus 4.7 and 9.2 μM, respectively) (Table I). Hst 4a, which included three additional amino acids at its N terminus (GYK), had equivalent candidacidal activity with Hst 4. Hst C, which represents the eight-amino acid C terminus of Hst 5, did not produce loss of yeast cell viability even at high concentrations (500 μM). These data demonstrate that the middle and N-terminal regions of full-length Hsts are required for optimal candidacidal activity and that the C-terminal region may not be essential for yeast killing and may even reduce Hsts’ fungicidal activity.

We next tested killing ability of the most potent Hst, Hst 5, on two strains of *S. cerevisiae* (BJ 2169 and TII#20). Both strains responded to Hst 5, although these yeast cells were less susceptible when compared with *C. albicans*. Concentrations of Hst 5 (31.25 μM) required to kill more than 95% of *C. albicans* induced only 34% and 67% loss of viability of the BJ 2169 and TII#20 cells, respectively (Fig. 1). Higher concentrations (125 μM) of Hst 5 did not further increase cell killing, suggesting that a subpopulation of *S. cerevisiae* cells was unresponsive to Hst 5.

Previous studies on Hst structure-function have indicated that the charge and weak amphipathicity of the molecule do not favor spontaneous insertion into yeast cells, thus implying that Hsts may require interactions with *C. albicans* cell surface components to mediate killing (37). We converted *C. albicans* cells to spheroplasts to test whether cell wall-free cells would still respond to Hst 5. Removal of the cell wall with Zymolase in the presence of 2-mercaptoethanol drastically reduced cell susceptibility to Hst 5. Spheroplasts were 14-fold less susceptible to Hst killing compared with whole cells (LD₅₀ = 25 μM versus 1.8 μM, respectively) (Fig. 1, Table I). These results suggest that either a cell wall component(s) is required for maximal Hst killing, or that spheroplasts are protected from Hst 5 lethal effects due to removal and/or alteration of membrane components during cell wall digestion with Zymolyase and 2-mercaptoethanol.

To test the specificity of Hsts as candidacidal molecules, three previously characterized proteins were selected for use in candidacidal assays. These molecules were salivary amylase, a 56-kDa protein that is found in *C. albicans* pellets formed in saliva (18); insulin β-chain, a 3.5-kDa protein with size similarity to Hst 5 (3.0 kDa); and hemoprotein cytochrome c (12.4 kDa), a highly basic protein (pI = 10.2) like Hst 5 (pI > 10). These proteins were tested at 500 μM, which is 16-fold greater than the concentration of Hst 5 required for complete killing of *C. albicans* cells (Table II). Neither salivary amylase nor insulin had any effect on cell viability; however, cytochrome c killed approximately 50% of cells. This is consistent with previous reports that cytochrome c caused release of nucleotide fractions and amino acid pools from *Candida utilis* leading to cell death (48).

Hsts Induce Loss of Intracellular Dye from Yeast Cells—Preincubation of *C. albicans* with a highly enriched fraction of histidine-rich polypeptides (HRPs) purified from human parotid saliva has been shown to produce rapid release of potassium from cells, thus implicating damage of the cell membrane as an early event in the HRP-induced killing (33). We examined the ability of synthetic Hsts to induce yeast cell membrane destabilization by measuring the release of the fluorescent dye calcine from yeast cells. *C. albicans* cells were loaded with calcine-AM, and fluorescent emission of the released free dye in response to Hsts was recorded over a period of 90 min.

Preliminary experiments were carried out to determine dye retention profiles in these cells. In contrast to the previous study (33), which reported 30% release of potassium from untreated cells, less than 1% of spontaneous dye release was detected over a period of 1 h. Evaluation of longer time periods
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**Table II**

| Inhibitor | 125I-Hst 5 binding | Loss of cell viability |
|-----------|-------------------|-----------------------|
| Hst 5     | 23 ± 3            | 100                   |
| Hst 3     | 27 ± 2            | 100                   |
| Hst 4     | 27 ± 5            | 100                   |
| Amylase   | 97 ± 8            | 0                     |
| Insulin   | 105 ± 18          | 0                     |
| Cytochrome c | 98 ± 10           | 54 ± 8                |
| Succinyl-REEDHELRRHHHQ | 96 ± 14         |                       |

N-terminal (3–17 amino acids) of MUC7, LD$_{50}$ = 39.1 µM (see Footnote 2).

b N-Succinyl derivative LD$_{50}$ = 305.3 µM (see Footnote 2).

showed a maximum base line of 2% dye released from cells after 6 h of incubation in phosphate buffer. This is in agreement with studies on human fibroblasts, in which calcein was well retained and not exported by multidrug transporters (49).

