AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Antifungal Mechanism of Action of Lactoferrin: Identification of H^+-ATPase (P_{3A}-Type) as a New Apoptotic-Cell Membrane Receptor

María T. Andrés,[a] Maikel Acosta-Zaldívar,[a,b] José F. Fierro[a,b]

Laboratory of Oral Microbiology, School of Stomatology, Faculty of Medicine, University of Oviedo, Oviedo, Spain[a]; Department of Functional Biology (Microbiology), Faculty of Medicine, University of Oviedo, Oviedo, Spain[b]

Human lactoferrin (hLf) is a protein of the innate immune system which induces an apoptotic-like process in yeast. Determination of the susceptibility to lactoferrin of several yeast species under different metabolic conditions, respiratory activity, cytoplasmic ATP levels, and external medium acidification mediated by glucose assays suggested plasma membrane Pma1p (P_{3A}-type ATPase) as the hLf molecular target. The inhibition of plasma membrane ATPase activity by hLf and the identification of Pma1p as the hLf-binding membrane protein confirmed the previous physiological evidence. Consistent with this, cytoplasmic ATP levels progressively increased in hLf-treated *Candida albicans* cells. However, oligomycin, a specific inhibitor of the mitochondrial F-type ATPase proton pump (mtATPase), abrogated the antifungal activity of hLf, indicating a crucial role for mtATPase in the apoptotic process. We suggest that lactoferrin targeted plasma membrane Pma1p H^+-ATPase, perturbing the cytoplasmic ion homeostasis (i.e., cytoplasmic H^+ accumulation and subsequent K^+ efflux) and inducing a lethal mitochondrial dysfunction. This initial event involved a normal mitochondrial ATP synthase activity responsible for both the ATP increment and subsequent hypothetical mitochondrial proton flooding process. We conclude that human lactoferrin inhibited Pma1p H^+-ATPase, inducing an apoptotic-like process in metabolically active yeast. Involvement of mitochondrial H^+-ATPase (nonreverted) was essential for the progress of this programmed cell death in which the ionic homeostasis perturbation seems to precede classical nonionic apoptotic events.

Lactoferrin (Lf) is a 77-kDa iron-binding glycoprotein of the transferrin family of proteins and an important effector molecule of innate immunity with antimicrobial activity in mammalian mucosal fluids (reviewed in reference 1). We have previously reported that human lactoferrin (hLf) induces apoptosis-like cell death of the pathogenic opportunistic yeast pathogen *Candida albicans*, demonstrating also that K^+ channel-mediated K^+ efflux is a common apoptotic event in yeast and metazoan cells (2). At present, the mechanism of action by which lactoferrin and other antimicrobial peptides induce apoptosis-like processes in yeast remains unknown (reviewed in reference 3). However, we have previously identified in bacteria the proton-translocating ATPase (H^+-ATPase) as the molecular target of lactoferrin, suggesting that the subsequent perturbation of the proton gradient (ΔpH) and intracellular pH (pH_i) will lead to cell death *in vitro* (4). This finding revealed lactoferrin as a natural extracellular inhibitor of the proton translocation mediated by H^+-ATPases, suggesting that the possible inhibition of a similar target also explains its antifungal activity. In this hypothetical case, the antifungal effect of lactoferrin might be due to altered pH homeostasis, as reported for bacteria (4).

In yeast, control of pH_i is critical for cell survival and is regulated through a concerted movement of protons out of the cytosol controlled by the P-type (i.e., Pma1p) and V-type (i.e., V-ATPase) H^+-ATPase pumps (H^+-ATPases), the major electrogenic pumps at the cytoplasmic and vacuolar membranes, respectively (5). Whereas Pma1p moves cytosolic protons out of the cell membrane, V-ATPase transfers protons into the vacuolar lumen. The pH_i is essential to protein folding and function, and proton gradients underlie the physiology of organelles such as mitochondria. Moreover, pH_i variations could be a trigger signal or constitute an optimal intracellular environment for proliferation, dimorphic switching, and virulence of pathogenic yeasts (5; reviewed in reference 6). Finally, pH_i decrease is an early apoptotic event observed in yeast and in the death receptor-mediated and mitochondrion-dependent apoptosis of higher eukaryotic cells (7, 8, 9; reviewed in reference 10).

The plasma membrane protein Pma1p (P_{3A}-type ATPase) is a single catalytic polypeptide (~100 kDa) that couples ATP hydrolysis to the expulsion of protons, generating an electrochemical proton gradient necessary for nutrient uptake and cellular ion balance (11, 12, 13, 14, 15, 16). This H^+-ATPase is a primary contributor to pH_i regulation and is crucial for cell survival, as shown by the potent fungicidal activity of certain Pma1p inhibitors. For example, the chemical drug omeprazole and some synthetic peptides which efficiently inhibit *C. albicans* Pma1p were proposed as new models to design more effective antifungal therapies (17, 18, 19; reviewed in reference 20). Besides this suggestion, H^+-ATPases have also emerged as potential therapeutic targets for treatments against bacterial infections. For example, bedaquiline, which belongs to the diarylquinoline class of drugs and efficiently inhibits the H^+-ATPase (F-type ATPase) of *Mycobacterium tuberculosis*, has been approved for treatment of multidrug-resistant tuberculosis (21). Despite the fact that Pma1p has

Received 2 February 2016 Returned for modification 24 April 2016
Accepted 27 April 2016
Accepted manuscript posted online 2 May 2016
Citation Andrés MT, Acosta-Zaldívar M, Fierro JF. 2016. Antifungal mechanism of action of lactoferrin: identification of H^+-ATPase (P_{3A}-type) as a new apoptotic-cell membrane receptor. Antimicrob Agents Chemother 60:4206–4216. doi:10.1128/AAC.03130-15.

Address correspondence to José F. Fierro, jffierro@uniovi.es.
M.T.A. and M.A.-Z. contributed equally to this work.

been validated as an antifungal molecular target, drugs based on inhibiting this H^+-ATPase, which is essential for yeast survival but is not present in human cells, have not yet been developed (17, 19, 20). Accordingly, the study of the possible interaction of lactoferrin with Pma1p could provide insight into strategies of the innate immunity for controlling pathogenic opportunists, which may be useful as a model for drug discovery and development of new therapeutic strategies. Moreover, the inhibition of fungal Pma1p H^+-ATPase, using lactoferrin or other specific inhibitors, became an attractive model to dissect apoptotic events in yeast.

In the present study, we have used classical cellular physiology and molecular approaches to assess whether Pma1p could be the molecular target of lactoferrin, thus representing the first cell surface receptor able to induce an apoptosis-like process in fungi. We also investigated intracellular mechanisms underlying this programmed cell death, which exhibit some apoptotic events similar to those characterized previously in higher eukaryotic cells (1, 7, 22).

MATERIALS AND METHODS

Materials. Recombinant human apo-lactoferrin was obtained from Ventria Bioscience (Sacramento, CA) and Sigma (St. Louis, MO). Goat anti-human lactoferrin biotinylated polyclonal antibody and mouse anti-Pma1p monoclonal antibody were obtained from Bethyl Laboratories (Montgomery, TX) and Acris Antibodies (Herford, Germany), respectively. Goat anti-mouse IgG secondary biotinylated antibody and anti-bovine serum albumin (BSA) antibody were purchased from Abnova Co. (Taipei, Taiwan). The ATPase assay kit and PiBin resin were purchased from Innova Biosciences (Cambridge, United Kingdom). The BacTiter-Glo microbial cell viability assay kit was purchased from Promega (Madison, WI). Annexin V-fluorescein isothiocyanate (FITC) was purchased from Thermo Fisher Scientific (Eugene, OR). All chemicals, unless otherwise noted, were supplied by Sigma-Aldrich. Nitrocellulose transfer membrane (0.45 μm) was obtained from Bio-Rad (Hercules, CA). Sabouraud-2% dextrose broth (SDB) was purchased from Difco Laboratories (Detroit, MI).

Strains and growth conditions. *C. albicans* ATCC 10231, *Candida glabrata* ATCC 2001, and *Saccharomyces cerevisiae* ATCC 7752 were routinely cultured in SDB for 16 to 20 h at 30°C and subcultured in SDB to mid-logarithmic growth phase. When required, *C. glabrata* and *S. cerevisiae* were grown under aerobiosis or anaerobiosis conditions in the presence of 2% glycerol or 2% glucose, respectively. The growth of yeast under strict anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) was performed in an anaerobic chamber (model 1024; Forma Scientific, Marietta, OH).

Fungicidal activity. Fungicidal activity was tested as previously described, except that Tris buffer (10 mM Tris-HCl, pH 7.4) was used (7).

