

## MUC7 20-Mer: Investigation of Antimicrobial Activity, Secondary Structure, and Possible Mechanism of Antifungal Action

Libuse A. Bobek\* and Hongsa Situ

Department of Oral Biology, University at Buffalo, The State University of New York,  
Buffalo, New York 14214

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**This study was aimed at examining the spectrum of antimicrobial activity of MUC7 20-mer (N-LAHQKP-FIRKSYKCLHKRCR-C; residues 32 to 51 of MUC7, the low-molecular-weight human salivary mucin, comprised of 357 residues) and comparing its antifungal properties to those of salivary histatin 5 (Hsn-5). We also examined the secondary structure of the 20-mer and the possible mechanism of its antifungal action. Our results showed that MUC7 20-mer displays potent killing activity against a variety of fungi and both gram-positive and gram-negative bacteria at micromolar concentrations. Time-dependent killing of *Candida albicans* and *Cryptococcus neoformans* by MUC7 20-mer and Hsn-5 indicated differences in killing rates between MUC7 20-mer and Hsn-5. The secondary structure prediction showed that MUC7 20-mer adopts an amphiphilic helix with distinguishable hydrophilic and hydrophobic faces (a characteristic that is associated with antimicrobial activity). In comparison to that of Hsn-5, the fungicidal activity of MUC7 20-mer against *C. albicans* seems to be independent of fungal cellular metabolic activity, as evidenced by its killing potency at a low temperature (4°C) and in the presence of inhibitors of oxidative phosphorylation in the mitochondrial system. Fluorescence microscopy showed the ability of MUC7 20-mer to cross the fungal cell membrane and to accumulate inside the cells. The internalization of MUC7 20-mer was inhibited by divalent cations. Confocal microscopy of cells doubly labeled with MUC7 20-mer and a mitochondrion-specific dye indicated that mitochondria are not the target of MUC7 20-mer for either *C. albicans* or *C. neoformans*.**

The need for efficient antimicrobial agents increases with the emergence of pathogens resistant to current therapies. Among the different approaches to finding novel, safe, and effective antimicrobial agents, the discovery and use of naturally occurring antimicrobial peptides are attracting increasing attention. This is so because, unlike many currently used antimicrobial compounds, they show little or no toxicity toward mammalian cells and a low tendency to elicit resistance. The functional and structural properties and therapeutic potential of naturally occurring antimicrobial peptides (ribosomally synthesized) were recently reviewed (17, 35). With respect to structure and function, magainins (39), dermaseptins (22), cecropins (31), and mammalian defensins (18) have been best characterized (3, 35). A small size, amphipathicity, and a net positive charge are the common features of most naturally occurring antimicrobial peptides (6). The majority of these peptides exhibit a random structure in water and a well-defined structure ( $\alpha$  helix,  $\beta$  sheet, extended structures, and loops) in a simulated membrane-like environment (17). Their mode of antimicrobial action is not well understood. In most instances, it is mediated not by receptors but rather through peptide-microbial cell membrane lipid interactions resulting in membrane permeation and cell lysis (28).

The human salivary nonimmune defense system includes antimicrobial components such as lactoferrin, lysozyme, and peroxidase (20) and the histidine-rich cationic peptides, histatins (23). Previous studies showed that histatin 5 (24-amino-

acid peptide) (Hsn-5) possesses potent fungicidal activity in vitro against *Candida albicans* (23, 36). Further studies showed that Hsn-5 is targeted to mitochondria and that its cytotoxic activity depends on the metabolic activity of *C. albicans* (12). The killing of *C. albicans* by Hsn-5 is accomplished by an increase in membrane potential and permeability and the subsequent release of intracellular ATP (15, 16). It was also shown that Hsn-5 and human neutrophil defensin 1 kill *C. albicans* via a shared pathway (4).

Our laboratory reported that MUC7 domain 1 (D1), a 51-amino-acid-residue peptide (Table 1) derived from the N terminus of the low-molecular-weight human salivary mucin, MUC7 (comprised of 357 residues), possesses antifungal activity that is comparable to or exceeds the antifungal activity of Hsn-5 (26). It was shown that this peptide is effective against wild-type,azole-resistant, and amphotericin B-resistant *C. albicans* and *Cryptococcus neoformans* and against *Candida glabrata*, *Candida krusei*, and *Saccharomyces cerevisiae* (29). It was implicated that the MUC7 D1 (MUC7 51-mer) net positive charge played a key role in its antifungal activity (26, 29). Another, much shorter peptide, MUC7 15-mer (amino acids 3 to 17 of MUC7) (Table 1), which was also derived from the MUC7 N terminus and which showed 53.3% sequence similarity to Hsn-5, was found to be at least sixfold less active against *C. albicans* than MUC7 51-mer (8). Because seven out of eight positively charged amino acid residues present in the rest of the MUC7 D1 sequence are located within its C-terminal 20 residues, we investigated this MUC7 20-mer peptide (amino acids 32 to 51 of MUC7) (Table 1). Indeed, our initial studies showed that MUC7 20-mer displayed fungicidal activities comparable to or better than those of MUC7 51-mer against *C. albicans* and *C. neoformans*, two medically important

\* Corresponding author. Mailing address: Department of Oral Biology, State University of New York at Buffalo, 109 Foster Hall, 3435 Main St., Buffalo, NY 14214-3092. Phone: (716) 829-2465. Fax: (716) 829-3942. E-mail: lbobek@acsu.buffalo.edu.

TABLE 1. Amino acid sequences and charges of peptides under study

| Peptide     | Amino acid sequence                                 | Charge |   |     |
|-------------|---|--------|---|-----|
|             |   | +      | - | Net |
| MUC7 51-mer | EGRERDHELRRRHHHQSPKSHFELPHYPLGLLAHQKPFIRKSYKCLHKRCR | 13     | 5 | +8  |
| MUC7 15-mer | RERDHELRRRHHHQ                                      | 5      | 3 | +2  |
| MUC7 20-mer | LAHQKPFIRKSYKCLHKRCR                                | 7      | 0 | +7  |
| Hsn-5       | DSHAKRHHGYKRKFHEKHHSHRGY                            | 7      | 2 | +5  |
| Ins-A       | GIVEQCCASVCSLYQLENYCN                               | 0      | 2 | -2  |

fungal pathogens. In addition, MUC7 20-mer also showed potent bactericidal activities against *Streptococcus mutans* (cariogenic bacteria) and *Streptococcus gordonii* (30).

In the present study, we examined the spectrum of antimicrobial activity of MUC7 20-mer, its secondary structure, and its possible mechanism of antifungal action. We focused on *C. albicans* and *C. neoformans* since candidiasis and cryptococcosis, caused by these organisms, are the most common opportunistic infections in immunocompromised patients, especially patients with human immunodeficiency virus or AIDS. MUC7 20-mer, like Hsn-5, is a basic salivary antimicrobial peptide (pI, 10.58). Thus, it was of interest to determine whether MUC7 20-mer and Hsn-5 kill fungi by similar mechanisms. We also examined and compared the dependence of 20-mer and Hsn-5 fungicidal activities on the metabolic state of the cells; the ability of the 20-mer to cross the plasma membrane and accumulate intracellularly; and the effects of temperature, metabolic inhibitors, and divalent cations on 20-mer internalization. Lastly, we studied the possible intracellular target(s) of the 20-mer.