Under conditions of the candidalass assays employed here and by others (36), the minimum concentration of Hst 5 required for complete killing of cells is 31.25 µM. Time course experiments of Hst 5-induced killing of C. albicans showed that 50% of the cells were killed following 10 min of Hst 5 exposure and complete killing was achieved following 90 min of incubation with Hst 5 (Fig. 2). Calcein release from cells following 10 min of incubation with 50 µM Hst 5 was about 2%. Approximately 10% of calcein release was detected over a period of 60 min, which corresponded to 86% cell killing in the candidalass assay; the maximum dye efflux from loaded cells was 12% during the 90-min exposure to Hst 5 (Fig. 2). Incubation of calcein-loaded cells with higher concentration of Hst 200 (200 µM) resulted in proportionally increased dye efflux. Hst 3, Hst 4, and Hst M induced dye efflux similar to that detected in response to Hst 5. This effect was not observed in response to Hst C, which also lacked candidalass activity. Although our dye release results suggest that cell membrane destabilization is a consequence of Hst activity, the time course and magnitude of calcein efflux indicate cell permeabilization and dye efflux are secondary effects associated with cell death rather than the primary cause of cell death. Consistent with findings that Hst-induced killing of C. albicans was significantly reduced when the yeast cell wall was removed, maximum calcein release in response to Hst was also dependent on the cell envelope integrity. C. albicans whole cells were loaded with calcein-AM, converted to spheroplasts, treated with Hst 5, and the release of the free dye recorded. As shown in Fig. 2, no significant release of calcein from spheroplasts was detected in response to Hst 5 over a 60-min period.

**Hsts Do Not Induce Leakage from Phospholipid Vesicles**—Candidalass and dye release assays suggested that C. albicans components in the cell envelope are required for optimal Hst activity. To determine whether Hsts can disturb a lipid bilayer that does not contain proteins, model membranes of differing compositions were constructed. The lysis of lipid vesicles was monitored by the quenching of fluorescence caused by leakage of encapsulated calcein, which is self-quenched inside liposomes. Bac 5, a proline- and arginine-rich polypeptide of bovine neutrophil granules with bactericidal activity against Gram-negative bacteria, was also tested in the dye release assay. We have previously shown that Bac 5 is a potent candidalass molecule and exhibits lytic effects on negatively charged liposomes (23). Increasing concentrations of Hsts and Bac 5 were mixed with calcein-containing small unilamellar vesicles of varying phospholipid composition. Membrane disruption or perturbation was measured by fluorescent intensity of dye release. Bac 5 caused rapid dye release from ePC liposomes; however, neither Hst 5 nor Hst M elicited significant increase in fluorescence when mixed with ePC liposomes at molar ratios examined (Fig. 3). No release of dye was detected with any Hst when mixed with ePC liposomes containing ergosterol or cholesterol, or PS + cholesterol. These data point to differing mechanisms of candidalass action between Hst and Bac 5. Bactericins have been shown to effect Gram-negative bacteria by increasing the permeability of inner and outer membranes and disrupting membrane potential (50, 51). In contrast, salivary Hsts do not interact spontaneously with the model membranes selected, in agreement with previous conformational studies of Hst M (37), and suggest that a protein or other cellular components may be crucial for Hst killing ability.

**Analysis of Hsts Binding to C. albicans Cells**—To test whether C. albicans express binding sites for Hsts, binding experiments were performed using 125I-Hst 5. Preliminary results were carried out to determine whether the iodinated Hst 5 retained biological activity. In a candidalass bioassay, 125I-Hst 5 in 10 mM phosphate buffer containing 2 mg/ml BSA had similar biological activity as the non-iodinated protein (data...
sites on analysis of the results indicated a class of saturable binding. Hst 3 and 4 competed for the same binding sites as 125I-Hst 5.