Starved cells were obtained by maintaining washed cells for 18 h in Tris buffer in an anaerobic chamber to deplete their carbon reserves and the oxygen present in the buffer. The killing assays with starved cells or with cells grown under anaerobic conditions were also performed inside the anaerobic chamber. All the media and buffers used in anaerobiosis were previously placed in the anaerobic chamber for 24 h.

Oxygen consumption measurement. Oxygen consumption was measured using a Clark-type electrode (dual digital-model 20; Rank Brothers Ltd., Cambridge, United Kingdom) at 25°C. *C. albicans* cells were grown to mid-logarithmic phase in SDB at 30°C, washed twice in Tris buffer, and resuspended in the same buffer. The assays were performed in 1.5 ml of Tris buffer at 25°C. Cell suspensions (5×10^6 cells/ml) were preincubated for 15 min at 37°C with hLf (25 μM) or piericidin A (32 μM).

Isolation of respiration-deficient yeast cells. *C. albicans* cells defective in mitochondrial respiration were induced by growing the cells at 42°C to stationary phase in the presence of acriflavine (100 μg/ml), as described previously (23, 24). Aliquots of the culture were plated on glucose-limited agar plates, and single colonies were transferred with sterile toothpicks to glucose- and glycerol-limited agar plates, the latter strictly supporting cellular respiration (24). After 3 days of incubation at 30°C, these plates were examined for the presence of colonies able to grow solely on glucose plates. The sensitive colonies (petite mutants) growing only on the fermentable carbon source (glucose) were verified as respiration-deficient mutants by testing their respiratory function with a Clark-type electrode and by the method for the color-based identification of RD mutants. For the color-based identification of respiration-deficient mutants, *C. albicans* cells were grown on plates containing the indicator medium [1.5 g KH_2PO_4, 1.5 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot H_2O$, 1.5 g peptone, 1.5 g yeast extract, 20 g glucose, 0.01 g eosin Y, 0.01 g trypan blue, 15 g agar, 1 liter H_2O] as described previously (24).

Glucose-dependent external acidification. A glucose-induced acid extrusion assay was performed as described by Monk et al. (25), with slight modifications. Briefly, cultures of *C. albicans* cells were grown to mid-log phase in SDB medium and washed twice in 10 mM Tris-HCl, pH 8.0. The cells were resuspended in 50 mM KCl and stored for 18 h at 4°C to deplete their carbon reserves. To determine the external acidification, the cells were concentrated ($\sim 10^7$ cells/ml) in 50 mM KCl, and lactoferrin (25 μM) or oligomycin (64 μg/ml) was immediately added. Cells were incubated for 15 min at 30°C, and the pH was adjusted to 6.7. Acidification was initiated by addition of glucose (final concentration, 2.5 mM) to the cell suspension (1 ml), and the time course was monitored using a SevenMulti S50-K pH meter (Mettler-Toledo, Greifensee, Switzerland) with constant stirring. To measure the extent of acidification of the external medium, the assay was performed in the absence of lactoferrin or oligomycin (control).

Plasma membrane isolation. Yeast plasma membranes were obtained as described previously (26), with slight modifications. Briefly, *C. albicans* cells were grown in SDB at 30°C with vigorous aeration and harvested by centrifugation (3,500 × *g*, 10 min, 4°C) in the late-log phase of growth. The cell pellet was washed twice with distilled water, suspended in fresh homogenization buffer (2.5 mM EDTA, 1 mM ethylene glycol tetraacetic acid [EGTA], 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and 50 mM Tris-HCl, pH 7.5), and disrupted by two passages at 19,500 lb/in^2 through a French pressure cell (SLM Aminco, Silver Spring, MD). Cell debris and unbroken cells were removed by centrifugation (3,000 × *g*, 10 min), and the supernatant was further centrifuged (14,000 × *g* for 20 min at 4°C) to obtain a clarified homogenate. The homogenate was centrifuged at 105,000 × *g* for 2 h at 4°C, and the resulting preparation (pellet) containing membrane fragments was resuspended in ice-cold membrane suspension buffer (1 mM EDTA, 1 mM PMSF, 10 mM Tris-HCl, pH 7.5) and centrifuged a second time at 105,000 × *g* for 2 h at 4°C. The sample was resuspended in the same buffer, and protein concentration was determined before the sample was stored at −80°C.

Estimation of intracellular ATP concentration. Measurements of the average intracellular ATP concentration were made using the BacTiter-Glo microbial cell viability assay kit according to the manufacturer's instructions. Briefly, cells were grown to mid-log phase in SDB at 30°C, washed twice in 10 mM Tris-HCl (pH 7.4), and resuspended at 10^5 cells/ml. Aliquots (1 ml) were incubated (90 min) with lactoferrin (5 μM) or oligomycin (32 μg/ml), and then 100 μl of cell suspensions was collected at various time points and immediately heat inactivated. Twenty-five microliters of cell lysates was transferred into 96-well opaque white plates, mixed with an equal volume of the BacTiter-Glo reagent, and incubated for 15 min in the dark. The reagent uses luciferase to produce luminescence in an ATP-dependent manner. The emitted luminescence was detected by using a Varioskan flash multimode reader (Thermo Scientific, Waltham, MA) and was expressed as relative luminescence units. A calibration curve was prepared for each experiment with ATP standards ranging from 0.1 to 100 nM.

Measurement of ATP hydrolysis. ATP hydrolysis was determined using a colorimetric ATPase assay kit and PiBind resin according to the

manufacturer's recommendations. All enzyme assays were performed with plasma membranes at a protein concentration of 40 μg/ml with 2 mM ATP at 30°C and pH 6.5. The amount of inorganic phosphate (P_i) released was calculated by spectrophotometry (A_{650}). For all experiments, calibration was performed using a standard range of P_i concentrations. To eliminate possible contributions from acid phosphatase as well as mitochondrial and vacuolar ATPases, 0.2 mM $(NH_4)_2MoO_4$, 5 mM NaN_3, and 50 mM KNO_3 were present in the assay mix. Residual activity represented specific ATP hydrolysis by Pma1p, as it was more than 90% inhibited by 1 mM diethylstilbestrol (DES), an inhibitor of fungal Pma1p H^+-ATPase (27). To determine the effect of lactoferrin on ATPase activity, the plasma membranes were preincubated with the protein for 30 min in the assay medium before initiating the reaction.

Far-Western blotting and immunodetection assays. Far-Western blotting was used as described previously, with some modifications (28). Briefly, cytoplasmic membrane protein samples, hLf, and molecular mass markers were separated by electrophoresis on SDS-PAGE gels and then electroblotted (100 V, 1 h, 4°C) to a nitrocellulose transfer membrane in a wet transfer system (Bio-Rad, Hercules, CA). Electroblotting was performed in transfer buffer (24 mM Tris-HCl, 190 mM glycine, 1.3 mM SDS, and 20% methanol). Transfer efficiencies were checked, and protein wells and molecular weight standards were marked with a soft pencil directly on the membrane after Ponceau-S staining. Following removal of Ponceau-S by rinsing (2×) in TBS buffer (20 mM Tris-HCl, 130 mM NaCl, pH 7.6), the membrane was blocked in TBS containing 5% (wt/vol) BSA for 13 h at 4°C. Strips containing the transferred plasma membranes then were incubated with hLf (1 mg/ml) using the same buffer as the diluent (5% BSA in TBS). After 90 min, the membrane was carefully washed twice with 0.1% (vol/vol) Tween 20 in TBS. It then was incubated with the anti-hLf biotinylated antibody (1:1,000) for 1 h. The membrane was subsequently washed twice more with TBS containing 0.1% Tween 20. In separated membrane strips, Pma1p H^+-ATPase was identified by incubation (1 h) of the blotted membrane proteins with a monoclonal anti-Pma1p antibody (1:1,000). Similarly, hLf alone was detected by incubation of the corresponding strips with the anti-hLf biotinylated antibody. As a negative control, duplicate electroblotted plasma membrane proteins were probed with BSA and detected with the anti-BSA antibody. Secondary antibody incubation (1 h) was performed with IgG biotinylated antibody (1:1,000). The binding of biotinylated antibodies was visualized by addition of streptavidin-horseradish peroxidase (HRP) polymer conjugate (1:10,000 dilution) and 3,3′,5,5′-tetramethylbenzidine (TMB), a chromogenic substrate.

MS-MRM analysis. The proteomic analysis was performed in the Proteomics Unit (PRB2-ISCIII; ProteoRed) of Complutense University of Madrid. The gel slices containing previously immunodetected hLf-binding protein were reduced, carbamidomethylated, and then digested with trypsin as described previously (29). The multiple reaction monitoring (MRM) method used to detect and confirm the presence of the Pma1p protein was developed by performing *in silico* analysis to predict the resulting trypsin-digested peptides and by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) of the trypsin-digested protein. Skyline (v. 3.1) was used to predict and optimize collision energies for the detection of each peptide. The resultant method was exported to Analyst 1.6 (SCIEX) mass spectrometer acquisition software.