#### MATERIALS AND METHODS

**Materials. (i) Peptides.** Unlabeled MUC7 20-mer and fluorescein isothiocyanate (FITC)-labeled MUC7 20-mer were purchased from Bio-Synthesis Inc., (Lewisville, Tex.). High-pressure liquid chromatography and mass spectrometry were performed by the company to analyze the purity of the peptides. Recombinant Hsn-5 was produced in *Escherichia coli* by using vector pET-30b(+). The cloning, expression, and purification of this peptide were done as previously described (34). Bovine insulin chain A (Ins-A) (21 amino acid residues) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

**(ii) Other materials.** Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and sodium azide were obtained from Sigma. MitoTracker Red CMXRos and MitoTracker Green FM dyes were purchased from Molecular Probes Inc. (Eugene, Oreg.). Sabouraud dextrose agar (SAB) and tryptic soy agar were obtained from Difco Laboratories (Detroit, Mich.).

**Yeast and fungal strains and culture conditions.** For the experiments reported in Table 2, the following strains were used. *C. albicans* strain DIS, a clinical isolate from a patient with denture-induced stomatitis, was provided by M. Edgerton (Department of Oral Biology, University at Buffalo), and an azole-resistant clinical isolate (no. 12-99) of *C. albicans* was a gift from Theodore C. White (University of Washington and Seattle Biomedical Research Institute, Seattle, Wash.). Azole-sensitive *C. glabrata* was purchased from ATCC (ATCC 90030), and its azole-resistant counterpart, clinical isolate 65C, was obtained from John E. Bennett (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). A clinical isolate of *C. krusei* was obtained from the Erie County Medical Center, Buffalo, N.Y. Amphotericin B-sensitive (CN2) and amphotericin B-resistant (CN2843) *C. neoformans* strains were obtained from AIDS patients with cryptococcal meningitis and were generously provided by John H. Rex (University of Texas Medical School, Houston). Additionally, *S. cerevisiae* strain S288C was provided by D. Kosman, Department of Biochemistry, University at Buffalo. All were streaked and grown on SAB plates at 37°C, except for *S. cerevisiae*, which was grown at 30°C, until large colonies formed. One colony was then picked and resuspended in 10 mM sodium phosphate buffer (pH 7.4), and

the concentration was adjusted to 10<sup>5</sup> cells/ml for the antifungal assay described below.

For all subsequent experiments, *C. albicans* (DIS) and *C. neoformans* (CN2) were used.

**Bacterial strains and culture conditions.** The following bacterial strains were tested: *S. mutans* GS-5, *S. gordonii* Challis, *E. coli* HB101, *Pseudomonas aeruginosa* ATCC 17648, *Actinobacillus actinomycetemcomitans* NCTC9710, and *Porphyromonas gingivalis* 381 and W50. *S. mutans*, *S. gordonii*, *A. actinomycetemcomitans*, and *P. aeruginosa* were grown anaerobically by candle jar extinction at 37°C on tryptic soy agar plates. *P. gingivalis* was grown in an anaerobic chamber at 37°C on sheep blood agar plates. *E. coli* was grown aerobically at 37°C on Luria-Bertani agar plates.

**Antifungal and antibacterial activity assays.** For killing assays, twofold serial dilutions of each peptide (100 to 1.56 μM) in 20 μl of 10 mM sodium phosphate buffer (pH 7.4) were incubated with equal volumes of bacterial or fungal cells (10<sup>5</sup> cells/ml in 10 mM sodium phosphate buffer [pH 7.4]) for 1.5 h at 30°C for *S. cerevisiae* and 37°C for the rest of the organisms tested. As controls, the cells were incubated without the peptides. At the end of the incubation, the samples were diluted 20-fold with the same buffer; aliquots (~120 cells) of each sample were plated on appropriate plates, depending on the organism tested, as indicated above. Plates were incubated for 1 to 7 days aerobically or anaerobically, depending on the organism tested. Colonies were then counted, and the loss of cell viability was plotted as a function of protein concentration. Fungicidal activities against *C. albicans* (DIS) and *C. neoformans* (CN2) were also tested at 4°C.

For assays of the time-dependent killing activity of peptides, *C. albicans* (DIS) and *C. neoformans* (CN2) fungal cells (10<sup>5</sup> cells/ml in 10 mM sodium phosphate

TABLE 2. ED<sub>50</sub>s of MUC7 20-mer and Hsn-5<sup>a</sup>

| Organism   | ED <sub>50</sub> (95% confidence limit), μM |                  |
|--|---|------------------|
|  | MUC7 20-mer                                 | Hsn-5            |
| <b>Fungi</b>                                       |   |                  |
| <i>C. albicans</i> (DIS)                           | 5.85 (4.17–8.67)                            | 6.68 (6.05–7.37) |
| <i>C. glabrata</i>                                 | 5.02 (3.75–8.22)                            | 38.7 (30.8–50.7) |
| <i>C. krusei</i>                                   | 5.16 (4.17–6.40)                            | 6.47 (5.79–7.55) |
| <i>C. neoformans</i> (CN2)                         | 4.05 (3.16–5.81)                            | 3.71 (1.92–5.60) |
| <i>S. cerevisiae</i>                               | 5.23 (4.01–6.76)                            | 74.0 (58.0–101.) |
| <i>C. albicans</i><br>(azole resistant)            | 2.40 (1.73–3.08)                            | 6.40 (5.59–7.32) |
| <i>C. glabrata</i><br>(fluconazole resistant)      | 12.3 (9.06–17.1)                            | 85.3 (78.4–94.0) |
| <i>C. neoformans</i><br>(amphotericin B resistant) | 4.29 (3.59–4.26)                            | 3.72 (2.90–4.87) |
| <b>Bacteria</b>                                    |   |                  |
| <i>A. actinomycetemcomitans</i> <sup>b</sup>       | 4.37 (3.74–5.04)                            | >100             |
| <i>E. coli</i>                                     | 1.61 (1.09–2.14)                            | Not tested       |
| <i>P. aeruginosa</i>                               | 4.41 (3.65–5.19)                            | Not tested       |
| <i>S. mutans</i> <sup>c</sup>                      | 1.39 (1.00–1.90)                            | 92.0 (85.1–99.3) |
| <i>S. gordonii</i>                                 | 2.43 (0.60–6.58)                            | Not tested       |
| <i>P. gingivalis</i>                               | <1.00                                       | Not tested       |

<sup>a</sup> For more details on organisms, see Materials and Methods. Ins-A showed no antimicrobial activity. Data for Hsn-5 and fungi are from reference 29.

<sup>b</sup> ED<sub>50</sub> of streptomycin, 2.7 μM.

<sup>c</sup> ED<sub>50</sub> of streptomycin, 64 μM.

buffer [pH 7.4]) were incubated with 6.5  $\mu\text{M}$  MUC7 20-mer or 6.5  $\mu\text{M}$  Hsn-5 (no peptide was added to control reactions). Aliquots of 40  $\mu\text{l}$  were taken at different times; after 20-fold dilution with phosphate-buffered saline to stop the reaction, the aliquots were plated on SAB plates.

To test the effects of inhibitors of oxidative phosphorylation in the mitochondrial system on peptide-induced killing, *C. albicans* (DIS) and *C. neoformans* (CN2) cells ( $10^7$  cells/ml in 10 mM sodium phosphate buffer [pH 7.4]) were preincubated with either 20 mM sodium azide or 300  $\mu\text{M}$  CCCP for 2 h at 37°C. Killing assays were then carried out as described above. In separate control experiments, both inhibitors were evaluated for their toxicity toward *C. albicans* and *C. neoformans* in the absence of peptides. At these concentrations, neither compound had any lethal effect.