Binding experiments were carried out on C. albicans cells with various concentrations of 125I-labeled Hst 5. Scatchard analysis of the results indicated a class of saturable binding sites on C. albicans detectable between 800 pm and 2.4 μM 125I-Hst 5 with an average equilibrium dissociation constant ($K_D$) of 9.5 ± 4.9 × 10^{-7} M (n = 5) and $B_{max}$ of 8.4 ± 3.8 pmol (Fig. 4). Binding assays were routinely performed on 10^6 C. albicans cells, and the detected binding level corresponded to 8.6 ± 3.9 × 10^5 binding sites/cell.

Specificity of 125I-Hst 5 Binding—Binding specificity was investigated using unlabeled Hst 5 and two related family members possessing candidacidal activities, Hst 3 and Hst 4, to compete with 125I-Hst 5 for the identified sites on C. albicans. Hst 3 and 4 competed for the same binding sites as 125I-Hst 5 with affinities ($K_i$) of 8.4 ± 3.6 × 10^{-7} M, n = 4 and 1.1 ± 0.28 × 10^{-6} M, n = 2, respectively) which were similar to the affinity of 125I-Hst 5 ($K_D$ = 9.5 ± 4.9 × 10^{-7} M) (Fig. 5). Moreover, the affinity of unlabeled Hst 5 ($K_i = 7.8 ± 1.4 × 10^{-7}$ M, n = 2) was very similar to the affinity of 125I-Hst 5, verifying that radiolabeled Hst 5 retained full binding ability. Although candidacidal assays showed differences in the potency of Hst 5, Hst 3, and Hst 4, data from competition binding experiments indicated these three Hsts have approximately the same affinities to the 125I-Hst 5 sites on C. albicans. We next tested whether salivary amylase, insulin, and cytochrome c, which were used in candidacidal assays, interact with binding sites on C. albicans (Table II). Even at high concentrations, salivary amylase and insulin did not produce a significant decrease in the total 125I-Hst 5 binding. In addition, cytochrome c, which induced 50% loss of C. albicans cell viability, did not inhibit 125I-Hst 5 binding.

Low molecular weight mucin (MUC7) is a prominent salivary glycoprotein, which contains in its protein core an N-terminal domain with 53% sequence homology to Hst 5.2 Although MUC7 is not a candidacidal molecule, a synthetic 15-amino acid histidine-rich N-terminal fragment of MUC7 has been recently shown to possess candidacidal activity, albeit significantly lower than Hst 5 (LD_{50} = 39.1 μM versus 1.5 μM, respectively, Tables I and II). This Hst 5-like domain of MUC7 and its N-succinyl derivative (LD_{50} = 305.3 μM, Table II), were tested in competition binding assays. Hst 5-like domain of

MUC7 decreased 125I-Hst 5 binding (38% inhibition of total binding), which is consistent with its reduced candidacidal activity compared with Hst 5. Moreover, the N-succinyl derivative, which is 170-fold less potent than Hst 5,2 did not produce significant inhibition of 125I-Hst 5 binding (Table II). The results from the competition binding experiments showed correlation between binding ability and killing activity and confirmed the specificity of 125I-Hst 5 binding to C. albicans, thus implying that the identified 125I-Hst 5 binding sites on C. albicans mediate its biological effect.

Because C. albicans spheroplasts were less sensitive to Hst 5 in killing assays, we tested whether 125I-Hst 5 binding sites were altered in the cell wall-free cells. In contrast to whole cells, specific binding of 125I-Hst 5 (800 pm to 2.4 μM) was not detected and binding was not saturable at the highest concentration of unlabeled Hst 5 used (800 μM). It is unlikely that the Hst 5 binding component was completely removed during

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spheroplast formation, inasmuch as spheroplasts, although less susceptible, were still killed by Hst 5. It is possible that Zymolase treatment of the cells in the presence of a reducing agent decreased the binding affinity of 125I-Hst 5 sites or that a high affinity binding component was removed, leaving a large number of low affinity sites that were not detected with the concentrations of 125I-Hst 5 used in the whole cell binding assay.