For the MRM assay, analyses were performed on an LC-MS/MS system, including an Eksigent nanoflow LC system coupled to a hybrid triple-quadrupole/ion trap mass spectrometer (QTRAP 5500; AB Sciex, Foster City, CA) equipped with a nanoelectrospray interface operated in the positive ion mode. The digested sample was directly loaded onto a NanoLC trap set (C_{18}, 3 μm, 120 Å; 350 μm by 0.5 mm; ChromXP) at 3 μl/min. After 5 min, the peptides were separated using an Eksigent column (75 by 15 cm; ChromXP NanoLC column) at 300 nl/min. Peptides were then electrosprayed into the QTRAP 5500, which was set to operate in the MRM mode. Data acquired were analyzed with Skyline software, and the MS/MS data were analyzed using ProteinPilot 5.0.1 (SCIEX) software to identify the peptides against DataBase with the FASTA sequence of the targeted protein. Peptide identifications were accepted if they could be established at a greater than 95% confidence interval ($P < 0.05$), and then they were used to generate a spectral library to ensure consistency between the transition and the sequences of peptide searched.

Test for apoptotic markers. Apoptotic markers, including annexin V-FITC labeling and the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) test, were performed with yeast cells after 2 h of hLf exposition as described previously (2).

Statistical analysis. Unless stated otherwise, all experiments were conducted in triplicate on separate occasions. Means and standard deviations (SD) were calculated and the Student *t* test performed to determine the significance of the data sets using the program GraphPad Prism v.6. A *P* value of ≤0.01 was considered significant.

RESULTS

The antifungal activity of hLf depends on the cells' energy metabolism. Previously, we have reported that the antimicrobial effect of lactoferrin involved an active cellular cooperation (4, 7). To examine the influence of energy metabolism on the antifungal effect of lactoferrin, we first performed killing assays in oxygenic and anoxic conditions (Fig. 1A). In the absence of oxygen, *C. albicans* cells (starved cells) were almost totally resistant (93% ± 7% cell survival) to different concentrations of lactoferrin (1.25, 2.5, and 5 μM), unlike nonstarved cells exposed to this protein under oxygenic conditions, which were susceptible in an hLf concentration-dependent way (68% ± 7%, 38% ± 4%, and 14% ± 6% cell survival). Increased resistance to lactoferrin under anoxic versus oxygenic conditions was also observed using *Candida glabrata* (88% ± 6%, 74% ± 8%, and 71% ± 9% versus 24% ± 4%, 17% ± 6%, and 4% ± 2% cell survival, respectively) and *Saccharomyces cerevisiae* cells (100%, 96% ± 4%, and 97% ± 3% versus 98% ± 2%, 52% ± 5%, and 36% ± 7% cell survival, respectively).

A second approach was used to assess the influence of the fermentative metabolism on the antifungal activity of lactoferrin. *C. albicans* is a Crabtree-negative species, a definition based on the observation that *C. albicans* and other yeast continue to respire in the presence of glucose (23, 30, 31). In contrast, the mitochondrial respiration of Crabtree-positive fungi (e.g., *C. glabrata* and *S. cerevisiae*) is repressed by fermentable substrates (32). The susceptibility of *C. albicans* cells to hLf was similar under respiratory and fermentative conditions obtained with cells grown in the presence of 2% glycerol (aerobiosis) or 2% glucose (anaerobic chamber), respectively (Fig. 1B). The percentages of viable cells exposed to 1.25, 2.5, and 5 μM hLf under fermentative conditions were 75% ± 6%, 50% ± 7%, and 12% ± 7% versus 57% ± 4%, 44% ± 12%, and 18% ± 5% of cell viability from assays performed in aerobiosis. In a similar way, Crabtree-positive yeasts performing oxygen respiration or fermentation showed a similar susceptibility to lactoferrin. Figure 1B shows data obtained from hLf-treated cells grown in the presence of a fermentation-inducing concentration of glucose as the sole carbon source (*C. glabrata*, 54% ± 6%, 32% ± 8%, and 12% ± 6% cell survival; *S. cerevisiae*, 84% ± 5%, 62% ± 3%, and 36% ± 8% cell survival) with respect to cells grown in the presence of glycerol (*C. glabrata*, 30% ± 5%, 22% ± 8%, and 17% ± 7% cell survival; *S. cerevisiae*, 100% ± 2%, 44% ± 7%, and 32% ± 4% cell survival).

The glycolysis inhibitor 2-deoxy-D-glucose (2-DG) was used to verify the dependence of hLf activity on the fermentative metabolism (Fig. 1B). *C. albicans* cells preincubated (30 min) with 10

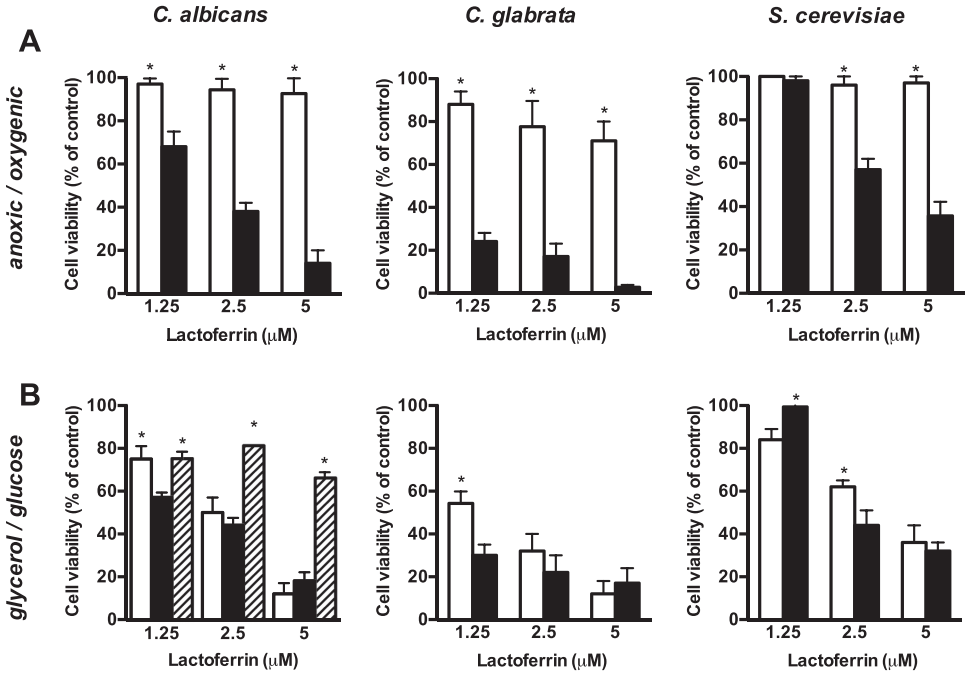


FIG 1 Influence of the energetic metabolism on the fungicidal activity of lactoferrin. (A) Viability of yeast cells (10^5 cells/ml) in Tris buffer incubated for 90 min at 37°C with three different concentrations of hLf under anoxic (white columns) and oxygenic (black columns) conditions. Assays under anoxic conditions were performed with starved yeast cells. (B) Viability of yeast cells grown in the presence of 2% glucose (fermentation) (white columns) or 2% glycerol (respiration) (black columns) incubated with hLf for 90 min at 37°C. *C. albicans* cells were incubated with the metabolic inhibitor 2-DG before the hLf exposition (dashed columns). Aliquots were plated and colonies were counted after 24 h. The results are the means ± SD from duplicates of at least three independent experiments. Statistical significance was assessed by Student's *t* test. *, $P < 0.01$.

mM 2-DG were less susceptible to 1.25, 2.5, and 5 μM lactoferrin (75% ± 6%, 81% ± 9%, and 66% ± 5% cell survival).

Programed cell death of hLf-treated cells was verified as previously described (2). After 2 h, samples of *C. albicans* cells incubated with lactoferrin exhibited peripheral fluorescence (annexin V-FITC) and TUNEL-positive phenotypes in 21% ± 5% and 48% ± 7% of the cells, respectively (data not shown). Less than 5% of lactoferrin-treated cells stained positively for propidium iodide, indicating a nonsignificant disruption of cytoplasmic membrane (data not shown).

Cellular respiration is not inhibited by lactoferrin. Oxygen utilization data obtained from cell suspensions exposed to a candidacidal concentration of lactoferrin (25 μM) showed a percentage of consumption of oxygen similar to that of the untreated cells (control) over a minimum of 30 min (Fig. 2A). In positive-control assays, cellular respiration was inhibited by 32 μM piericidin A, an inhibitor of the NADH dehydrogenase (complex I) of the respiratory chain.

