The Cellular Target of Histatin 5 on \textit{Candida albicans} Is the Energized Mitochondrion* \\

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Histatin 5 is a human basic salivary peptide with strong fungicidal properties \textit{in vitro}. To elucidate the mechanism of action, the effect of histatin 5 on the viability of \textit{Candida albicans} cells was studied in relation to its membrane perturbing properties. It was found that both the killing activity and the membrane perturbing activity, studied by the influx of a DNA-specific marker propidium iodide, were inhibited by high salt conditions and by metabolic inhibitors, like sodium azide. In addition, exposure to histatin 5 resulted in a loss of the mitochondrial transmembrane potential \textit{in situ}, measured by the release of the potential-dependent distributional probe rhodamine 123. Localization studies using tetramethylrhodamine isothiocyanate-labeled histatin 5 or fluorescein isothiocyanate-labeled histatin 5 showed a granular intracellular distribution of the peptide, which co-localized with mitotracker orange, a permeant mitochondria-specific probe. Like the biological effects, uptake of labeled histatin 5 was inhibited by mitochondrial inhibitors and high salt conditions. Our data indicate that histatin 5 is internalized, and targets to the energized mitochondrial.

It is generally recognized that basic peptides are important constituents of natural defense systems of most living organisms, including bacteria, plants, insects, and mammals. Examples of such antibiotic peptides are magainins, secreted by the skin of \textit{Xenopus laevis} (1), defensins from the human neutrophils (2), and histatins from human saliva (3–5). Common features of anti-microbial peptides, which manifest great diversity in primary structure, are a net positive charge at physiological pH and the ability to adopt amphipathic α-helix and β-sheet conformations in hydrophobic solvents (6). It is assumed that the activity of these peptides is directed against the cellular membranes, mainly the acidic lipid matrices of the target cells (7). In addition, the susceptibility towards some of these peptides is controlled by the metabolic state of the target cell (8, 9). The mechanism underlying this phenomenon, however, is not understood. Olson et al. (10) reported the importance of metabolic activity in the susceptibility of \textit{Candida albicans} cells to basic proteins, like protamine, by showing that uncoupling of oxidative phosphorylation by chemicals or by genetic mutation, protected the cells against their lethal activity. The requirement for mitochondrial activity was also found for HNP-1, a defensin peptide (11). It has been speculated that energy-dependent processes, e.g. receptor-mediated uptake, or the maintenance of the cell membrane electronegative potential would be implicated in the peptide-mediated killing, but up to now there is no experimental evidence to support this hypothesis. The aim of the present study was to elucidate the mechanism of action of the human salivary antifungal peptide, histatin 5. We found that non-respiring yeast cells were protected against histatin 5 killing activity. In localization studies we identified the energized mitochondrion as the target of histatin 5 in the yeast cell. The direct association with the mitochondrion and the requirement of mitochondrial activity provide a new explanation for the energy-dependent activity of antimicrobial peptides against \textit{C. albicans}.

EXPERIMENTAL PROCEDURES

\textbf{Yeast Culture and Test Conditions—}\textit{C. albicans} (ATCC 10231) was grown for 48 h at 30 °C on a Sabouraud dextrose agar. For use in killing and permeabilization studies, cells were picked from plate and suspended to \(10^7\) colony-forming units/ml in 1 mM potassium phosphate buffer (PPB), pH 7.0. The killing activity of histatin 5 against \textit{C. albicans} was investigated as described previously (12). The killing activity against non-respiring cells was performed in an anaerobic cabinet, essentially according to Lehrer et al. (11).

Histatin 5 (DSHAKRRHGYKRKFHEKHHSHRGY), and two unrelated control peptides, dcysSA (WSQFEEDRIEGLG) and dcysSS (SSSKEENRIIPGGI), were made by solid phase peptide synthesis procedures as described previously (13).

\textbf{Permeabilization Assays—}Permeabilization of whole \textit{C. albicans} cells by histatin 5 was studied using the DNA-staining fluorescent probe propidium iodide (PI) (14). Permeabilization of the yeast mitochondria \textit{in situ} by histatin 5 was studied using the membrane potential-dependent distributional probe 2-(6-amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester (rhodamine 123) (15). PI and rhodamine 123 were obtained from Molecular Probes, Inc. (Eugene, OR).

