Different Anti-

Candida Activities of Two Human Lactoferrin-Derived Peptides, Lfpep and Kaliocin-1

Mónica Viejo-Díaz,1,2† María T. Andrés,2† and José F. Fierro1,2*

Department of Functional Biology (Microbiology), Faculty of Medicine,1 and Laboratory of Oral Microbiology, School of Stomatology,2 University of Oviedo, 33006 Oviedo, Spain

Received 15 December 2004/Returned for modification 18 January 2005/Accepted 4 April 2005

The synthetic peptides Lfpep and kaliocin-1 include the sequences from positions 18 to 40 and 153 to 183 of human lactoferrin, respectively. Lfpep is a cationic peptide with bactericidal and giardicidal effects, whereas kaliocin-1 is a novel bactericidal peptide that corresponds to a highly homologous sequence present in the transferrin family of proteins. Both peptides presented fungicidal activity against Candida spp., including fluconazole- and amphoterin B-resistant clinical isolates. Lfpep exhibited higher antifungal activity (8- to 30-fold) and salt resistance than kaliocin-1. The killing activity of Lfpep was mediated by its permeabilizing activity on Candida albicans cells, whereas kaliocin-1 was unable to disrupt the cytoplasmic membrane, as indicated by its inability to allow permeation of propidium iodide and the small amount of K+ released. The amino acid sequence of kaliocin-1 includes the “multidimensional antimicrobial signature” conserved in disulfide-containing antimicrobial peptides and a striking similarity to brevinin-1Sa, an antimicrobial peptide from frog skin secretions, exhibiting a “Rana box”-like sequence. These features may be of interest in the design of new antifungals.

Mucosal and systemic infections caused by Candida albicans are the most frequent human fungal infections, especially in immunocompromised patients (15). The toxicities of antifungals and the emergence of strains that are resistant to conventional drugs (16) have renewed interest in the development of novel approaches to the design of peptide-based antifungals that are less harmful for host cells and that possess a low tendency to select for resistant strains (21, 35). Human mucosal secretions contain some innate defense peptides and proteins (9) that may be cleaved by proteolytic enzymes in vivo, resulting in small peptides with similar or enhanced antimicrobial activities compared with those of their parent proteins (3, 5, 24, 26, 28). It has thus been suggested that optimized analogues of these resulting peptides could be used as alternative agents to classical antifungals (21, 26, 28) or as adjunctive topical therapy to decrease the doses of conventional drugs, thus minimizing their toxicities (18, 32).

Human lactoferrin (hLf) is a major protein of mucosal innate host defenses (11) that includes different antimicrobial amino acid sequences, such as human lactoferrin (lactoferricin H) and kaliocin-1 (4, 33). The first reported bactericidal hLf-derived peptide was lactoferricin H (residues 1 to 47), obtained by enzymatic hydrolysis of lactoferrin, which exhibits antimicrobial activity greater than that of the intact protein (3, 4). Furthermore, a variety of shorter antimicrobial synthetic peptides (lactoferricins) derived from the original sequence of lactoferricin H have been described (3, 11, 20, 24, 25). For example, Lfpep (residues 18 to 40 of hLf) is a lactoferricin H derived-peptide that possess giardicidal and bactericidal effects and the ability to permeabilize bacterial membranes, thus leading to cell death (1, 31). We recently reported on a novel antifungal hLf-derived peptide (residues 153 to 183 of hLf), termed kaliocin-1. This peptide differs from lactoferricin H (Table 1) and exhibits a bactericidal effect at low salt concentrations without causing membrane permeabilization (33).

The aims of the present study were to determine the anti-Candida activities of two distinct human lactoferrin-derived synthetic peptides (Lfpep and kaliocin-1), as well as to conduct a comparative study of the optimal candidacidal conditions of both peptides and their effects on the cytoplasmic membrane of C. albicans cells.

MATERIALS AND METHODS

Materials. Recombinant human lactoferrin (rhLf) was provided by Ventria Bioscience (Sacramento, CA). Amphotericin B, fluconazole, and nystatin were obtained from Sigma (St. Louis, MO). 3,3-Dipentetoxycarbonylanine iodide (DiOC6(3)) and propidium iodide (PI) were purchased from Molecular Probes, Inc. (Eugene, OR). Bacto peptone, Sabouraud dextrose broth (SDB), and Sabouraud dextrose agar (SDA) were purchased from Difco (Detroit, MI).