Lactoferrin activity depends on cellular respiration. Two experimental approaches were used to confirm whether the activity of lactoferrin was dependent on respiratory cellular function. First, the candidacidal effect of lactoferrin was tested using *C. albicans* cells preincubated (15 min at 37°C) with 4 or 8 μM piericidin A. The piericidin A-treated cells, exposed for 90 min to different hLf concentrations (2.5 and 5 μM), were less susceptible to this protein (Fig. 2B). For example, cells preincubated with 8 μM piericidin A were less susceptible to 5 μM hLf (94% ± 5% cell survival) than cells not treated with piericidin A (17% ± 4% cell survival), which were used as a control (Fig. 2B). Second, the fungicidal activity of lactoferrin was assessed using a respiration-deficient (RD) mutant (Fig. 2C). Data from killing assays showed that this mutant strain was more resistant to hLf (2.5 and 5 μM) than the wild-type strain (100% and 97% ± 8% versus 29%± 9% and 19% ± 8% cell survival, respectively). The RD mutant strain was obtained by acriflavine exposition, and its respiration-deficient phenotype was demonstrated by (i) the absence of oxygen consumption by cell suspensions measured using a biological oxygen monitor (Fig. 2D), (ii) the deep-violet color of RD mutant colonies grown on indicator medium (Fig. 2E), and (iii) the absence of growth in medium containing glycerol as a nonfermentable carbon source (Fig. 2F).

Mitochondrial H^+-ATPase is essential for the antifungal activity of lactoferrin. Previous reports have demonstrated that mitochondria play an essential role in other apoptosis-like processes of yeast, as occurs in higher eukaryotic cells (33, 34, 35). To determine the role of mitochondria in the hLf-induced apoptosis, mitochondrial function was inhibited with different concentrations (4, 8, 16, and 32 μg/ml) of oligomycin, a specific inhibitor of mitochondrial F_1F_0-ATPase (mtATPase), in the presence of lactoferrin. Figure 3 shows that the preincubation (15 min) of *C. albicans* cells with oligomycin (4 μg/ml) markedly decreased the antifungal activity of lactoferrin (1.25, 2.5, and 5 μM). Inhibition (72% ± 4% cell survival) was obtained in the presence of 5 μM hLf and oligomycin (4 μg/ml), and similar percentages of inhibition were obtained with 8 to 32 μg/ml oligomycin (data not shown).

In yeast, vacuolar H^+-ATPase (V-type ATPase) contributes to vacuolar and cytosolic pH control and is susceptible to the mac-

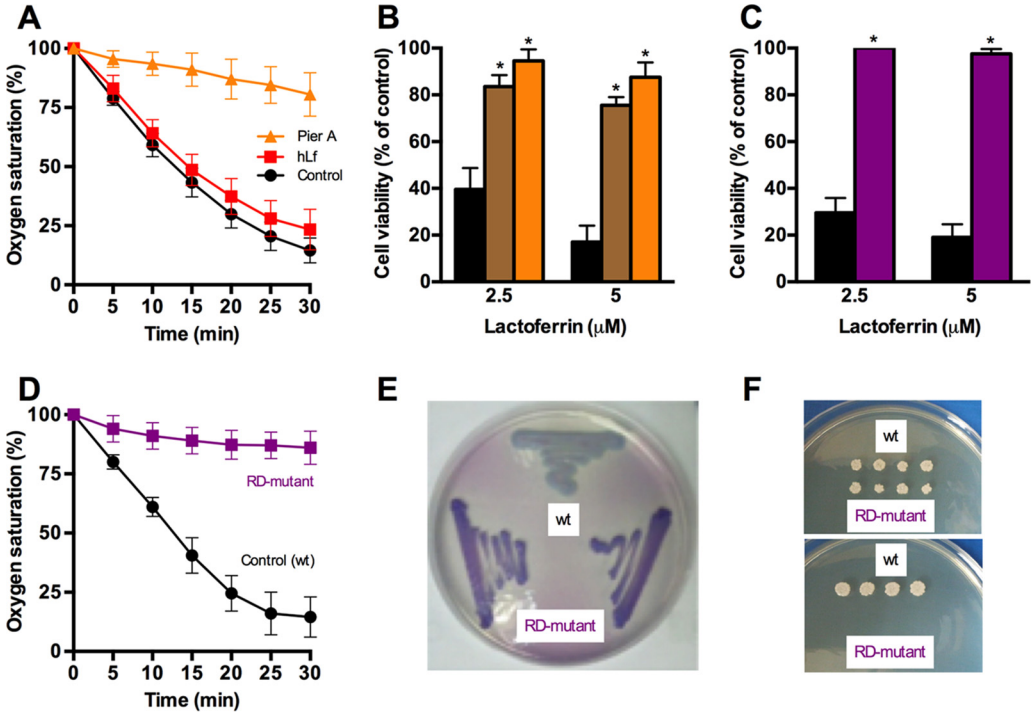


FIG 2 Effect of cellular respiration on the candidacidal activity of lactoferrin. (A) Consumption of oxygen in *C. albicans* cells. Black, control cells; orange, cells incubated with 32 μM piericidin A; red, cells treated with 25 μM hLf. The respiration inhibitor piericidin A was added 15 min before the addition of lactoferrin. (B) Viability of *C. albicans* cells treated with different concentrations of hLf (black) or preincubated with 4 μM (brown) or 8 μM (orange) piericidin A before the addition of hLf. (C) Susceptibility to hLf of wild-type (black) and respiration-deficient mutant (RD mutant) cells (purple). (D) Comparison of percentage of O_2 consumption by wild-type (black) and RD mutant (purple) strains. (E) Color differentiation of wild-type (wt) and RD mutant strains on an indicator plate containing eosin and trypan blue. On this medium, metabolically active wild-type cells were grown as pale-bluish colonies, while the RD mutant strains were grown as deep-violet colonies. (F) Growth characteristics of wild-type (wt) and RD mutant strains on agar plates. Cells were incubated on glucose-limited agar plates (upper) or on glycerol-limited agar plates (lower) for 72 h. The results are the means ± SD from duplicates of at least three independent experiments. Statistical significance was assessed by Student's *t* test. *, $P < 0.01$.

rolide antibiotic bafilomycin A_1 (5). Bafilomycin A_1 is a highly specific inhibitor of V-ATPase at nanomolar concentrations, but P-ATPases are also sensitive to micromolar concentrations (36). Low concentrations of bafilomycin A_1 (1, 10, and 100 nM) slightly increased the candidacidal activity of lactoferrin. Figure 3 shows the effect of the lowest drug concentration (1 nM) assayed in the presence of lactoferrin. A similar increased killing effect was observed with 10 and 100 nM bafilomycin A_1 (data not shown).

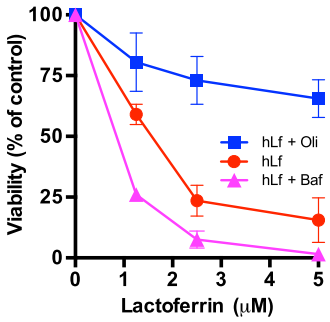


FIG 3 Influence of mitochondrial and vacuolar H^+-ATPases on candidacidal activity of lactoferrin. Viability of *C. albicans* cells (10^5 cells/ml) treated with different concentrations of hLf (red) for 90 min at 37°C or preincubated for 15 min with 4 μg/ml oligomycin (Oli; blue) or 1 nM bafilomycin A1 (Baf; magenta) before the addition of hLf. Aliquots were plated and colonies were counted after 24 h. The results are the means ± SD from duplicates of at least three independent experiments.

Intracellular ATP concentration is increased in hLf-treated cells. Intracellular ATP content was measured luminometrically to determine whether the cell death induced by hLf was an energy-dependent process, as apparently suggested by the protective effect of oligomycin. Interestingly, *C. albicans* cells treated with 5 μM hLf resulted in a time-dependent incremental change of the ATP concentration, displaying a more than 6-fold increase (90 min) compared with control cells (Fig. 4). Oligomycin (32 μg/ml) abrogated the increment of intracellular ATP induced by hLf, indicating the mitochondrial origin of the ATP accumulated in the hLf-treated cells. In parallel, the viability of hLf-treated cells decreased in a time-dependent way, showing a direct relationship between the accumulation of ATP and the loss of cell viability (Fig. 4). In addition, the increase of ATP content in hLf-treated cells indicated that hLf was unable to reach the mtATPase of complete cells.

Lactoferrin interacts with Pma1p H^+-ATPase. The data obtained with *C. albicans* cells indicated that neither mitochondrial nor vacuolar H^+-ATPases of complete cells were inhibited by lactoferrin. Consequently, we assumed that plasma membrane H^+-ATPase is the molecular target of lactoferrin, and different assays were performed with *C. albicans* cells and isolated plasma membranes to assess this assumption.