Identification of a C. albicans Hst 5-binding Protein—We next examined whether Hst 5 binds to C. albicans proteins using an overlay assay. C. albicans cells were disrupted with glass beads and proteins from whole cell lysates and crude membrane fractions were separated by gel electrophoresis and analyzed on nitrocellulose membranes with radiolabeled Hst 5. The results revealed that 125I-Hst 5 bound to a C. albicans protein with an apparent molecular mass of 67,000 ± 4000 (n = 4). This protein was detected in C. albicans whole cell lysate and crude membrane fraction and was the only protein consistently observed to bind Hst 5 (Fig. 6, lanes 1 and 2). In some experiments, a similar sized protein was found in the high speed supernatant containing the soluble post-membrane fraction (data not shown). 125I-Hst 5-binding proteins were not detected from Zymolase C. albicans cell wall digest or lysates prepared from human embryonic kidney epithelial (HEK 293) cells or neutrophil plasma membranes, even in longer exposures (data not shown). Interestingly, when proteins from Hst 5-susceptible yeast S. cerevisiae strain TII#20 were incubated with 125I-Hst 5 on overlay assays, a doublet with relative molecular mass of 70,000 and 87,000 was observed (Fig. 7, lanes 2). The faster migrating component was of similar size and may be related to the C. albicans Hst 5 binding component, or alternatively, may be a degradation fragment of the slower migrating protein in S. cerevisiae.

Cross-linking of iodinated Hst 5 was next employed to study the functional interaction of Hst with C. albicans leading to cell killing. C. albicans cells were incubated with 100 nm 125I-Hst 5 and proteins were covalently linked to the iodinated factor with a membrane-permeable, non-cleavable cross-linking reagent, DSS. In order to extract yeast cell proteins, cell wall from

![Image](image_url)

**Fig. 6. Overlay assay of the binding of 125I-Hst 5 to proteins from yeast cells.** C. albicans and S. cerevisiae (strain TII#20) were disrupted with glass beads and whole cell lysates and crude membrane fractions were prepared as described under “Experimental Procedures.” Solubilized proteins were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated for 4 h with 50 nM 125I-Hst 5 in binding buffer. Lane 1, C. albicans whole cell lysate; lane 2, C. albicans crude membrane fraction; lane 3, S. cerevisiae strain (TII#20) whole cell lysate. The molecular sizes (× 103) of protein standards are indicated by bars to the left and the position of 125I-Hst 5-binding proteins by arrows.

![Image](image_url)

**Fig. 7. Cross-linking of 125I-Hst 5 to C. albicans cells.** C. albicans cells were incubated for 20 min at room temperature with 125I-Hst 5 in the presence (lane 2) or absence (lanes 1 and 3) of excess of unlabeled Hst 5. Cells were then placed on ice and incubated for 20 min with 0.9 mM non-cleavable membrane-permeable cross-linking reagent DSS. Cells were processed as described under “Experimental Procedures,” and cell lysates (lanes 1 and 2) or cell wall material (lane 3) were subjected to SDS-PAGE and analyzed by autoradiography. Bars indicate the position of molecular size protein standards (× 103), and the arrow indicates the cross-linked complex.

**DISCUSSION**

The family of salivary Hsts consists of structurally related, low molecular weight histidine rich proteins, which are part of the non-immune host defense system of the oral-esophageal area. The finding that Hsts are potent in vitro antifungal agents, while nontoxic to human cells, provided promise for their therapeutic potential in patients with oral candidiasis. Despite the characterization of Hsts at the structural and conformational level (38), at present little is known about their fungicidal mechanism of action and the basis for selectivity between eukaryotic fungal and host mammalian cells. This study represents the first description of Hst 5 binding sites on C. albicans that mediate cell killing, and identifies a 67-kDa yeast Hst 5-binding protein. We report here that Hst-induced killing is initiated upon binding to a candidal receptor rather than spontaneous permeabilization and destabilization of cell membranes based on biological and fluorescent dye release assays as well as Hst 5 binding and cross-linking studies. Hst binding to a specific component on target yeast cells may be the basis for their selectivity and lack of toxicity to human host cells.