In addition, the fungicidal activities of MUC7 20-mer (25  $\mu\text{M}$ ) and of Hsn-5 (25  $\mu\text{M}$ ) in 10 mM sodium phosphate buffer (pH 7.4) in the presence of various concentrations (1 to 50 mM) of  $\text{Ca}^{2+}$  (as  $\text{CaCl}_2$ ) or  $\text{Mg}^{2+}$  (as  $\text{MgCl}_2$ ) were examined.

**Statistical analysis.** Molar concentrations of peptides required to kill half the maximal number of cells ( $\text{ED}_{50}$ s) and 95% confidence limits of  $\text{ED}_{50}$ s were determined by the PROBIT procedure (SPSS software package 6.1.2 for Macintosh). A time course study of peptide-induced killing was statistically analyzed with the Student *t* test.

**Secondary structure prediction.** For secondary structure prediction, we used the PSI Pred graphical viewer from the Brunel Bioinformatics Group of Brunel University (Uxbridge, United Kingdom). The helical wheel projections of the predicted helical region of each peptide were made by using Genetics Computer Group sequence analysis software.

**Fluorescence light microscopy studies.** *C. albicans* (DIS) and *C. neoformans* (CN2) cells,  $10^7$  in 100  $\mu\text{l}$  of 10 mM sodium phosphate buffer (pH 7.4), were treated with 50  $\mu\text{M}$  FITC-MUC7 20-mer for 45 to 90 min at 37 or 4°C. In separate experiments, the cells were first preincubated with 20 mM sodium azide or 300  $\mu\text{M}$  CCCP for 2 h at 37°C and then treated with 50  $\mu\text{M}$  FITC-MUC7 20-mer. In other experiments, the cells were incubated simultaneously in the presence of 50 mM  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  and 50  $\mu\text{M}$  FITC-MUC7 20-mer. The cells were then extensively washed with the same phosphate buffer, concentrated by centrifugation, and resuspended in the same buffer. The cell suspension was quickly mounted on slides with sealed coverslips. Fluorescence light micrographs were made on a Nikon Optiphot microscope with a fluorescent light source.

**Confocal fluorescence microscopy studies.** The intracellular localization of MUC7 20-mer was investigated in a double-labeling experiment with FITC-MUC7 20-mer and a MitoTracker Red CMXRos mitochondrial probe. *C. albicans* (DIS) and *C. neoformans* (CN2) cells ( $10^7$  in 100  $\mu\text{l}$  of 10 mM sodium phosphate buffer [pH 7.4]) were incubated with 500 nM MitoTracker Red CMXRos for 15 min at room temperature, washed three times with the same buffer, and subsequently incubated with 50  $\mu\text{M}$  FITC-MUC7 20-mer. After 20 min of incubation at 37°C, the suspension was washed extensively with phosphate buffer. For control experiments, the same numbers of cells in the same buffer as that described above were incubated with two mitochondion-specific dyes: first, in 500 nM MitoTracker Red CMXRos, and second, in 500 nM MitoTracker Green FM. The cell suspensions (2  $\mu\text{l}$ ) were quickly mounted on slides with sealed coverslips. Confocal fluorescence microscopy was performed by using a Bio-Rad MRC-1024 confocal microscope system with a krypton-argon laser, which outputs 488-, 568-, and 647-nm excitation lines, and a Nikon upright epifluorescence microscope (Zeiss  $\times 60$  oil immersion planApp objective with a 1.4 numerical aperture).

## RESULTS

**Antifungal and antibacterial activities of MUC7 20-mer.** MUC7 20-mer (see Table 1 for the sequence) showed potent activity against a variety of fungi (Table 2), including *C. albicans*, *C. glabrata*, *C. krusei*, *C. neoformans*, and *S. cerevisiae*. In addition, it was also active against several drug-resistant strains, including azole-resistant *C. albicans*, fluconazole-resistant *C. glabrata*, and amphotericin B-resistant *C. neoformans*. Its potency against all fungi tested was comparable to or exceeded that of full-length MUC7 D1 (51-mer; see Table 1 for the sequence) (29). In order to rule out a nonspecific nature of this activity, Ins-A, an unrelated peptide with a similar chain length (21 residues) (Table 1), was assayed. This peptide had no fungicidal activity (data not shown). In comparison to

TABLE 3. Time-dependent killing activity of MUC7 20-mer and Hsn-5

| Time (min) | % Loss of viability of the following cells in the presence of the indicated agent <sup>a</sup> : |            |                            |            |
|------------|--|------------|----------------------------|------------|
|            | <i>C. albicans</i> (DIS)   |            | <i>C. neoformans</i> (CN2) |            |
|            | 20-Mer   | Hsn-5      | 20-Mer                     | Hsn-5      |
| 5          | 31 $\pm$ 8   | 0 $\pm$ 0  | 97 $\pm$ 1                 | 9 $\pm$ 2  |
| 15         | 42 $\pm$ 5   | 1 $\pm$ 3  | 97 $\pm$ 2                 | 43 $\pm$ 3 |
| 30         | 52 $\pm$ 1   | 11 $\pm$ 4 | 96 $\pm$ 2                 | 59 $\pm$ 5 |
| 45         | 64 $\pm$ 3   | 34 $\pm$ 2 | 98 $\pm$ 1                 | 64 $\pm$ 1 |
| 90         | 74 $\pm$ 6   | 42 $\pm$ 3 | 100 $\pm$ 0                | 71 $\pm$ 7 |

<sup>a</sup> Peptide concentration, 6.25  $\mu\text{M}$ . Data are reported as mean and standard deviation. *P* values for 20-mer versus Hsn-5 were  $<0.05$ .

Hsn-5, MUC7 20-mer showed especially high activity against *C. glabrata* and *S. cerevisiae*.

Time-dependent killing of *C. albicans* (DIS) and of *C. neoformans* (CN2) indicated differences in killing rates between MUC7 20-mer and Hsn-5 (Table 3). For these experiments, 6.5  $\mu\text{M}$  concentrations of MUC7 20-mer and Hsn-5 were used. For *C. albicans*, both peptide concentrations fall within the  $\text{ED}_{50}$ s, which are 5.85  $\mu\text{M}$  (range, 4.17 to 8.67) for the MUC7 20-mer and 6.68  $\mu\text{M}$  (range, 6.06 to 7.37) for Hsn-5 (Table 2). For *C. neoformans*, 6.5  $\mu\text{M}$  concentrations of the peptides used are somewhat higher than their  $\text{ED}_{50}$ s, which are 4.05  $\mu\text{M}$  (range, 3.16 to 5.8) for the MUC7 20-mer and 3.71  $\mu\text{M}$  (range, 1.92 to 5.60) for Hsn-5 (Table 2). The results indicated that at equal molar concentrations (6.5  $\mu\text{M}$ ), MUC7 20-mer kills both *C. albicans* and *C. neoformans* faster than Hsn-5 does ( $P < 0.05$ ) (Table 3).