Peptides. The human lactoferrin-derived peptides kaliocin-1 and Lfpep were synthesized according to standard 9-fluorenylmethoxy carbonyl chemistry by Bachem Bioscience (King of Prussia, PA).

Yeast strains and growth conditions. The strains used for determination of candidacidal activity included Candida albicans ATCC 10231 (American Type Culture Collection, Manassas, VA) and clinical isolates obtained from M. Alvarez (Hospital Universitario Central de Asturias, Oviedo, Spain). The cells were grown to stationary phase in SDB and subcultured (1:400) in the same medium to the mid-logarithmic growth phase at 37°C. After the blastoconidia were centrifuged and washed twice with the same buffer or medium used to perform the assay, their concentrations were adjusted as appropriate.

Candidacidal assays. The anti-Candida effects of the peptides were monitored by using cell suspensions (106 cells/ml) of C. albicans ATCC 10231 in 10 mM

* Corresponding author. Mailing address: Department of Functional Biology (Microbiology), Faculty of Medicine, University of Oviedo, C/ Julian Claveria, 6, 33006 Oviedo, Spain. Phone: 34-985-103643. Fax: 34-985-103533. E-mail: jffierro@uniovi.es.
† M.V.-D. and M.T.A. contributed equally to this report.
Tris-HCl (pH 7.4) incubated with the peptides at 37°C. At the indicated times after peptide addition, serial dilutions prepared in the same buffer were spread onto SDA plates. Colonies were counted after 24 h of incubation at 37°C, and the results were expressed as the percentages of the colony counts of bacteria not exposed to the peptides.

To determine the optimal pH value, candidacidal assays were performed in Tris buffer at different pHs (range, 5.5 to 7.5) by incubation of the cells with Lfpep (50 μM) or kaliocin-1 (150 μM) at 37°C for 1 h. Similar assays were performed in Tris buffer (pH 7.4) to determine the differences in the activity at 4°C and 37°C.

Broth microdilution assays. Cell suspensions (10⁵ cells/ml) of Candida spp. were suspended in PG broth (0.3% Bacto peptone, 2% glucose) in 96-well plates. The growth-inhibitory concentration was determined by using twofold serial dilutions of Lfpep (range, 0.6 to 75 μM), kaliocin-1 (range, 0.6 to 300 μM), rhLf (range, 0.125 to 25 μM), and magainin 2 (range, 0.6 to 150 μM). The plates were incubated at 37°C for 24 h, and the growth-inhibitory concentration was the one at which there was no visible growth. Resistance to fluconazole (≥2 μg/ml) and amphotericin B (>2 μg/ml) was determined in RPMI medium following CLSI (formerly NCCLS) guidelines (23).

Permeability of cytoplasmic membrane. The permeabilization of the membrane of C. albicans cells was investigated by flow cytometric analysis by using PI, as described previously (20). Cell suspensions (10⁵ cells/ml) in Tris-HCl (pH 7.4) were incubated at 37°C with the peptide, and samples taken at different times were incubated with 9 μM PI for 5 min. Cell fluorescence was recorded with a flow cytometer (Cytoron Absolute; Ortho Diagnostics Systems Inc., Raritan, NJ).

Measurement of ions. Determinations of K⁺ release were performed as described previously (2, 34). Briefly, cell suspensions were centrifuged (2,000 × g, 2 min) and then washed twice with ice-cold MgCl₂ (20 mM) containing an isosmotic concentration of sorbitol. The cells (10⁵ cells/ml) were resuspended in 5 mM sodium phosphate buffer (pH 7.4), and the peptides or nystatin (100 μg/ml) was added. The mixture was incubated at 37°C, samples (0.5 ml) were taken at different times (15, 30, 45, and 60 min) and centrifuged, and the supernatant was collected to determine the extracellular K⁺ content by flame photometry. The total K⁺ content of the cellular suspension was estimated after K⁺ was released from the cells by treatment with 0.5% (vol/vol) perchloric acid and heat (95°C for 1 h) and centrifugation to remove the cell debris. The supernatant was then analyzed for the intracellular K⁺ content. The percentages of K⁺ were calculated on the basis of the total cellular K⁺ content of untreated cells.

Measurement of electrical potential in C. albicans cells. Membrane potential was measured with the membrane potential-sensitive fluorescent probe DiOC₃(3), as described previously (17). Cells (10⁵ cells/ml) in Tris-HCl (pH 7.4) were incubated with the peptide at 37°C. The samples were reincubated for an additional 10 min with 0.5 μM DiOC₃(3) and were immediately analyzed by cytofluorometry.