This study used three major salivary Hsts and the middle active region (Hst M), which represent truncated variants of 125I-Hst 5-cross-linked cells was removed and separated from the wall-free cells by centrifugation. Proteins from osmotically sensitive cells were then solubilized in SDS-containing buffer and a 125I-Hst 5-containing complex with a relative molecular mass of 73,000 ± 1000 (n = 2) was resolved after SDS-PAGE and autoradiography (Fig. 7, lane 1). The labeling of this component was specific, as the complex was not detected when cells were incubated with an excess of unlabeled Hst 5 during the binding (Fig. 7, lane 2). Consistent with the overlay data, 125I-Hst 5 cross-linked proteins were not detected in the cell wall material (Fig. 7, lane 3), thus implying that Hst 5-binding proteins are not likely to be located on the yeast cell wall. Together, these results suggest Hst 5 interacts with at least one C. albicans-binding protein.
full-length salivary Hst 3. The candidacidal activity of the synthetic peptides, shown here, is consistent with the reported activities of naturally occurring (6, 11, 15), synthetic (17), and recombinant (18, 36) salivary Hsts. Presence of the C-terminal portion of the molecule reduced the fungicidal activity, as demonstrated by the lower activity of Hst 3 and Hst 4 when compared with structurally equivalent Hst 5 and Hst M, which lack the C-terminal region (Table I). Thus, full-length Hst 3 may be a precursor form of Hsts and protolytic cleavage of the C-terminal region represents a post-translational processing step for activation of the protein. This concept is in agreement with the findings of Perinpanayagam et al. (14) that Arg$^{25}$ is a primary cleavage site of Hsts in vivo. This cleavage site resides at the junction of M and C regions defined in the present study, and may have functional significance in protolytic processing to produce a functional molecule. In contrast, protolytic cleavage at other sites in the defined M and N-terminal regions may produce a less active Hst. We have shown that C. albicans cells have the ability to degrade $^{125}$I-Hst 5 (45). In this respect, less active or inactive Hst fragments detected in vivo (14) and generated by protolytic cleavage at sites Lys$^5$, Arg$^6$, Lys$^{12}$, His$^{18}$, and Arg$^{22}$ may represent a microbial protective mechanism against the cytotoxic Hsts.

Many polypeptide antibiotics exert their activity through spontaneous insertion into microbial membranes forming ion channels or pores, thereby disrupting cell structure and integrity. If Hsts function primarily as membrane lytic agents, direct exposure to the plasma membrane of wall-free spheroplasts should enhance Hst candidacidal activity. However, treatment of C. albicans cells with Zymolase in the presence of a reducing agent to generate wall-free cells resulted in a 14-fold reduction of cellular sensitivity to Hst 5 compared with intact cells (Table I and Fig. 1). Zymolase-20T preparation contains a mixture of hydrolytic enzymes including $\beta$-1,3-laminaripentaoylhydrolase, $\beta$-1,3-glucanase, protease, and mannanse (52). Therefore, either the yeast cell wall is necessary for Hst killing, or an enzymatic cleavage and/or alteration of specific yeast membrane component critical for optimal killing accounts for decreased sensitivity of spheroplasts to Hst 5. Similarly, Lactococcus lactis spheroplasts or trypsin-pretreated whole cells were less sensitive to the bacteriocin Lactostrepcin 5, thus implicating a putative membrane receptor mediating Lactostrepcin 5 and Lactococcin A effects (53). Driscoll and colleagues (54) found that lyticase treatment of $^{125}$I-Hst 5 revealed that the weak amphipathic character of the helical structure precludes spontaneous insertion into microbial membranes and formation of pores or ion channels across the membrane (37, 38). Hsts’ inability to disrupt lipid membranes, measured here by the release and dequenching of calcein, further supports these observations. Unlike Bac 5, which was effective in disrupting artificial membranes (Fig. 3) (23), Hst 5 and Hst M do not display membrane lytic activities to any of the membranes selected. Furthermore, analysis of the magnitude and time course of Hst 5-induced calcein release from C. albicans cells (Fig. 2) showed that loss of cell integrity is a secondary effect following cell death rather than the result of primary disruption of the membrane by channel or pore formation. Together, these results suggest a selectivity of Hst fungicidal activity that is exerted through interaction with a specific yeast component(s). An early study utilizing a partially purified fraction of HRP s from human parotid saliva reported 90% release of potassium from C. albicans cells after a 30-min exposure to HRPs (33). Although this work correlated HRP-induced loss of cell viability with loss of cell membrane integrity, our calcein-release experiments utilizing pure synthetic Hst 5 indicate that cell death is not correlated with leakage of the dye, e.g. with Hst 5-induced yeast plasma membrane disruption. The observed difference in the response of C. albicans cells to a mixture of purified HRPs reported previously (33) as compared with pure Hst 5 in the present study is difficult to explain. The use of purified mixtures of HRPs in the earlier studies suggests the possibility of the presence of additional salivary components in the mixture, which could be responsible for potassium leakage from the cells. Moreover, the correlation of candidacidal activity and the leakage of potassium ions from yeast cells using a mixture of Hsts that exhibit variations in their candidacidal potency and multifunctional properties may not be unequivocal.