MUC7 20-mer displayed potent antibacterial activity (Table 2) against *S. mutans*, *S. gordonii*, *E. coli*, and *P. gingivalis*, with  $\text{ED}_{50}$ s of less than 3  $\mu\text{M}$ , and *A. actinomycetemcomitans* and *P. aeruginosa*, with  $\text{ED}_{50}$ s of less than 5  $\mu\text{M}$ . On the other hand, Hsn-5 had an  $\text{ED}_{50}$  of 92  $\mu\text{M}$  against *S. mutans* and induced only a 24% loss of cell viability at the highest concentration tested (100  $\mu\text{M}$ ) against *A. actinomycetemcomitans*. Streptomycin and Ins-A were used as positive and negative controls, respectively, in the experiments with *S. mutans* and *A. actinomycetemcomitans*. No killing was detected with Ins-A. The  $\text{ED}_{50}$ s of streptomycin against *A. actinomycetemcomitans* and *S. mutans* were 2.7 and 64  $\mu\text{M}$ , respectively.

**Effects of temperature on MUC7 20-mer-induced killing.** Previous studies reported that no killing of *C. albicans* by Hsn-5 was detected at 4°C (9) and that *C. albicans* killing by histatin 3 was highly attenuated at 0°C (37). We performed parallel lethal activity assays at 4°C with Hsn-5 and MUC7 20-mer using *C. albicans* and *C. neoformans*. Our results (Fig. 1) confirmed the limited potency of Hsn-5 against *C. albicans* at 4°C and revealed that MUC7 20-mer sustained almost 90% killing activity against *C. albicans*. The results also showed that the activities of both MUC7 20-mer and Hsn-5 against *C. neoformans* at 4°C were barely affected.

**Effects of CCCP and sodium azide on MUC7 20-mer-induced killing.** CCCP is an uncoupler of oxidative phosphorylation in the mitochondrial system, and sodium azide inhibits ATP synthesis by inhibiting cytochrome oxidase. It was previously reported that both CCCP and sodium azide were able to

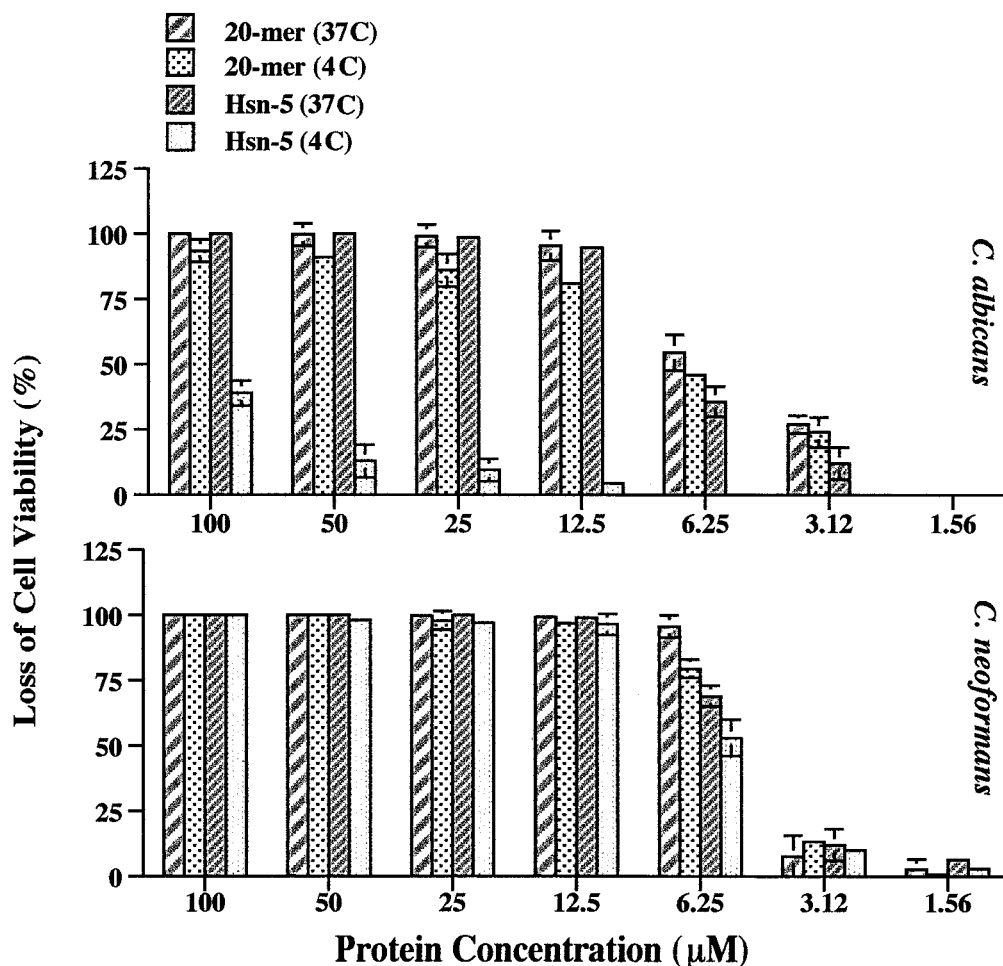


FIG. 1. Effects of temperature on peptide-induced killing of *C. albicans* (DIS) and *C. neoformans* (CN2). Fungal cells ( $2 \times 10^3$  in 20  $\mu$ l of 10 mM sodium phosphate buffer) were incubated with 20  $\mu$ l of MUC7 20-mer or Hsn-5 peptide (at concentrations ranging from 1.56 to 100  $\mu$ M in 10 mM sodium phosphate buffer) for 1.5 h at 4 and 37°C. Cell viability was determined by plating on SAB plates. Results represent the mean and standard deviation of duplicate or triplicate experiments.

protect *C. albicans* from Hsn-5 killing (10, 16). Our results showed that pretreatment of *C. albicans* or *C. neoformans* with CCCP or sodium azide did not protect either organism from killing by MUC7 20-mer (Fig. 2). These results, together with the killing properties at 4°C (Fig. 1), demonstrated that the fungicidal activity of MUC7 20-mer, unlike that of Hsn-5, does not require an active cell metabolic state. Interestingly, though, we found that the killing of *C. albicans* and *C. neoformans* by Hsn-5 in the presence of CCCP and sodium azide showed distinct features (Fig. 2)—namely, both CCCP and sodium azide rescued *Candida* cells from killing by Hsn-5 but were unable to do the same for *Cryptococcus* cells.

**Effects of divalent cations on MUC7 20-mer-induced killing.** Previous studies showed that divalent cations ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) adversely affected the candidacidal activities of human neutrophilic granulocyte defensins (18) and of Hsn-5 (24). Thus, we tested the effects of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on the fungicidal activity of MUC7 20-mer. The addition of 1 mM  $\text{Ca}^{2+}$  (as  $\text{CaCl}_2$ ) or  $\text{Mg}^{2+}$  (as  $\text{MgCl}_2$ ) to sodium phosphate buffer (10 mM, pH 7.4) had a limited effect on MUC7 20-mer anticandidal activity (Fig. 3); however, increases in the concentrations

of these ions greatly reduced MUC7 20-mer potency. The inhibitory effects of these divalent cations on the antifungal activity of MUC7 20-mer were more pronounced for *C. albicans*. MUC7 20-mer sustained 90 and 84% killing of *C. neoformans* in the presence of 10 mM  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , respectively, while it sustained only 10% anticandidal activity.