Sequence analysis. Comparison of amino acid sequences was performed by using the ClustalW software (30). The similarity of the amino acid sequences was calculated by use of the program EMBoss-Align at the homepage of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/align).

Statistical analysis. The data are presented as the means ± standard errors of the means from at least three sets of independent experiments. The Mann-Whitney U test was performed by using GraphPad InStat v. 3.06 (GraphPad Software, San Diego, CA). A P value <0.05 was considered statistically significant.

RESULTS

Candidacidal activity. The susceptibility of C. albicans to both peptides was concentration and time dependent (Fig. 1A and B). The influence of NaCl on the candidacidal activities of both peptides was tested in 10 mM Tris-HCl buffer (pH 7.4) containing various concentrations of NaCl (Fig. 1C). The increase in the ionic strength of the buffer, in which the isomolarity was preserved by means of sorbitol, progressively de-

![FIG. 1. Candidacidal activities of kaliocin-1 and Lfpep. (A and B) Dose- and time-dependent reductions in the number of viable C. albicans cells (10⁵ cells/ml) incubated with kaliocin-1 (A) or Lfpep (B) in 10 mM Tris-HCl (pH 7.4) at 37°C. (C) Effect of NaCl on the candidacidal activities of 150 μM kaliocin-1 and 50 μM Lfpep tested for 1 h in 10 mM Tris-HCl (pH 7.4) containing different concentrations of NaCl. The percentage of viable cells was determined relative to that for cells incubated without peptides. The results are the means ± standard deviations of at least three separate experiments.](http://aac.asm.org/)
creased the candidacidal activity of kaliocin-1 (150 μM), which was totally inhibited by 30 mM NaCl. In contrast, a different pattern of inhibition was obtained for cells treated with Lfpep (50 μM). Candidacidal activity decreased with ≥60 mM NaCl, and maximum inhibition (100%) was observed with about 100 mM NaCl. The inhibitory effect of NaCl on the killing activity was not reversed by increased concentrations of kaliocin-1 (up to 300 μM) or Lfpep (up to 150 μM). In addition, the candidacidal effect of neither peptide was significantly modified in the presence of 0.32 M sorbitol (data not shown).

At pH 5.5, the lethal effect of Lfpep was inhibited by 17% ± 2% (n = 3) with respect to that observed at pH 7.5. The maximal killing activity of kaliocin-1 was observed at pH 5.5, showing an increase of 16% ± 2% (n = 5). The candidacidal activity of kaliocin-1, although not that of Lfpep, decreased slightly (11% ± 4%; n = 3) at 4°C.

Comparative growth inhibition. Concentrations over a range of 0.6 to 150 μM of kaliocin-1 were inactive against Candida spp. when standard MIC determinations were performed in RPMI 1640 or PG broth (containing 1% peptone) (data not shown). A similar inhibitory effect, caused by medium components, was previously observed when other antifungal peptides were tested and was solved by using non-standardized synthetic media (26, 27). However, these alternative media were not suitable for testing the susceptibility of the Candida strains to kaliocin-1 due to the presence of an inhibitory salt concentration. Consequently, the assays were performed in PG broth prepared with 0.3% peptone that contained low Na⁺ and K⁺ concentrations, as determined by flame photometry (data not shown). In this medium, the growth of all Candida strains was supported and the killing activity of the peptide was retained, thus allowing the comparative assessment of the in vitro antifungal efficacies of the peptides. Lfpep and kaliocin-1 were active against all Candida strains, including fluconazole- and amphotericin B-resistant clinical isolates (Table 2). Lfpep was more active than kaliocin-1 against C. albicans (8-fold) and Candida spp. (8- to 30-fold). The corresponding values for rhLf and the antimicrobial peptide magainin 2 are also reported for the sake of comparison.

To test the effect of the reduction of disulfide bonds on the antifungal activities of both peptides, similar assays in which 15 mM dithiothreitol (DTT) was added to the cell suspensions were conducted. The candidacidal activity of kaliocin-1 (upon 300 μM) was abolished in the presence of this reducing agent, whereas the activity of Lfpep was not modified significantly under similar conditions (Table 2). The cell viability was not decreased by the presence of 15 mM DTT alone.