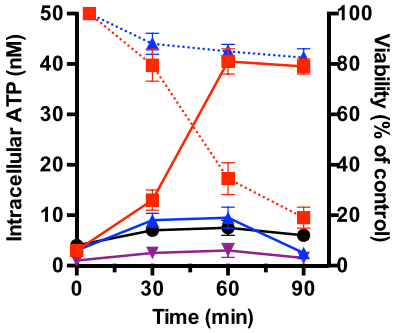


FIG 4 Kinetics of intracellular ATP content and viability of hLf-treated cells. Measurement of intracellular ATP levels of cells of *C. albicans* resuspended in Tris buffer incubated for 90 min at 37°C (black; control) or incubated with 5 μM hLf (continuous red line), 32 μg/ml oligomycin (purple), an inhibitor of mitochondrial F_1F_0-ATPase (mtATPase), or oligomycin and lactoferrin (continuous blue line). Cell viability of the cell suspensions treated with hLf in the absence (dashed red line) or in the presence (dashed blue line) of oligomycin was determined using a plate count method as described in Materials and Methods. Data are the means (±SD) from at least three experiments.

(i) Lactoferrin inhibits the glucose-induced acidification of external medium. To test whether lactoferrin interacts with the plasma membrane Pma1p H^+-ATPase of *C. albicans* cells, the effect of this protein on the glucose-stimulated acidification of unbuffered medium (50 mM KCl) was monitored. In *Candida* cells depleted of their carbon sources, the addition of glucose induces a proton-carbohydrate symport driven by the proton motive force (PMF). The PMF is generated by the proton gradient maintained by the Pma1p H^+-ATPase, which couples the hydrolysis of ATP to the outward translocation of protons. The extrusion of protons that previously had entered the cytosolic compartment together with the glucose is the main cause of the external acidification and can be measured with a pH electrode (15, 18, 25, 37).

Figure 5 shows that the addition of glucose to glucose-starved cell suspensions caused a rapid fall (1.37 ± 0.07 pH units in 15 min) in the external pH (control assay). However, a significant inhibition (0.14 pH ± 0.04 pH units in 15 min) of glucose-induced acidification was observed in cell suspensions treated with 25 μM lactoferrin. As expected, oligomycin (64 μg/ml) had no influence on the external acidification promoted by the presence of glucose (Fig. 5). Under these experimental conditions (50 mM KCl), cell viability was unaltered by hLf (data not shown), as previously reported (7). In these assays and before the addition of glucose, a slow cellular H^+ influx was manifested by a slow increase in medium pH. The addition of lactoferrin did not change the rate of this influx, excluding the possibility that lactoferrin increased the passive permeability of cell membranes to protons (data not shown). This observation agrees with our previous reports showing that lactoferrin is unable to permeabilize the cell plasma membrane (7). It was assumed that the cells used in these assays were efficiently starved of glucose because they showed no basal medium acidification properties but rapidly acidified the medium following addition of glucose, as previously described (25).

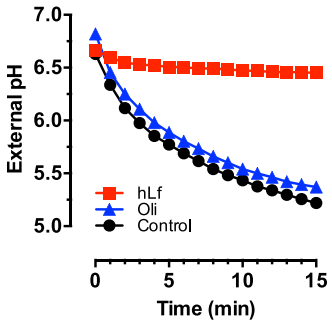


FIG 5 Effect of lactoferrin on glucose-dependent external acidification. The proton pumping activity of *C. albicans* was determined by monitoring glucose-induced acidification of the external medium by measuring the pH by means of an electrode as described in Materials and Methods. Starved cells (10^7 cells/ml) resuspended in 50 mM KCl were preincubated with 25 μM lactoferrin (red) or 64 μg/ml oligomycin (blue) for 15 min. Glucose (final concentration of 2.5 mM) then was added to induce the proton efflux mediated by the Pma1p H^+-ATPase, as indicated by the external acidification. The glucose-induced acidification of the external medium without hLf or oligomycin was measured in control experiments (black line). Only the mean data ($n = 3$) are shown, and the bars representing ± standard errors (coefficient of variation of <10%) are omitted for clarity.

TABLE 1 Effect of lactoferrin on plasma membrane ATPase activity

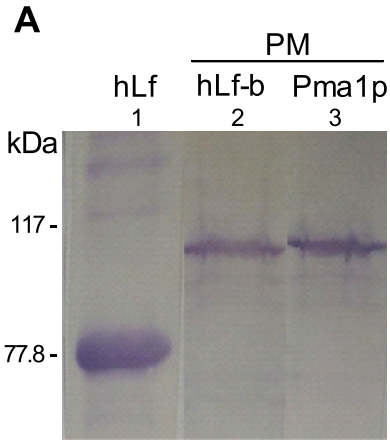
Treatment	ATPase activity[a] (%)
Control	1.73 ± 0.27 (100)
DES (1 mM)	0.15 ± 0.03* (8.6)
Lactoferrin	
5 μM	0.77 ± 0.02* (44.5)
10 μM	0.64 ± 0.04* (36.9)
25 μM	0.55 ± 0.07* (31.7)

[a] ATPase activity of isolated plasma membranes expressed as μmol P_i min^{-1} (mg protein)$^{-1}$. Values in parentheses are percentages of activity with respect to the control. ATPase activity in the presence or absence of lactoferrin was assayed in a buffer containing 0.2 mM $(NH_4)_2MoO_4$, 5 mM NaN_3, and 50 mM KNO_3 to inhibit acid phosphatase and mitochondrial and vacuolar ATPases, respectively. Residual activity represents specific ATP hydrolysis by the Pma1p H^+-ATPase, as it was more than 90% inhibited by 1 mM diethylstilbestrol (DES). Values are means ± SDs from at least three experiments. *, $P < 0.01$.

This result strongly suggested an inhibition of the plasma membrane Pma1p H^+-ATPase by lactoferrin.

(ii) Inhibition of the plasma membrane ATPase activity by lactoferrin. To determine the possible interaction of lactoferrin with Pma1p H^+-ATPase, ATP hydrolysis catalyzed by the membrane fraction of *C. albicans* cells in the presence or the absence of lactoferrin was investigated. In the presence of vacuolar and mitochondrial ATPase inhibitors, plasma membrane preparations were preincubated with lactoferrin or left untreated, and the amounts of inorganic phosphate (P_i) released were quantified. In the absence of lactoferrin (control), residual activity [1.73 ± 0.27 μmol P_i min^{-1} (mg protein)$^{-1}$, $n = 3$] represented specific ATP hydrolysis by the Pma1p H^+-ATPase, as it was more than 90% inhibited by 1 mM diethylstilbestrol (DES). Table 1 shows that the ATPase activity was progressively inhibited when plasma membrane samples were preincubated with increasing lactoferrin concentrations before initiating the reaction with ATP. The initial rate of ATPase activity significantly decreased to 44.5%, 36.9%, and 31.7% in the presence of 5, 10, and 25 μM lactoferrin, respectively.

(iii) Identification of the lactoferrin binding protein as Pma1p H^+-ATPase. To identify the lactoferrin-binding protein(s), cytoplasmic membrane preparations were submitted to far-Western blot analysis.



B

Peptide sequence	MW precursor	m/z	Transitions No.	MS/MS (dotp)
AVPEELLQTDPR	1367.71	984.36 (+2)	6	No
VGLTDDEVTK	1076.54	538.77 (+2)	5	Yes (0.92)
TLANSALVVR	1043.62	522.31 (+2)	5	Yes (0.89)
VIEFQPFDPVSK	1405.73	703.37 (+2)	7	Yes (0.79)
VIEFQPFDPVSK	1405.73	469.25 (+3)	6	No
GAPLFVLK	844.52	422.76 (+2)	4	Yes (0.97)
QLGLGTNIYDADR	1435.71	718.36 (+2)	5	No
YNAVEILQSR	1192.53	596.81 (+2)	5	Yes (0.86)
ADTGIAVEGATDAAR	14717.7	709.35 (+2)	4	No
SAADIVFLAPGLSAIIDALK	1985.13	662.38 (+3)	7	Yes (0.66)

FIG 6 Identification of the plasma membrane lactoferrin-binding protein of *C. albicans*. (A) Far-Western blot of interaction between lactoferrin and an ~100-kDa protein. Partial SDS-PAGE images show the protein bands corresponding to the bands detected by far-Western blotting. Lane 1, human lactoferrin (hLf). Lane 2, plasma membrane (PM) proteins incubated with hLf. hLfb, hLf bound to an ~100-kDa protein. Lane 3, PM proteins incubated with anti-Pma1p. Far-Western membranes were stripped and challenged with either anti-hLf biotinylated antibody (lanes 1 and 2) or mouse anti-Pma1p monoclonal antibody (lane 3). The location of anti-Pma1p using a secondary antibody (IgG biotinylated antibody) is shown. Binding of biotinylated antibodies was visualized by addition of streptavidin-HRP polymer conjugate and a chromogenic substrate. (B) Identification of the plasma membrane hLf-binding protein by LC-MS/MS MRM. Nine trypsin peptides were detected by LC-MS/MS analysis corresponding to the *C. albicans* protein Pma1p (UniProt entry P28877; PMA1_CANAX). MW, molecular weight; *m/z*, experimentally determined mass-to-charge ratio; dotp, dot product.