**Secondary structure prediction.** Figure 4 shows the secondary structure prediction for MUC7 20-mer in comparison to those for MUC7 15-mer (histatin-like domain, amino acids 3 to 17 of native MUC7) (Table 1) and Hsn-5 and the helical wheel projections of the predicted helical region of each peptide. As shown at the top of Fig. 4, a certain portion of each peptide sequence was predicted to have a helical structure; the amino acid stretches involved consist of 12 residues out of 20 in MUC7 20-mer, 7 out of 15 in MUC7 15-mer, and 9 out of 24 in Hsn-5. The rest of the sequence exhibits a coil structure. No  $\beta$ -sheet structure was predicted for any of the three peptides. The helical wheel projections of the predicted helical region of each peptide are shown below the secondary structures in Fig. 4. Most antimicrobial peptides exhibit an amphiphilic character. However, neither Hsn-5 nor its active domain (residues 11

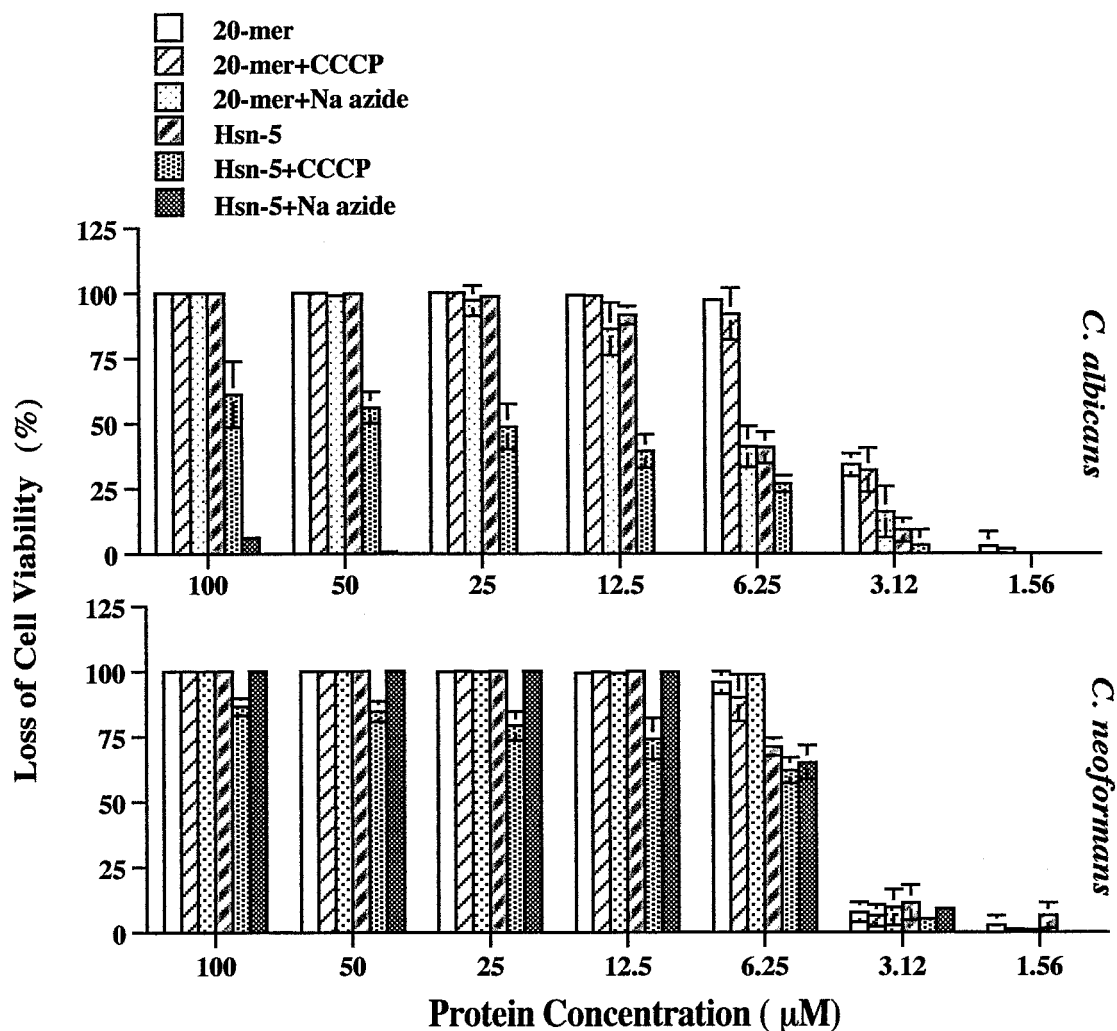


FIG. 2. Effects of CCCP and sodium azide on peptide-induced killing of *C. albicans* (DIS) and *C. neoformans* (CN2). Fungal cells were incubated with 20 mM sodium azide or 300 µM CCCP for 2 h at 37°C. Cells without any pretreatment were used as controls. A 20-µl aliquot ( $2 \times 10^3$  cells) of treated fungal cells was then incubated with 20 µl of MUC7 20-mer or Hsn-5 peptide (1.56 to 100 µM in 10 mM sodium phosphate buffer) for an additional 1.5 h at 37°C. Cell viability was determined by plating on SAB plates. Results represent the mean and standard deviation of duplicate or triplicate experiments.

to 24) adopts amphiphilic structures (8, 35), and this finding was confirmed by our study. On the other hand, the secondary structure prediction results obtained for MUC7 20-mer indicated that this peptide adopts an amphipathic helix with distinguishable hydrophilic and hydrophobic faces. The hydrophobic face is made up of C<sup>14</sup>, F<sup>7</sup>, R<sup>18</sup>, S<sup>11</sup>, L<sup>15</sup>, I<sup>8</sup>, and Y<sup>12</sup>, with five hydrophobic residues (C<sup>14</sup>, F<sup>7</sup>, S<sup>11</sup>, I<sup>8</sup>, and Y<sup>12</sup>). The hydrophilic face is formed by K<sup>10</sup>, K<sup>17</sup>, K<sup>13</sup>, R<sup>9</sup>, and H<sup>16</sup>. We also acquired peptide conformations from far-UV circular dichroism spectra in an aqueous solution and a nonpolar medium (organic solvent trifluoroethanol [TFE] which simulates a membrane environment) (data not shown). The analysis of these spectra indicated that in the aqueous medium, all peptides exist in a random conformation, and that in TFE, they adopt a helical structure; these results are consistent with the secondary structure prediction.

**Internalization and localization of MUC7 20-mer.** In order to visualize the association of MUC7 20-mer with fungi or

bacteria and determine its fungal cellular localization, FITC-labeled MUC7 20-mer was used. The fungicidal activity of FITC-MUC7 20-mer is about 10% lower than that of unlabeled MUC7 20-mer (data not shown). Figure 5 shows the fluorescence light micrographs of fungi (*C. albicans* and *C. neoformans*) incubated with FITC-MUC7 20-mer. The fluorescent active peptide was visible on the cell membrane and intracellularly in both *Candida* and *Cryptococcus*, indicating that MUC7 20-mer penetrated the cell wall and cell membrane and accumulated inside the cells. A low incubation temperature (4°C) (Fig. 5B) and pretreatment of cells with sodium azide (Fig. 5C) and CCCP (Fig. 5D) did not affect the uptake of MUC7 20-mer. FITC-MUC7 20-mer was seen within all of the cells with a fluorescence intensity similar to that seen in untreated cells. However, the uptake of the peptide was significantly limited in the presence of the divalent cations Mg<sup>2+</sup> and Ca<sup>2+</sup> (Fig. 5E and F, respectively). FITC-MUC7 20-mer was seen within only a very small number of cells. The fluo-