Cytoplasmic membrane permeabilization. To determine the effects of kaliocin-1 and Lfpep on cell membrane integrity, C. albicans cells that had been treated or not treated with either of the peptides for different times (15, 30, 60, 90, and 120 min) were exposed to the fluorescent probe PI and analyzed by flow cytometry. PI fluorescently stains the nucleic acids of cells that suffer a loss of membrane integrity. Figure 2A shows typical results for C. albicans cells exposed to a candidacidal concentration of kaliocin-1 (150 μM) for 30 min, indicating the inability of this peptide to permeabilize the cytoplasmic membrane of C. albicans cells. Similar results were obtained after cell incubation with kaliocin-1 for 60, 90, and 120 min. In contrast, cells incubated with Lfpep (50 μM) showed a rapid increase in fluorescence due to intracellular PI accumulation, indicative of membrane permeabilization (Fig. 2A). A similar permeabilization was observed in control assays with the membrane-perturbing antifungal drug amphotericin B.

Potassium release. The ability of kaliocin-1 or Lfpep to disturb the integrity of the cytoplasmic membrane of C. albicans cells was also assessed by measuring the K⁺ released in cell suspensions exposed to candidacidal concentrations of these peptides. The K⁺ release induced by kaliocin-1 (150 μM) reached a maximal rate the first time tested (15 min) and was estimated to be 22% ± 2% (n = 6) of the total K⁺ content. However, C. albicans cells treated with Lfpep (50 μM) showed a rapid and higher rate of K⁺ release (93% ± 3%; n = 6) at the same time (15 min), a rate similar to that caused by nystatin (94% ± 1%; n = 4), used in control assays.

Effect of rhLf-derived peptides on the C. albicans membrane potential. Peptide-induced changes in membrane potential were measured with the potentiometric dye DiOC₃(3), a cationic membrane-permeant probe that accumulates inside the cell on polarized membranes. The fluorescence of the probe thus decreases upon membrane depolarization. Addition of candidacidal concentrations of kaliocin-1 (150 μM) or Lfpep (50 μM) to a C. albicans suspension caused an immediate decrease in fluorescence intensity, indicative of membrane depolarization. A similar decrease of the membrane potential

---

**TABLE 2. Susceptibilities of Candida spp. to lactoferrin-derived peptides, lactoferrin, and magainin 2**

<table>
<thead>
<tr>
<th>Yeast strain&lt;br&gt;</th>
<th>Growth-inhibitory concn (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lfpep</th>
<th>Lfpep&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Kaliocin-1</th>
<th>Kaliocin-1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>rhLf</th>
<th>Magainin 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans ATCC 11231</td>
<td>150</td>
<td>300</td>
<td>&gt;300</td>
<td>0.6</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans (AmB&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>150</td>
<td>300</td>
<td>&gt;300</td>
<td>0.6</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans (Flu&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>150</td>
<td>300</td>
<td>&gt;300</td>
<td>1.1</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. glabrata</td>
<td>150</td>
<td>300</td>
<td>&gt;300</td>
<td>1.1</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. guilliermondii</td>
<td>150</td>
<td>300</td>
<td>&gt;300</td>
<td>1.1</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. krusei</td>
<td>150</td>
<td>300</td>
<td>&gt;300</td>
<td>0.6</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>150</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>0.6</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>150</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>1.1</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Determined in PG broth (0.3% peptone, 2% glucose). The values were the result of at least three independent assays and never differed by more than 1 twofold dilution.
* AmB<sup>c</sup> and Flu<sup>c</sup>, resistance to amphotericin B and fluconazole, respectively.
* The determinations were performed in the presence of the reducing agent DTT (15 mM).
was observed at 15, 30, 60, 90, and 120 min. Figure 2B shows representative results obtained with cells exposed to the indicated candidacidal concentrations of both peptides and to amphotericin B (positive control) for 30 min.

DISCUSSION

Comparison of the killing activities of the peptides showed that Lfpep was more effective (8- to 30-fold) than kaliocin-1. Both peptides were effective against fluconazole- and amphotericin B-resistant clinical isolates of *C. albicans* but differed in their potencies. The different killing activities could be due to the greater positive net charge of Lfpep (+7) with respect to that of kaliocin-1 (+1), resulting in differential affinities for the binding site on cells. This assumption also seems to be supported by the different inhibition patterns of NaCl on the candidacidal activities exerted by these peptides, indicative of the involvement of charge interactions. Similar salt-sensitive antimicrobial activity has been reported for many antimicrobial peptides and proteins, including defensins, histatin 5, lactoferricin, LL-37, lactoferrin, and synthetic antimicrobial peptides such as novispirin G10 (4, 6, 13, 14, 19, 29, 33).