\textbf{FITC and TRITC Labeling of Peptides—}Tetramethylrhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) were both purchased from Sigma. Labeling was conducted essentially as described by Lane et al. (16). In brief, TRITC or FITC were freshly dissolved in Me2SO to 1 mg/ml, and added to 2 mg/ml of histatin 5 or dcysS in 50 mM potassium phosphate buffer (final pH 7.6) to a final concentration of 25 \(\mu\)g/ml. The calculated molar ratio of TRITC or FITC to histatin 5 was 0.08 and 0.10, respectively. After incubation for 16 h in the dark at 4 °C,

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RESULTS

Killing and Permeabilization of C. albicans by Histatin 5—The effect of histatin 5 on the membrane integrity was studied by monitoring the influx of PI, a DNA-staining fluorescent probe, into C. albicans cells. Fig. 1 shows the results of the FACScan analysis of cells incubated with 4.1 mM histatin 5 or a negative control peptide, in the presence of 9 μM PI. After 10 min of incubation with histatin 5, the majority of the cells were fluorescently labeled. To investigate in more detail the relationship between the effect of histatin 5 on PI permeabilization and the fungicidal activity of histatin 5, cells were incubated with varying concentrations of the peptide, and concomitantly tested for PI labeling (by fluorescence microscopy) and cell viability (in a killing assay). Fig. 2A indicates that killing and PI labeling display a similar dose response. These two parameters also showed a striking similarity with respect to their ionic strength dependence (Fig. 2B). At phosphate buffer concentrations exceeding 20 mM, both PI permeability and killing of C. albicans by histatin 5 were completely abolished.

Influence of Cellular Respiration on Histatin 5 Activity—It was further investigated whether the metabolic state of the yeast cell influences its susceptibility to histatin 5. For this purpose, cells were incubated with histatin 5 in the presence of several agents and conditions that inhibit mitochondrial respiration, including azide, cyanide, and anaerobic incubation. In a control experiment, it was found that temporarily blocking respiration by mitochondrial inhibitors during the time course of the killing experiment (1.5 h) did not influence the viability of the cells. Fig. 3 shows that blocking of the mitochondrial respiration protected C. albicans against the fungicidal activity of histatin 5. A concentration of 5 mM cyanide, which blocks the conventional respiratory pathway of C. albicans, induced over a 6-fold increase in IC50 value, shifting from 3 μM to 20 μM (Fig. 3A). In buffer containing 5 mM azide, which blocks both the conventional and the alternative respiratory pathway of C. albicans cells (17, 18), no histatin 5 killing activity was observed (IC50 > 65 μM). Another way to block cellular respiration is by creating anaerobic conditions. Cysteine at a concentration of 2.5 mM reduces the redox potential of a solution to ~220 mV, which is the most useful measure for the degree of anaerobiosis (19). This was verified by the color change of a redox-potential dependent indicator, resazurin, added to a control vial. Cells incubated in buffer containing cysteine or azide did not accumulate rhodamine 123 in their mitochondria, indicating that under these conditions the mitochondrial transmembrane potential was dissipated (data not shown). In buffer containing cysteine, histatin 5-induced killing was completely abolished (IC50 > 65 μM, Fig. 3B). In Fig. 3C, it is shown that anaerobic conditions protect C. albicans cells against histatin 5 (IC50 > 65 μM), although at high concentrations of peptide 40% killing was observed, probably due to the presence of trace amounts of oxygen in the anaerobic cabinet. These results indicate that mitochondrial respiration is a prerequisite for histatin-induced killing.

Effect of Histatin 5 on Mitochondrial Membrane Integrity—C. albicans cells were incubated with the fluorescent dye rhodamine 123, which accumulates specifically in intact mitochondria in direct proportion to the mitochondrial transmembrane potential. Cells preloaded with rhodamine 123 released the dye upon a 10-min incubation period with 17 μM histatin 5. Microscopic analysis revealed that the typical granular appearance of the probe, indicative of a mitochondrial localization, was abolished upon incubation with histatin 5 (Fig. 4A), but preserved in the cells treated with the negative control peptide (Fig. 4B). This was corroborated by FACScan analysis, indicating that histatin 5 reduced the cell-associated rhodamine fluorescence intensity by more than 90%.

Internalization and Localization of Histatin 5—The experiments of Fig. 4 indicated that, upon incubation of C. albicans with histatin 5, the mitochondrial membrane potential was dissipated. To study whether this was due to a direct association with the mitochondria, the cellular localization of histatin 5 was investigated by confocal fluorescence microscopy and FACScan using histatin 5 coupled to FITC or TRITC. The fungicidal activity of histatin 5 was not affected by the labeling, as was verified in a killing assay (data not shown). Fig. 5 shows a confocal fluorescence micrograph of cells incubated with TRITC-histatin 5. A granular, intracellular staining pattern was observed, indicating the uptake of the peptide into the cell. A similar fluorescence pattern was obtained with FITC-hista-
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In this study, we addressed the mechanism of action of histatin 5, a basic amphipathic antifungal peptide from human saliva. In line with previous observations (20), we found that FITC-histatin 5 is internalized by C. albicans cells. The uptake is dependent on the metabolic state of the cell, and is directly related to the fungicidal activity of histatin 5. Non-respiring C. albicans cells are protected against histatin 5 killing activity, against its membrane perturbing activity, and do not internalize the peptide.