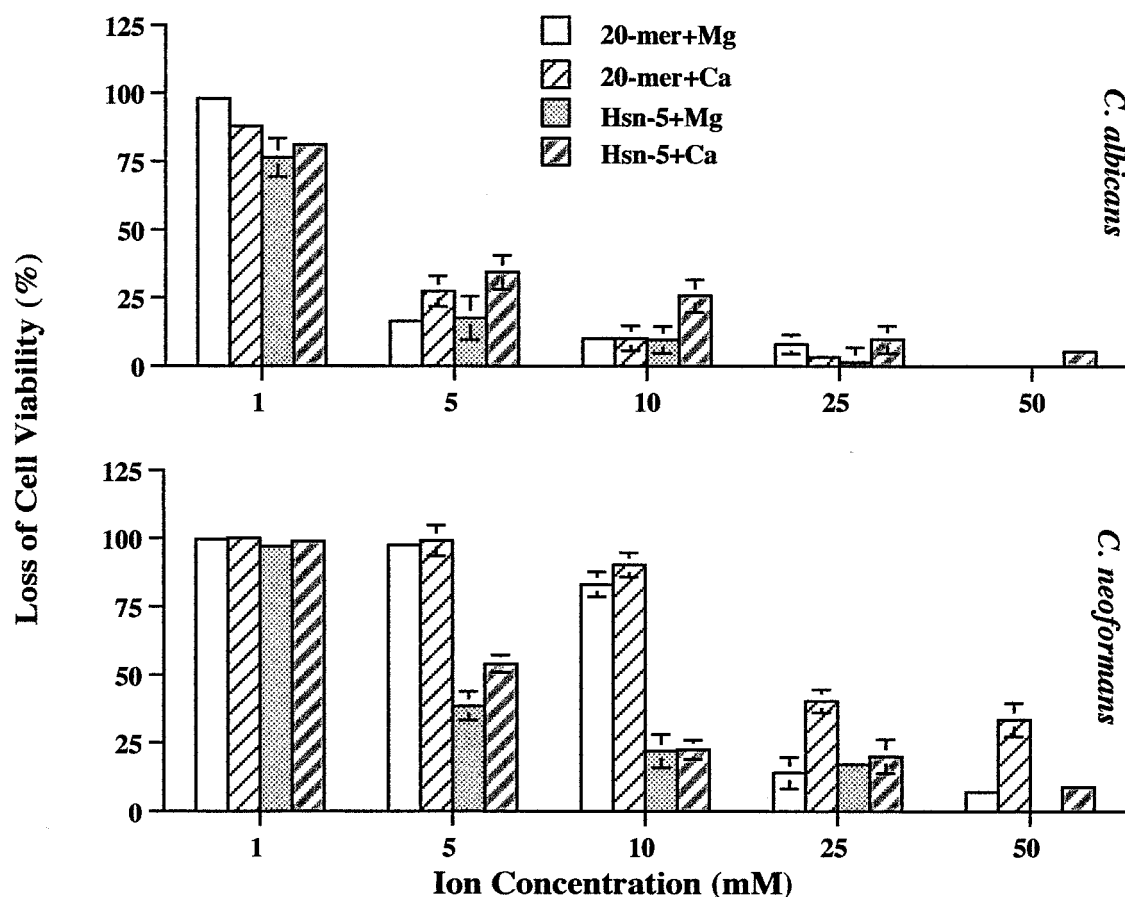


FIG. 3. Effects of cations on peptide-induced killing of *C. albicans* (DIS) and *C. neoformans* (CN2). Fungal cells ( $2 \times 10^3$  in 20  $\mu$ l of 10 mM sodium phosphate buffer) were incubated with 20  $\mu$ l of MUC7 20-mer or Hsn-5 peptide (final concentrations, 25  $\mu$ M) in 10 mM sodium phosphate buffer in the presence of either  $Mg^{2+}$  or  $Ca^{2+}$  at concentrations ranging from 1 to 50 mM for 1.5 h at 37°C. Cell viability was determined by plating on SAB plates. Results represent the mean and standard deviation of duplicate or triplicate experiments. At a 25  $\mu$ M concentration of peptides, without the presence of cations, 100% peptide-induced killing was obtained.

rescence intensity was also low. An interesting observation was that FITC-MUC7 20-mer was visualized on the circumference of *C. neoformans* in the presence of cations; this characteristic was not seen with *C. albicans*, indicating that the binding of the peptide to *C. neoformans* was affected much less than that to *C. albicans*. A higher fluorescence signal was detected within *C. neoformans* cells treated with cations than within *C. albicans* cells so treated. FITC-labeled peptide was also seen on the cell membrane of and inside *A. actinomycescomitans* cells (data not shown), indicating internalization of MUC7 20-mer in bacterial cells as well.

In addition, confocal fluorescence microscopy was used to study the intracellular localization of MUC7 20-mer. Double labeling with FITC-MUC7 20-mer and MitoTracker Red CM-SRos, a mitochondrion-specific dye, did not show overlapping patterns, suggesting that mitochondria are not the intracellular target of MUC7 20-mer in *C. albicans* or *C. neoformans* (Fig. 5G). In the control experiment with two mitochondrion-specific dyes (MitoTracker Red CM-SRos and MitoTracker Green FM), both dyes localized to the mitochondria of both fungi, as shown by identical (overlapping) staining patterns (Fig. 5H).

## DISCUSSION

Human saliva is fundamentally important in the host innate nonimmune defense system against oral pathogens. Salivary mucins, together with other groups of salivary proteins (proline-rich proteins, cystatins, statherins, and amylase), protect the oral cavity from microbial infections through more general protective mechanisms rather than the direct killing of microorganisms. The antimicrobial effect of mucins has been attributed largely to agglutination activity—trapping and clearing the microorganisms from the system. The selective binding of salivary mucins to microbial adhesins prevents the subsequent attachment of microorganisms to host surfaces (32, 33). Conversely, mucins bound to tissues may serve as docking ports facilitating the colonization of microorganisms such as *Streptococcus* (7). Thus, salivary mucins play an intriguing, paradoxical role in the dynamics of the oral flora. This study, together with two previous studies (26, 29), further complicates the unique relationship of MUC7 and microbial pathogens because it was shown that MUC7-derived peptides (possibly generated in vivo by proteolytic enzymes) can directly kill bacteria (this study) and fungi (26, 29; this study), making MUC7 a