Although similar experimental low-salt conditions are not encountered in biological fluids, which brings into question the potential therapeutic application of these compounds, most peptides exhibit antimicrobial activities in vivo (24, 27). It will be necessary to perform in vivo assays with Lfpep and kaliocin-1 to determine their potential value as therapeutic agents.

Kaliocin-1 was inactive (at 300 μM) under reducing conditions, suggesting that the disulfide bridges have an important effect on the candidacidal activity of this peptide. However, the activity of Lfpep under similar reducing conditions was not changed, in agreement with previous reports showing that the absence of intramolecular disulfide bonds is not critical for the antimicrobial activities of lactoferricins (3). Work is being conducted with linearized synthetic peptides to determine the role of disulfide bonds on the antimicrobial activity of kaliocin-1.

We have previously shown that Lfpep and kaliocin-1 are able to permeabilize liposomes (1, 33) and planar membranes (unpublished data). Although this fact suggests that cell death might be a consequence of the disturbance of membrane functions, this mode of action is probably true only for Lfpep, due to the loss of cell viability correlated with the rapid intracellular accumulation of propidium iodide, high K⁺ release (~90%), and collapse of the membrane potential. These effects are similar to those previously reported for other lactoferricin peptides with permeabilizing activities (11). In contrast, the killing mediated by kaliocin-1 was not accompanied by membrane permeabilization for propidium iodide, and a lower percentage (~20%) of the intracellular K⁺ released was observed. Moreover, the dissociation between the loss of viability and membrane depolarization suggests that these effects are unrelated. Although we cannot draw a definitive conclusion with regard to the mechanism underlying the killing effect of kaliocin-1, the ability of this peptide to permeabilize artificial membranes (34), although not cell membranes, differs from that of pore-forming peptides and hypothetically suggests a different mechanism of action for kaliocin-1, such as the putative interaction of kaliocin-1 with a structural element of the membrane (e.g., protein), which induces K⁺ efflux and membrane depolarization, as was reported for the candidacidal peptide histatin 5 (17), among other possible mechanisms.

Interestingly, kaliocin-1 contains the recently reported amino acid sequence of the “multidimensional antimicrobial signature,” including the characteristic CXG motif (Table 3), which corresponds to a structure present only in cysteine-containing peptides that exhibit antimicrobial activity (36). Since this model may be used to predict the unforeseen antimicrobial activities of peptides from phylogenetically distant organisms,
the observed antibacterial (33) and antifungal activities of kaliocin-1 support that suggestion.

The structural and functional similarities between kaliocin-1 and brevinin-1, a family of natural antimicrobial peptides isolated from frog skin secretions (22), are also noteworthy. The primary structure of the brevinin-1 group is characterized by a sequence of 24 residues and the presence of a Cys motif located in the C terminus, This Cys motif was termed the “Rana box” or the “insect box” (10) due to the presence of homologous structures in antimicrobial peptides isolated from frog skin secretions (brevinins-2, esculentins-1, gaegurins, rana-lexin, ranatuerins, and rugosins) and insects (e.g., thanatin), respectively. These “boxes” are defined by two cysteines flanking a sequence of five (brevinins) or six (thanatin) amino acids characterized to include a central Thr or Ser residue (10).

Although kaliocin-1 is a human lactoferrin-derived peptide, it contains the “multidimensional antimicrobial signature” (36) and even the motif CXG and include a primary structure pattern similar to the “Rana box.” Moreover, kaliocin-1 particularly retains the cidal effect of lactoferrin only in a low ionic environment, as described for transferrins (33). Overall, these observations are compatible with our previous hypothesis in which we suggested that sequences homologous to the kaliocin-1 sequence present in transferrins may be involved in the well-known antimicrobial activities of these evolutionarily ancient proteins (33).

In conclusion, two novel human lactoferrin-derived peptides with different anti-Candida activities are described. These results suggest an attractive approach to the search for cell targets for antifungals and novel antimicrobial synthetic peptides containing conserved sequences throughout evolution.

ACKNOWLEDGMENT

This work was supported by the University of Oviedo (CN-96-133-BI/Laboratorio de Microbiología Oral).

REFERENCES


ACKNOWLEDGMENT

This work was supported by the University of Oviedo (CN-96-133-BI/Laboratorio de Microbiología Oral).

REFERENCES

1. Aguilerà, O., H. Ostolaza, L. M. Quíroz, and J. F. Fierro. 1999. Permea-


5. Devine, D. A. 2003. Antimicrobial peptides in defence of the oral and respira-


12. Divine, D. A. 2003. Antimicrobial peptides in defence of the oral and respira-