Human lactoferrin and cytoplasmic membrane proteins were resolved by gel electrophoresis and transferred to a nitrocellulose membrane. This membrane was cut into three strips (Fig. 6), corresponding to human lactoferrin (lane 1) and plasma membranes of *C. albicans* cells (lanes 2 and 3). While all of the proteins blotted to the nitrocellulose membrane were readily detected by Ponceau staining, only an ~100-kDa protein band was visualized in the strip containing plasma membranes that were previously incubated with hLf and detected with an anti-hLf biotinylated antibody (lane 2). A band with a similar molecular mass (~100 kDa) was immunodetected using the monoclonal anti-Pma1p antibody in an independent strip (lane 3). Blotted lactoferrin (control) was visualized as a unique ~80-kDa stained band on a separate strip (lane 1). This result strongly suggested that the identified hLf-binding protein corresponded to Pma1p H^+-ATPase. Nonspecific binding of the streptavidin-HRP polymer conjugate or BSA was not detected in duplicate control blotted membrane proteins (strips) probed only with the labeled streptavidin or with the anti-BSA antibody. Lactoferrin binding to immobilized plasma membrane protein was detected using an anti-human lactoferrin antibody and a secondary anti-hLf biotinylated antibody.

The identity of the ~100-kDa protein targeted by lactoferrin in the immunodetection assays was determined by performing an MRM-MS analysis. The MRM-MS methodology provides the ability to rapidly search for candidate peptides obtained from the mass fingerprints that match with the target of interest.

Twelve peptides, with parent and daughter ions, were included in the MRM method. Nine of these possible peptides corresponding to Pma1p were detected using a QTRAP 5500 mass spectrometer, and six of them were confirmed by MS/MS sequence identification. Figure 6B shows the peptides detected and identified by MRM-MS. Although two peptides typically are sufficient to confidently identify a protein using MRM-MS, six matching peptides (70 amino acids) were conclusively obtained ($P < 0.05$), indicating high confidence in the identity of the ~100-kDa protein as Pma1p H^+-ATPase (PMA1_CANAX; UniProt entry P28877).

DISCUSSION

In this study, we present data from a variety of cellular and molecular techniques supporting the idea that the plasma membrane Pma1p H^+-ATPase is targeted by human lactoferrin, inducing an apoptotic-like process previously described in yeast (2). First, the susceptibility to lactoferrin was tested using three yeast species under different metabolic conditions to elucidate whether the killing activity was an energy-dependent process or a disturbance of the cellular ion homeostasis. The latter was reported in bacteria where the blocking effect of lactoferrin on H^+-ATPase induced an imbalance of the proton gradient and a lethal pH_i perturbation (4).

Lactoferrin was active on three different species of yeast (*C. albicans*, *C. glabrata*, and *S. cerevisiae*) under respiratory or fermentative conditions but was almost inactive on starved cells. Under aerobic conditions, the candidacidal effect of lactoferrin was a respiratory-dependent process, as indicated by the decreased activity of this protein in the presence of piericidin A, an inhibitor of type I NADH dehydrogenase of *Candida* spp. Similarly, a respiratory-deficient mutant strain showed high resistance to lactoferrin. These results indicated that mitochondrial respiration was an important function for the antifungal activity of lactoferrin, suggesting a key role of these organelles in this apoptotic-like process, as was previously observed in apoptotic higher eukaryotic cells (33, 35). However, the oxygen consumption of hLf-treated cells was unmodified with respect to control cells, indicating that components of the respiratory chain were not targeted by lactoferrin.

The antifungal activity was almost abolished using starved cells, suggesting that the candidacidal effect of lactoferrin was an energy-dependent process requiring ATP consumption. This was supported by the fact that oligomycin, an inhibitor of mitochondrial F_1F_0-ATPase (mtATPase), and 2-deoxy-D-glucose (2-DG), an inhibitor of the glycolytic pathway, mitigated the killing activity of lactoferrin on respiring and fermenting cells, respectively. In contrast, the cytoplasmic ATP level of respiring *C. albicans* cells incubated with lactoferrin progressively and significantly in-

creased with respect to the control, indicating that, as was found in bacteria, the cidal activity of this protein was not an energy-consuming process but required an active cellular metabolism (7). Furthermore, a similar increment of cytosolic ATP levels in apoptosis of higher eukaryotic cells was previously reported and was even interpreted as a prerequisite for the progression of the apoptotic process (38, 39). Previous reports suggested that partial inhibition of plasma membrane Pma1p H^+-ATPase increases mitochondrial F_1F_0-ATPase activity (40). Similarly, the increment of synthesized ATP observed in hLf-treated yeast cells is in agreement with those observations, suggesting the inhibition of the Pma1p H^+-ATPase.

Taken together, all of the above-described observations were compatible with an ion homeostasis disturbance mediated by an active metabolism rather than an energy-dependent process. Moreover, this idea was congruent with the previously reported K^+ efflux, pH_i decrease, and observed changes of transmembrane electrical potential ($\Delta\psi$) in hLf-treated *C. albicans* cells (2, 7, 22). Since Pma1p H^+-ATPase is the main cellular component responsible for the regulation of pH_i and for $\Delta\psi$ maintenance in fungi, all of the above-described results were again in accordance with the possible involvement of this proton pump in the apoptotic-like process induced by lactoferrin.

In bacteria, two representative members of the transferrin family of proteins, lactoferrin and transferrin, target H^+-ATPase. Consequently, the proton gradient and pH_i are perturbed, leading to cell death (4). Despite the structural differences between the plasma membrane H^+-ATPases of bacteria (F-type ATPases) and fungi (P-type ATPases), they share an ancestral origin, and some amino acid sequences have been conserved throughout evolution (41). Therefore, we hypothesized that plasma membrane Pma1p H^+-ATPase is a target for lactoferrin that is accessible on the cell surface of *C. albicans* cells, and this assumption was assessed experimentally.

In yeast, the measurement of the glucose-induced acidification of the external medium is used for the determination of the activity of Pma1p H^+-ATPase of complete cells and to identify drugs that may inhibit this proton pump (15, 18, 25, 37). In the presence of lactoferrin, a clear inhibition of the H^+-ATPase-mediated acidification of the external medium by *C. albicans* cells was observed, supporting the hypothesis that Pma1p is the cellular target of this protein. As expected, oligomycin alone was unable to inhibit the extracellular glucose-induced acidification, a result which agreed with the known oligomycin resistance of fungal P-type and V-type ATPases. This result suggested that oligomycin, which inhibited the candidacidal activity of lactoferrin, and lactoferrin targeted different types of H^+-ATPases. Moreover, the protector effect of oligomycin on the candidacidal activity of lactoferrin again supported the idea that the mitochondrial F_1F_0-ATPase (mtATPase) was involved in the apoptosis-like process triggered by lactoferrin. Recently, we reported a similar involvement of mtATPase in the apoptosis-like process induced by hLf of *S. cerevisiae* cells (42).

To validate the above-described physiological data supporting Pma1p H^+-ATPase as the target of lactoferrin, evidence of the ability of lactoferrin to interact with this H^+-ATPase was provided by the inhibitory effect of this protein on the ATPase activity of plasma membrane fractions of *C. albicans* cells. This interaction also was suggested by immunodetection methods showing the binding of lactoferrin to an ~100-kDa protein. The electrophoretic migration of this plasma membrane protein was similar to that of Pma1p detected with a monoclonal antibody specific for this H^+-ATPase. Finally, this protein was identified by MRM-MS analysis as Pma1p, confirming the previous physiological evidence. To the best of our knowledge, other natural proteins or peptides with inhibitory activity on Pma1p H^+-ATPase have not been described previously.

In mammalian and yeast cells, a causal relationship between the F_1F_0-ATPase function and execution of the apoptotic mitochondrial cell death pathway has been previously demonstrated, but the role of the mitochondrial F_1F_0-ATPase in this process remains unclear (reviewed in reference 34). A reverse operation of the F_1F_0-ATPase proton pump, effluxing protons, and consuming ATP in apoptotic yeast cells expressing the mammalian proapoptotic Bax protein has been hypothesized (8, 34, 35). However, in our case, the progressive increase of the ATP content in hLf-treated cells with respect to the control, which was abrogated by oligomycin, suggested that mitochondrial F_1F_0-ATPase function was not reverted. Interestingly, hLf-treated viable cells increased in the presence of either oligomycin or piericidin A, showing a correlation between mtATPase activity and candidacidal effect. We interpret this result as a consequence of the ability of these drugs to directly or indirectly interrupt the flow of protons via mtATPase to the mitochondrial matrix.