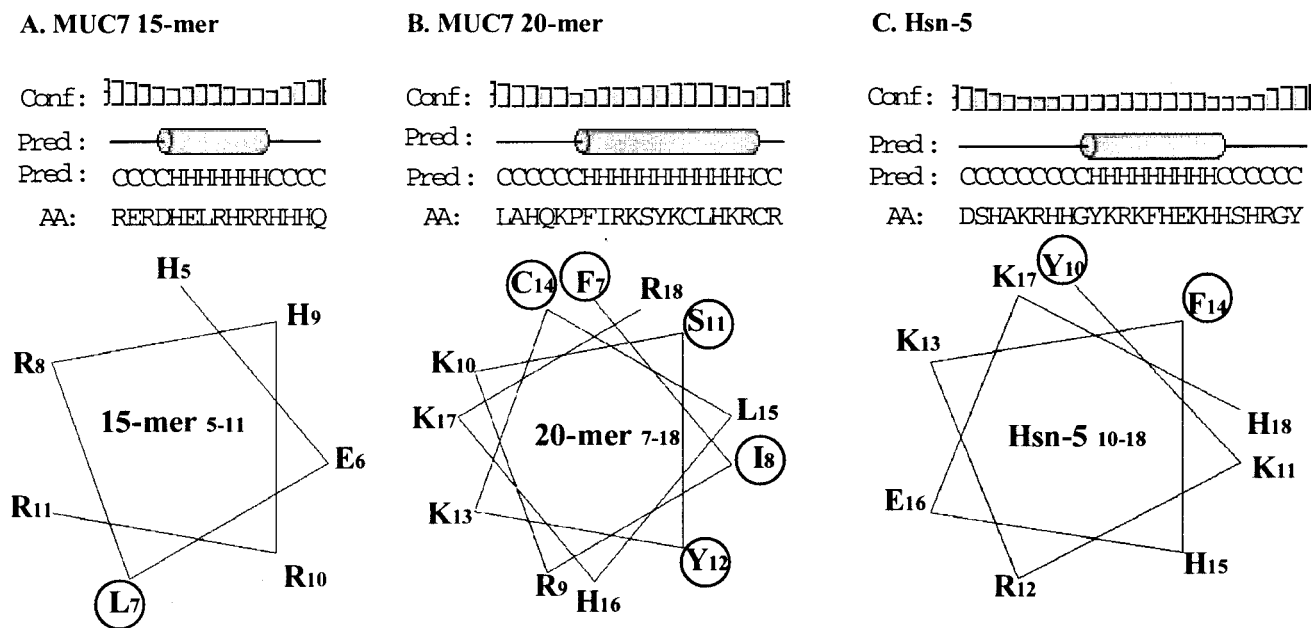


FIG. 4. Secondary structure predictions and helical wheel projections for MUC7 15-mer, MUC7 20-mer, and Hsn-5. For the secondary structure prediction (shown at the top), a PSI Pred graphical viewer was used. Conf, confidence of prediction; Pred, predicted secondary structure; AA, target sequence. The cylinder denotes helix structure (H), and the line denotes coil structure (C). The helical wheel projections of the predicted helical region of each peptide were made by using Genetics Computer Group sequence analysis software. Hydrophobic residues are displayed in circles.

multifaceted, critical component of oral defense mechanisms. Another study (19) showed that the N-terminal region of MUC7 (amino acids 1 to 144) contains a structural determinant for the binding of oral streptococci and exhibits candidacidal activity.

It is important to note that the broad-spectrum antimicrobial activity of MUC7 20-mer reported here is specific, since the control peptide, Ins-A (composed of 21 amino acid residues), showed no lethal activity toward fungi or bacteria. MUC7 20-mer displayed both antifungal and antibacterial activities at micromolar concentrations, and the activities were comparable. Hsn-5 required more than a 10-fold-higher concentration for killing bacteria than for killing fungi (Table 2).

Antimicrobial peptides are classified according to their mode of action as inhibitors of protein biosynthesis, inhibitors of DNA or RNA synthesis, lytic peptides that disrupt the membrane, and inhibitors of microbial cellular metabolism (1, 27, 38). Earlier studies showed that Hsn-5 is taken up into the intracellular space possibly by means of a receptor-mediated mechanism, after which it interacts with an intracellular target, mitochondria (5, 12). A recent study demonstrated that it subsequently inhibits respiration and induces the formation of reactive oxygen species, leading to cell death (13). We have demonstrated, by using lethal assays, metabolic inhibitors, and confocal laser microscopy, that MUC7 20-mer and Hsn-5 may have different targets. Our preliminary data also showed that both peptides cause disturbances of *C. albicans* and *C. neoformans* plasma membrane potentials, indicating membranolytic activity (data not shown). FITC-MUC7 20-mer not only was associated with the plasma membrane but also was internalized, suggesting that its mechanism of killing does not involve a direct effect on the membrane alone. Other studies showed

that cell membrane permeabilization (depolarization) by itself is not necessarily the crucial event in the killing of microorganisms. To explain this finding, the “aggregate channel model” (of pore formation) has been postulated (11). In this model, the peptides are allowed to cross the membrane through pores formed by the peptides but also have an intracellular target(s) on which to exert their killing activities. MUC7 20-mer may work via such a mechanism.

In contrast to the characteristics of Hsn-5, 20-mer peptide internalization and killing potency were not affected (blocked) by a low temperature or the presence of inhibitors of ATP synthesis (CCCP and sodium azide). These results indicate that its action is not dependent on cellular metabolic activity (respiration). The 20-mer does not target mitochondria, as shown by double labeling and confocal microscopy. In agreement with the characteristics of Hsn-5, internalization and killing were affected by the addition of the divalent cations  $Ca^{2+}$  and  $Mg^{2+}$ . We speculate that, as with Hsn-5, the fungicidal activity of the 20-mer may depend on the initial interactions between the positively charged 20-mer molecules and the negatively charged head groups of the yeast cell membrane bilayer. The presence of divalent cations reduces the 20-mer interaction with fungal cells and its internalization. However, binding of the 20-mer to *C. neoformans* was affected much less than was that to *C. albicans* in the presence of the cations. These results were evidenced by the detection of a higher fluorescence signal associated with the membrane of *C. neoformans* than with that of *C. albicans*. These observations, coupled with the lethal assay results, further suggest that the interaction of the peptide with the cell membrane and the further internalization of the 20-mer are crucial for the 20-mer to exert its fungicidal activity. The dependence of Hsn-5 on