A direct involvement of the mitochondrion in the killing process is demonstrated by co-localization of histatin 5 with a mitochondria-specific probe, revealing that it binds to mitochondria in situ. In addition, histatin 5 dissipates the mitochondrial transmembrane potential in situ, monitored by rhodamine 123 efflux. Together, these results strongly suggest that killing activity of histatin 5 is directly related to perturbation of the mitochondrial membrane.

These results imply the following sequence of events: inter-
action of histatin 5 with the plasma membrane of the yeast cell, translocation across the plasma membrane, release into the intracellular compartment, followed by targeting to and permeabilization of the mitochondria.

Under conditions when histatin 5 was internalized, no labeling of the plasma membrane by TRITC-histatin 5 could be detected (Fig. 7), suggesting that the binding to the plasma membrane is a transient event. Nevertheless, it seems a crucial step in the killing process, since high ionic strength conditions, which have been reported to abolish the initial interaction of basic peptides with fungal cellular membranes (21), prevent both killing and intracellular accumulation of TRITC-histatin 5 (this study).
It is still unclear by which mechanism histatin 5 is transported across the fungal membrane into the cytoplasm. It has been suggested that basic peptides bound to the membrane are subsequently electrophoresed into the membrane under the influence of an inside-negative transmembrane potential (6). Maduke and Roise (22) described the potential-dependent import into protein-free phospholipid vesicles of synthetic peptides similar in length to histatin 5. Matsuzaki et al. (23, 24) showed that magainin 2 was inserted and translocated across artificial phospholipid bilayers to the inner leaflet. These studies suggest that translocation and internalization of basic peptides would be possible without the intervention of membrane-bound permeases. Apparently, from our results, translocation of histatin 5 is attended with the concomitant influx of propidium iodide, suggesting that the uptake of histatin 5 leads to plasma membrane perturbations.

With regard to the targeting of histatin 5 to the mitochondrion, it is important to note that histatin 5 shows structural and functional resemblances with mitochondrial precursors, i.e. terminal amino acid extensions that target mitochondrial proteins from the cytosol to the mitochondrial. Like histatin 5 and other basic antimicrobial peptides, these matrix-targeting sequences of mitochondrial precursor proteins are basic peptides and adapt amphipathic helix structures in hydrophobic environments (25). It is believed that the divalent negative phospholipid cardiolipin, which is abundantly present in the mitochondrial membrane, especially supports the initial attraction of mitochondrial precursors (25). The same mechanism may apply for histatin 5.

The insertion process of mitochondrial precursors into the mitochondrion has been shown to be independent of mitochondrial surface proteins (26), and to occur in a potential-dependent manner (22, 27). It has been reported that an inside-negative membrane potential also enhances the activity of membrane-directed basic antimicrobial peptides against living bacterial cells (28), against isolated energized mitochondria (29), and against model membrane vesicles (23). It is conceivable that factors that diminish the mitochondrial transmembrane potential will reduce histatin 5 binding and the cytotoxic consequences. This is in agreement with our results that the presence of a mitochondrial transmembrane potential is a prerequisite for the antifungal activity of histatin 5, as revealed by the protective efficacy of mitochondrial inhibitors or anaerobic conditions. Previously, Lehrer et al. (11) demonstrated that respiratory activity was necessary to sensitize C. albicans to the lethal effects of a defensin peptide HNP-1, suggesting a mechanism of antifungal action similar to that of histatin 5. Interestingly, it was found recently that cardiolipin, which is enriched in the mitochondria, plays a key role in the membrane perturbing activity of rabbit neutrophil defensins (30).

Nicolay et al. (25) demonstrated the destructive effect of amphipathic peptides derived from the presequence of the cytochrome c oxidase subunit IV on the permeability barrier of isolated mitochondria. These peptides also display antimicrobial activity (31). Likewise, Westerhoff et al. (29, 32) showed that magainins dissipated the mitochondrial potential of isolated rat liver mitochondria and postulated that this would be the mechanism of antibacterial activity, because of the similar organization of membrane-linked energy coupling in bacteria and mitochondria. Whether these antimicrobial peptides share other mechanistic properties with mitochondrial precursors, like translocation to the mitochondrial inner membrane and processing by proteolytic enzymes, are interesting subjects for further study.

Based on our results, we propose that the energized mitochondrion, which might be considered as an endosymbiont of bacterial origin (33), is the cellular target of histatin 5. This may offer a unifying principle for the mechanism of action of basic antifungal peptides that depend on active cell metabolism.

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FIG. 7. Confocal fluorescence microscopy of C. albicans cells labeled with FITC-histatin 5 and a mitochondria-specific marker. Cells were incubated for 0.5 h at 37 °C with 150 nM FITC and the mitotracker orange CM-H 2-TMRos in 1 mM PIP, pH 7.0, washed once, and subsequently incubated for 15 min at 37 °C with 17 μM FITC-histatin 5. Microscopic pictures show the localization of FITC-histatin 5 (A), the localization of mitotracker orange (B), and the double labeling (C). There was no cross-talk between the FITC and the mitotracker orange signal. Bar = 5 μm.
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