More work needs to be done to elucidate the mechanism by which yeast undergo apoptosis induced by Pma1p inhibition. In order to integrate the present results with previously reported results (2, 7, 22), we propose a hypothetical mechanism for the apoptosis-like process induced by lactoferrin (Fig. 7). Briefly, the inhibition of Pma1p H^+-ATPase will cause two types of intracellular events, including (i) cytoplasmic ionic events, where the accumulation of cytoplasmic protons (7), consequence of an active metabolism, and the subsequent pH_i decrease will promote the K^+ channel-mediated K^+ efflux and the membrane depolarization previously reported (2, 7). In this model, cytoplasmic acidification will be the first intracellular apoptotic event leading to (ii) the mitochondrial ionic events, where the previous cytoplasmic K^+ efflux could facilitate the K^+ efflux from the mitochondrial matrix, for example, through the mitochondrial K^+/H^+ antiporter (43). This will facilitate the acidification of the mitochondrial matrix, because the mitochondrial effluxed K^+ could be replaced by massive H^+ reentry via mtATPase (ATP synthase), establishing two coupled cytoplasmic and mitochondrial K^+/H^+-positive feedback loops. The supposed intramitochondrial acidification (mitochondrial proton flooding) will be a second and critical triggering signal leading to the subsequent nonionic steps of apoptotic cell death. Evidence implicating the dysregulation of cellular pH as an early event for the induction of apoptosis involving mitochondria has been reported (reviewed in reference 8).

Under our experimental conditions, the cell is unable to use alternative mechanisms for proton homeostasis. Consequently, the inability to extrude protons across Pma1p H^+-ATPase leads to cytoplasmic acidification, which will be the first triggering signal of apoptosis induced by lactoferrin. Accordingly, the increased candidacidal activity of lactoferrin in the presence of bafilomycin A_1, an inhibitor of the vacuolar H^+-ATPase (V-type ATPase), could be due to the additional cytoplasmic accumulation of protons which were not pumped to the vacuole of bafilomycin-treated cells, leading to an acceleration of the cell death process.

Lactoferrin seems to interact specifically with at least two different proton pump classes (F- and P-type ATPases) of the ATPase

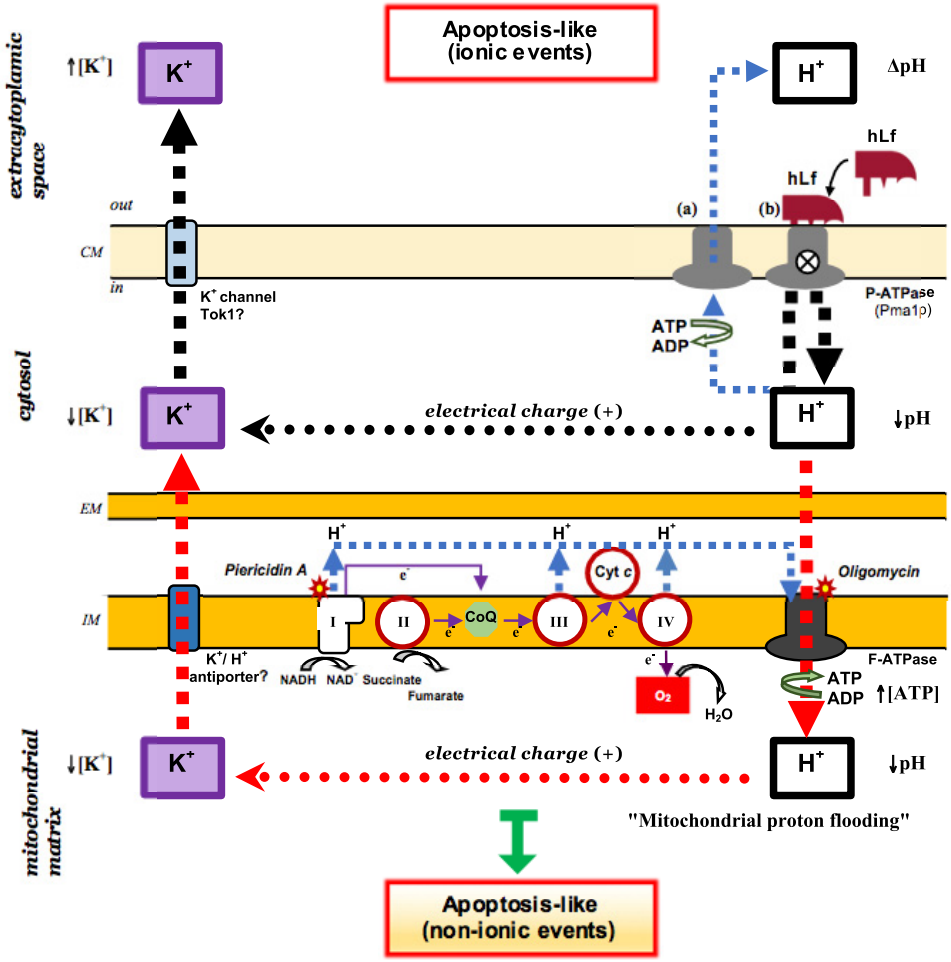


FIG 7 Schematic diagram illustrating the mechanism by which lactoferrin hypothetically induces an apoptosis-like process in yeast. Previously reported data on hLf-induced cell death have been incorporated into this model (2, 7, 22). In *C. albicans* cells, proton pumping through the Pma1p H^+-ATPase (a) is essential for generation of a proton gradient (ΔpH) across the cytoplasmic membrane (CM) and for pH homeostasis, which is critical for cell survival. Under our experimental conditions, the blocking effect of hLf on Pma1p H^+-ATPase (b) seems to induce a lethal perturbation in ionic homeostasis in two hypothetical coupled phases. Phase 1 is cytoplasmic ionic events, such as the intracellular accumulation of protons generated by an active metabolism due to the blocking effect of hLf on Pma1p H^+-ATPase. To balance the electrical charge, accumulated protons pull potassium ions outside the cell through K^+ channels. These previously reported ion-mediated events (2, 7, 22) will lead to phase 2, mitochondrial ionic events, where the previous cytoplasmic K^+ efflux could facilitate a loss of mitochondrial potassium ions and the simultaneous reemplacement of mitochondrial potassium ions by protons entering via mtATPase, as suggested by incremental ATP synthesis. The coupled cytoplasmic and mitochondrial K^+/H^+ loops are indicated by the black and red thick arrows, respectively. Supporting this hypothesis, cellular protection was observed when the accumulation of protons in the mitochondrial matrix via mtATPase was prevented by (i) inhibition of the mtATPase with oligomycin and (ii) inhibition of proton translocation mediated by the respiratory chain, as suggested by the results obtained using piericidin A, anoxic conditions, or a respiration-deficient mutant. The hypothetical mitochondrial proton flooding process mediated by mtATPase could trigger the subsequent nonionic apoptotic events.

family (4). Therefore, the presence of a common structural motif in these ATPases with affinity for a specific region of lactoferrin can be postulated. This suggestion implies the existence in lactoferrin, and probably other related members of the transferrin family of proteins, of a complementary structure which would interact with the predicted motif of H^+-ATPases. Supporting this, we have previously identified a structural domain in lactoferrin that could interact with the postulated H^+-ATPase-interacting region (44, 45, 46). This hLf region, when synthesized as a peptide (hLf UniProt entry P02788|171-201; kaliocin-1), retained the antimicrobial activity of lactoferrin under similar experimental conditions (45). Indeed, kaliocin-1 exhibited high identity and homology to the antimicrobial multidimensional signature named gamma-core (γ-core), an evolutionarily well-conserved structural motif found in all natural antimicrobial Cys peptides (46, 47). Moreover, the γ-core motif of lactoferrin also includes the *Rana* box and CXG motifs previously identified in other natural antimicrobial peptides, adding support to this structure being a candidate for the interaction of lactoferrin with H^+-ATPases (45). Studies are in progress to identify this putative receptor region of the ATPase family and to verify the hypothesis that the identified γ-core motif is the complementary lactoferrin-ligand region.