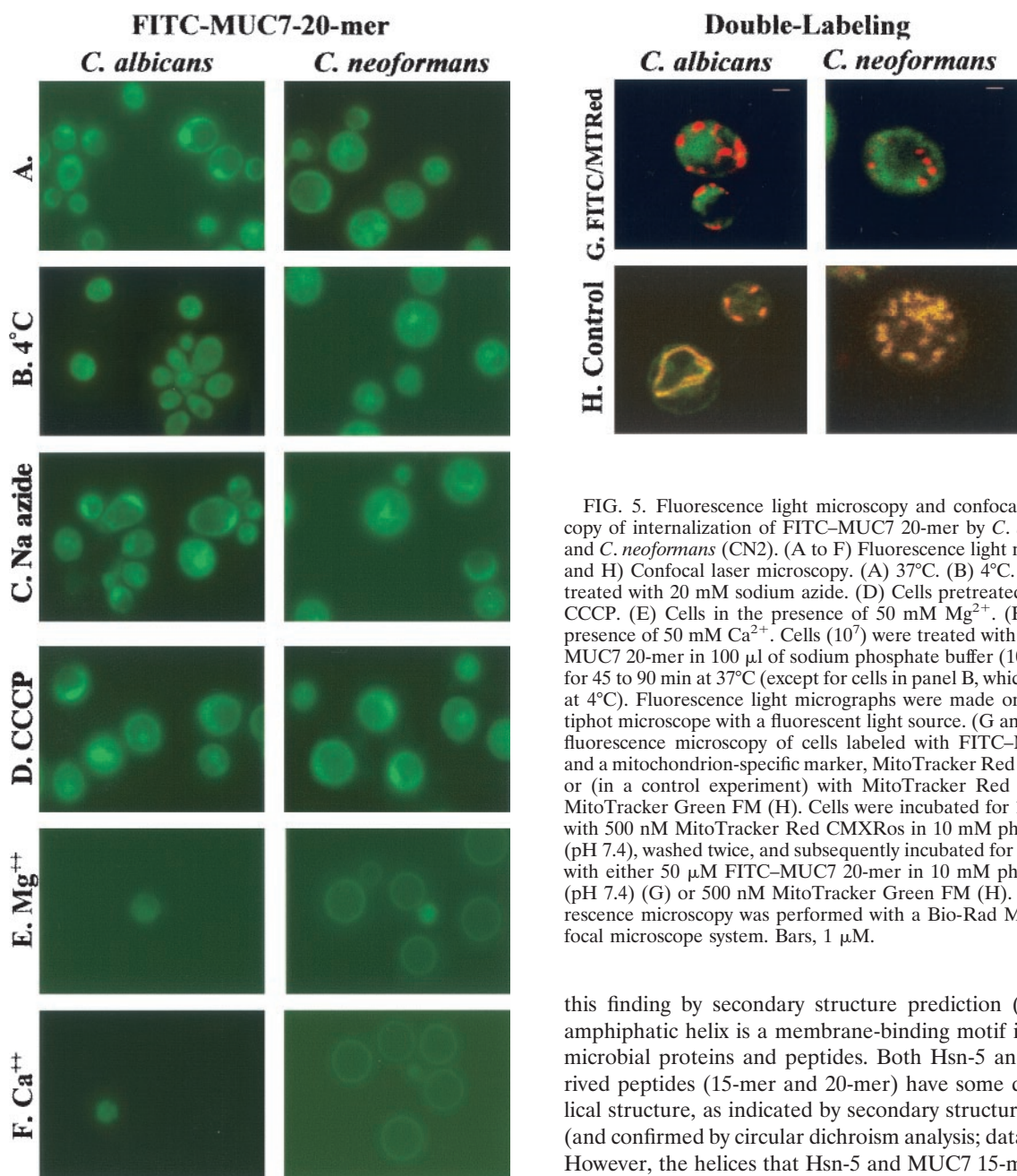


FIG. 5. Fluorescence light microscopy and confocal laser microscopy of internalization of FITC-MUC7 20-mer by *C. albicans* (DIS) and *C. neoformans* (CN2). (A to F) Fluorescence light microscopy. (G and H) Confocal laser microscopy. (A) 37°C. (B) 4°C. (C) Cells pretreated with 20 mM sodium azide. (D) Cells pretreated with 300  $\mu$ M CCCP. (E) Cells in the presence of 50 mM Mg<sup>2+</sup>. (F) Cells in the presence of 50 mM Ca<sup>2+</sup>. Cells (10<sup>7</sup>) were treated with 50  $\mu$ M FITC-MUC7 20-mer in 100  $\mu$ l of sodium phosphate buffer (10 mM, pH 7.4) for 45 to 90 min at 37°C (except for cells in panel B, which were treated at 4°C). Fluorescence light micrographs were made on a Nikon Optiphot microscope with a fluorescent light source. (G and H) Confocal fluorescence microscopy of cells labeled with FITC-MUC7 20-mer and a mitochondrion-specific marker, MitoTracker Red CMXRos (G), or (in a control experiment) with MitoTracker Red CMXRos and MitoTracker Green FM (H). Cells were incubated for 15 min at 37°C with 500 nM MitoTracker Red CMXRos in 10 mM phosphate buffer (pH 7.4), washed twice, and subsequently incubated for 20 min at 37°C with either 50  $\mu$ M FITC-MUC7 20-mer in 10 mM phosphate buffer (pH 7.4) (G) or 500 nM MitoTracker Green FM (H). Confocal fluorescence microscopy was performed with a Bio-Rad MRC-1024 confocal microscope system. Bars, 1  $\mu$ M.

*Candida* cellular metabolic activity to exert killing is quite unique, as evidenced by our results demonstrating that the Hsn-5-induced killing of *Cryptococcus* and the 20-mer-induced killing of both *Candida* and *Cryptococcus* are not dependent on cellular metabolic activity. In addition, a recent study (25) showed that after pretreatment of *C. albicans* cells with sodium azide (blocking cellular respiration), the uptake of Hsn-5 variant dhvar4 and consequently the killing by dhvar4 were barely affected, and those of variant dhvar5 were affected only moderately.

Differences in the activities of MUC7 20-mer and Hsn-5 are also correlated with their structural differences. We obtained

this finding by secondary structure prediction (Fig. 4). The amphipathic helix is a membrane-binding motif in many antimicrobial proteins and peptides. Both Hsn-5 and MUC7-derived peptides (15-mer and 20-mer) have some degree of helical structure, as indicated by secondary structure predictions (and confirmed by circular dichroism analysis; data not shown). However, the helices that Hsn-5 and MUC7 15-mer adopt are weakly amphipathic, in contrast to that of MUC7 20-mer, which is strongly amphipathic. While many antimicrobial peptides are characterized as having helices, this characteristic is not a prerequisite for activity, as demonstrated by the relative lack of activity of MUC7 15-mer, a peptide with a significant  $\alpha$ -helical structure (8). The stronger amphipathicity of MUC7 20-mer may be the key to understanding its potency. Having the polar side chain aligned along one side and the hydrophobic residues aligned along the opposite side of the helical wheel allows an optimal interaction of the peptide with the cell membrane and facilitates the entrance of the peptide into the cytoplasm.

Most natural antimicrobial peptides are also positively charged. The specific role of a positive peptide charge for the interaction with the negatively charged membrane has long



been recognized and has been documented by a variety of attempts to induce or to improve antimicrobial activity by charge modification (2, 21). It would also seem that this charge could account for the greater susceptibility of *C. neoformans* to small cationic peptides, since its unique polysaccharide capsule contributes to its increased negative charge. At a physiologic pH (7.4), MUC7 15-mer contains five positively and three negatively charged residues, so that it carries a net positive charge of 2. MUC7 20-mer contains seven positively and no negatively charged residues, for a net positive charge of 7. Hsn-5 also carries seven positively charged residues but carries two negatively charged residues, which decrease its net positive charge to 5. Here we showed that MUC7 20-mer, which is much more amphipathic than Hsn-5 and which has a higher net positive charge, is also more potent against many of the fungi and bacteria tested (lower ED<sub>50</sub>s) (Table 2). Other investigators showed that a histatin variant with increased amphipathicity (and net positive charge) had enhanced candidacidal activity (14). It is inferred from these data that amphipathicity and higher charge are characteristics associated with potency. Recent data on the antifungal activity of MUC7 12-mer (12 C-terminal residues of the 20-mer; RKSYPK-CLHRKCR) also support this inference (L. A. Bobek, S. Mashhoon, and H. Situ, *J. Dent. Res.*, abstr. 1798, 2002). MUC7 12-mer has a net positive charge of 6 (versus 7 in the 20-mer). It also exhibits an amphipathic helix. Its ED<sub>50</sub> against *C. albicans* is 2.2 μM (versus 5.8 μM for the 20-mer). When the first and second positive amino acid residues (R and K) were substituted with A, the ED<sub>50</sub> increased to 14.6 μM. This substitution decreases the net positive charge to 4, and even though it adds two more hydrophobic residues, these disrupted the hydrophilic phase of the helix. These results indicate that the amphipathic nature of the peptide and the higher net positive charge are important for activity.

In summary, MUC7 20-mer is potent against a broad range of microorganisms, including fungi, that are resistant to conventional clinically used drugs, and it appears to work via a mechanism distinct from that of Hsn-5. These characteristics are those which would make 20-mer an ideal drug for patients who have developed resistance to conventional antimicrobial agents. It is also possible that 20-mer could act synergistically with other antimicrobial agents in saliva (e.g., Hsn-5), making it an important ingredient of artificial saliva formulations. Finally, at this stage, it is not known whether 20-mer exerts similar antimicrobial activities in vivo. Although some important questions and concerns remain regarding the expression of its activity in physiological and pathological situations, 20-mer represents a prototypic molecule useful for the development of new antimicrobial therapeutic agents.

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