$^{125}$I-Hst 5 equilibrium binding results indicated that C. albicans cells express a class of saturable binding sites for Hst 5, Hst 3, and Hst 4, numbering 8.6 $\times$ 10$^5$/cell (Fig. 4). The affinity binding constant ($K_d$) is in agreement with the active concentrations on the candidacidal assay (LD$_{50}$ = 1.8 $\mu$m) (Table I) and is similar to the $K_d$ for $^{125}$I-Hst 5 in equilibrium binding studies on Porphyromonas gingivalis (K$_d$ = 1.5 $\mu$m) (56). Although Hst 5 bactericidal activity to P. gingivalis has not been reported, Hst 5 has been shown to be effective in inhibition of aggregation of these cells with other bacteria by binding to specific sites numbering 3600/cell.

Analysis of Hst binding and killing of C. albicans spheroplasts further suggested a correlation between binding and killing ability. Specific $^{125}$I-Hst 5 binding was not detected to spheroplasts, which were 14-fold less susceptible to killing, compared with intact cells (Table I). The appearance of lower affinity and/or a larger number of binding sites for Hst 5 not detected with the concentrations of $^{125}$I-Hst 5 used in the whole cell binding assays could explain our inability to measure specific Hst 5 binding to spheroplasts. Hst 3 and Hst 4 recognized $^{125}$I-Hst 5 sites on C. albicans with very similar affinities (Fig. 5), although they were 5- and 2.5-fold, respectively, less potent than Hst 5 on the candidacidal assays (Table I). Observed differences in candidacidal activities of Hst 3, Hst 4, and Hst 5 may reflect different biological effects including killing, inhibition of germination, or increased proteolysis. We selected three proteins for use in our killing and binding assays to address the specificity of Hst 5 candidacidal and binding activities: $\alpha$-amylase, a salivary protein; insulin-$\beta$-chain with a similar size; and cytochrome c, which has a high pI. Amylase and insulin neither induced cell killing nor competed for Hst 5 binding sites (Table II). Cytochrome c ($500\mu$m) did not inhibit $^{125}$I-Hst 5 binding; however, it induced about 50% loss of C. albicans cell viability, thus suggesting a different mechanism for Hst and
cytochrome $c$ yeast cell killing. Earlier experiments have shown that cytochrome $c$ exhibits lytic activity to $C.\textit{utilis}$, perhaps due to electrostatic interactions with the yeast cell membrane (47). Recent studies have indicated an involvement of cytochrome $c$ in apoptosis of eukaryotic cells by its release from the mitochondrial intermembrane space into the cytosol (57).

Overlay assays demonstrated the existence of a candidal 67-kDa $^{125}$I-Hst 5-binding protein (Fig. 6). This protein was detected in whole cell lysates and crude membrane fractions prepared from glass-bead disrupted $C.\textit{albicans}$ cells, but not in the yeast cell wall fraction. Moreover, cross-linking experiments revealed the presence of a specific 73-kDa $^{125}$I-Hst 5-containing complex (Fig. 7). This complex may contain one or two molecules of $^{125}$I-Hst 5 covalently linked to the 67-kDa binding protein, detected in the overlay assays. Because of the small size of Hst 5 (~3 kDa), our experiments did not clarify the exact stoichiometry of the binding event. The absence of cross-linked products in the cell wall material further confirmed overlay results that the Hst 5-binding protein is not located on the cell wall. $^{125}$I-Hst 5-binding protein of similar size was also observed in susceptible $S.\textit{cerevisiae}$ strain TI#20 (Fig. 6), but it was not detected in extracts from human kidney epithelial cells and neutrophils, suggesting that the presence of a yeast receptor for Hst may be the basis for its lack of toxicity to human cells. The presence of an additional higher molecular mass binding protein in $S.\textit{cerevisiae}$ TI#20 cells may account for the differences in the sensitivity of $C.\textit{albicans}$ and $S.\textit{cerevisiae}$ TI#20 to Hst 5. Interestingly, preliminary overlay assay data showed that Bac 5 partially competed with labeled Hst 5 for the $C.\textit{albicans}$-binding protein. Although Bac 5 differs from Hst 5 in its ability to lyse lipid membranes, it is possible that its killing mechanism may overlap that of Hst 5. Additional experiments are needed to support this assertion.

Although the exact location of the Hst 5-binding protein is yet to be determined, our data show that it is not located within the yeast cell wall. Removal of the cell wall with 2-mercaptoethanol and Zymolase, a preparation containing a variety of hydrolytic enzymes including a protease, produced cells with decreased Hst 5 sensitivity, suggesting alteration of a cell membrane protein as the source of reduced Hst 5 effect. Preliminary cross-linking experiments that employed membrane-impermeable cross-linking reagent BS to covalently link $^{125}$I-Hst 5 to $C.\textit{albicans}$ cells detected a $^{125}$I-Hst 5-containing complex of similar size (data not shown) to the one formed with its membrane-permeable analog DSS (Fig. 7). These results, together with the detection of Hst 5-binding protein in the $C.\textit{albicans}$ crude membrane fraction (Fig. 6, lane 2), provide evidence that Hst 5-binding protein may be located on the plasma membrane.

$C.\textit{albicans}$ has been shown to express several adhesins that are receptors for fibronectin, fibrinogen, laminin, collagen, and complement receptors for C$_{3}\text{d}$ and C$_{3}\text{b}$ (1, 58–62). These receptors uniformly have a yeast cell wall location and are associated with pathogenicity of $C.\textit{albicans}$ species as related to adherence to epithelial and endothelial tissues. It has been suggested that similarities between these receptors may in fact reflect a single $C.\textit{albicans}$ surface mannoprotein with a number of binding activities (59). The cell wall location and binding characteristics of these candidal mannoprotein receptors ($K_{d}$ ~1 nM, numbering about 5000/cell) differ from the presently characterized Hst 5-binding protein. Hst binding characteristics reported here are more similar to those described for the pore-forming colicins (63). These bacterial toxins kill susceptible Escherichia coli by formation of ion channels in the inner membrane of the target cells via binding to specific outer membrane receptors. The density of OmpF, the receptor for colicin N, has been evaluated at approximately $4 \times 10^{5}$ copies/E. coli with a binding affinity $K_{d}$ ~1 nM (64, 65). Following binding, colicins are translocated across the cell envelope by helper proteins, which belong to nutrient uptake systems.

It is currently unclear how association of Hsts with the yeast-binding protein leads to cell death. Hsts may utilize an existing yeast membrane transport system for vital nutrients to gain illicit access into the cell where secondary effects occur. Membrane-bound transport proteins have been described for both $C.\textit{albicans}$ and $S.\textit{cerevisiae}$, which are involved in uptake of amino acids, peptides, and sugar molecules (66, 67). Yeasts also express drug efflux pumps which have been divided into two major classes: membrane proteins belonging to the ABC (ATP binding cassette) or MSF (major facilitator superfamily) families, which share structural and presumably functional homology to bacterial components involved in the active transport of a wide range of molecules including oligopeptides, histidine, maltose, ribose, phosphate, vitamin B$_{12}$, and hemolysin) (68, 69). Intracellularly, Hsts may effect sites such as mitochondria to inhibit energy production, induce release of cytochrome $c$, a process characteristic for apoptosis, or inhibit DNA or RNA synthesis. Finally, interaction with the yeast receptor may be required for correct orientation or oligomerization of Hst following insertion into the membrane. In this respect, $\alpha$-hemolysin, a toxin secreted from the human pathogen Staphylococcus aureus binds to the membrane as a monomer, forms a heptamer and then inserts and lyses human platelets, erythrocytes, and endothelial cells (70).

In conclusion, major disadvantages of currently used anti-fungal drugs are their toxicity and the development of resistant yeast strains (71). In contrast, salivary Hsts are nontoxic to humans and yet potent candidicidal agents even with drug-resistant strains. Although the therapeutic potential of Hsts is becoming apparent, particularly against azole-resistant strains, the exact mechanism of cell killing must be elucidated to fully utilize them as therapeutic agents. The identification of a specific Hst 5-binding protein on $C.\textit{albicans}$ in this study provides insight into the potential mechanism of yeast killing and suggests a basis for the nontoxic nature of Hsts. Finally, the binding characteristics of Hst 5, described here, are in agreement with the observed potency of its biological effect and further provide crucial information to the use of Hst 5 as a therapeutic agent.

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