The induction of fungal apoptosis by different external inducers (i.e., peptides and chemical-apoptotic triggers) have been reported, but the intracellular mechanisms have not been elucidated (reviewed in references 3 and 48). To our knowledge, the plasma

membrane Pma1p H^+-ATPase is the first cell membrane apoptotic receptor identified in yeast. The hLf-induced programmed cell death shows striking similarities to the intrinsic mitochondrial apoptosis type (intrinsic pathway) described for higher eukaryotic cells due to mtATPase being essential for progression to cell death (34). These results illustrate the potential application of lactoferrin and other selective Pma1p H^+-ATPase inhibitors as molecular tools to gain insight into fungal apoptotic-intracellular events. Furthermore, the identification of the fungal target of lactoferrin confirms previous proposals of Pma1p H^+-ATPase as an antifungal target of therapeutic interest (19, 20) and may improve our knowledge on the defensive role of this effector molecule of innate immunity in the context of host-parasite interactions.

ACKNOWLEDGMENTS

We are indebted to Carlos Bousoño (HUCA, Oviedo) for support and stimulating discussions.

FUNDING INFORMATION

Funding was provided by Fondo de Investigaciones Sanitarias and Fundación Sira Carrasco para la Ayuda a la Fibrosis Quística (grants FIS PI05-2588 and SV-03-FSCARRASCO to J.F.F.), as well as Servicio para el Control de la Esterilización (grant CN-04-005 to M.T.A. and M.A.-Z.). M.A.-Z. was a Research Fellow of the University of Oviedo (UNOV-10-BECDOC; 2011 to 2014).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. **García-Montoya IA, Cendón TS, Arévalo-Gallegos S, Rascón-Cruz Q.** 2012. Lactoferrin a multiple bioactive protein: an overview. Biochim Biophys Acta **1820:**226–236. http://dx.doi.org/10.1016/j.bbagen.2011.06.018.
2. **Andrés MT, Viejo-Díaz M, Fierro JF.** 2008. Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of K^+-channel-mediated K^+-efflux. Antimicrob Agents Chemother **52:**4081–4088. http://dx.doi.org/10.1128/AAC.01597-07.
3. **Choi H, Lee W, Lee DG.** 2013. A new concept on mechanism of antimicrobial peptides: apoptosis induction, p 775–785. *In* Méndez-Vilas A (ed), Microbial pathogens and strategies for combating them: science, technology and education, vol 2. Formatex Research Center, Badajoz, Spain.
4. **Andrés MT, Fierro JF.** 2010. Antimicrobial mechanism of action of transferrins: selective inhibition of H^+-ATPase. Antimicrob Agents Chemother **54:**4335–4342. http://dx.doi.org/10.1128/AAC.01620-09.
5. **Martínez-Muñoz GA, Kane P.** 2008. Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. J Biol Chem **283:**20309–20319. http://dx.doi.org/10.1074/jbc.M710470200.
6. **Orij R, Brul S, Smits GJ.** 2011. Intracellular pH is a tightly controlled signal in yeast. Biochim Biophys Acta **1810:**933–944. http://dx.doi.org/10.1016/j.bbagen.2011.03.011.
7. **Viejo-Díaz M, Andrés MT, Fierro JF.** 2004. Modulation of *in vitro* fungicidal activity of human lactoferrin against *Candida albicans* by extracellular cation concentration and target cell metabolic activity. Antimicrob Agents Chemother **48:**1242–1248. http://dx.doi.org/10.1128/AAC.48.4.1242-1248.2004.
8. **Matsuyama S, Llopis J, Deveraux QL, Tsien RY, Reed JC.** 2000. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. Nat Cell Biol **2:**318–325. http://dx.doi.org/10.1038/35014006.
9. **Sokolov S, Knorre D, Smirnova E, Markova O, Pozniakovsky A, Skulachev V, Severin F.** 2006. Ysp2 mediates death of yeast induced by amiodarone or intracellular acidification. Biochim Biophys Acta **1757:**1366–1370. http://dx.doi.org/10.1016/j.bbabio.2006.07.005.
10. **Lagadic-Gossmann D, Huc L, Lecureur V.** 2004. Alterations of intracellular pH homeostasis in apoptosis: origins and roles. Cell Death Diff **11:**953–961. http://dx.doi.org/10.1038/sj.cdd.4401466.
11. **Axelsen KB, Palmgren MG.** 1998. Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol **46:**84–101. http://dx.doi.org/10.1007/PL00006286.
12. **Bublitz M, Morth JP, Nissen P.** 2011. P-type ATPases at a glance. J Cell Sci **124:**2515–2519. http://dx.doi.org/10.1242/jcs.088716.
13. **Hubbard MJ, Surarit R, Sullivan PA, Sheppherd MG.** 1986. The isolation of plasma membrane and characterisation of the plasma membrane ATPase from the yeast *Candida albicans*. Eur J Biochem **154:**375–381. http://dx.doi.org/10.1111/j.1432-1033.1986.tb09408.x.
14. **Serrano R.** 1988. Structure and function of proton translocating ATPase in plasma membranes of plants and fungi. Biochim Biophys Acta **947:**1–28. http://dx.doi.org/10.1016/0304-4157(88)90017-2.
15. **Serrano R, Kielland-Brandt MC, Fink GR.** 1986. Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+, K^+), K^+- and Ca^{2+}-ATPases. Nature **319:**689–693. http://dx.doi.org/10.1038/319689a0.
16. **Monk BC, Kurtz MK, Marrinan JA, Perlin DS.** 1991. Cloning and characterization of the plasma membrane H^+-ATPase from *Candida albicans*. J Bacteriol **173:**6826–6836.
17. **Monk BC, Niimi K, Lin S, Knight A, Kardos TB, Cannon RD, Parshot R, King A, Lun D, Harding DRK.** 2005. Surface-active fungicidal D-peptide inhibitors of the plasma membrane proton pump that block azole resistance. Antimicrob Agents Chemother **49:**57–70. http://dx.doi.org/10.1128/AAC.49.1.57-70.2005.
18. **Soteropoulos P, Vaz T, Santangelo R, Paderu P, Huang DY, Tamás MJ, Perlin DS.** 2000. Molecular characterization of the plasma membrane H^+-ATPase, an antifungal target in *Cryptococcus neoformans*. Antimicrob Agents Chemother **44:**2349–2355. http://dx.doi.org/10.1128/AAC.44.9.2349-2355.2000.
19. **Yatime L, Buch-Pedersen MJ, Musgaar M, Morth JP, Lund Winther AM, Pedersen BP, Olesen C, Andersen JP, Vilsen B, Schiøtt B, Palmgren MG, Møller JV, Nissen P, Fedosova N.** 2009. P-type ATPases as drug targets: tools for medicine and science. Biochim Biophys Acta **1787:**207–220. http://dx.doi.org/10.1016/j.bbabio.2008.12.019.
20. **Perlin DS, Seto-Young D, Monk BC.** 1997. The plasma membrane H^+-ATPase of fungi. A candidate drug target? Ann N Y Acad Sci **834:**609–617. http://dx.doi.org/10.1111/j.1749-6632.1997.tb52330.x.
21. **Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V.** 2005. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science **307:**223–227. http://dx.doi.org/10.1126/science.1106753.
22. **Viejo-Díaz M, Andrés MT, Fierro JF.** 2004. Effects of human lactoferrin on the cytoplasmic membrane of *Candida albicans* cells related with its candidacidal activity. FEMS Immunol Med Microbiol **42:**181–185. http://dx.doi.org/10.1016/j.femsim.2004.04.005.
23. **Aoki S, Ito-Kuwa S.** 1982. Respiration of *Candida albicans* in relation to its morphogenesis. Plant Cell Physiol **23:**721–726.
24. **Gyurko C, Lendenmann U, Troxler RF, Oppenheim FG.** 2000. *Candida albicans* mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5. Antimicrob Agents Chemother **44:**348–354. http://dx.doi.org/10.1128/AAC.44.2.348-354.2000.
25. **Monk BC, Mason AB, Abramochkin G, Haber JE, Seto-Young D, Perlin DS.** 1995. The yeast plasma membrane proton pumping ATPase is a viable antifungal target. I. Effects of the cysteine-modifying reagent omeprazole. Biochim Biophys Acta **1239:**81–90.
26. **Manavathu EK, Dimmock JR, Vashishtha SC, Chandrasekar PH.** 1999. Proton-pumping-ATPase-targeted antifungal activity of a novel conjugated styryl ketone. Antimicrob Agents Chemother **43:**2950–2959.
27. **Serrano R.** 1988. H^+-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. Methods Enzymol **157:**533–544. http://dx.doi.org/10.1016/0076-6879(88)57102-1.
28. **Edmondson DG, Dent SYR.** 2001. Identification of protein interactions by Far Western analysis. Curr Protoc Protein Sci **25:**19.7.1–19.7.10.
29. **Sechi S, Chait BT.** 1998. Modification of cysteine residues by alkylation. A tool in peptide mapping and protein identification. Anal Chem **70:**5150–5158.
30. **Helmerhorst EJ, Stan M, Murphy MP, Sherman F, Oppenheim FG.** 2005. The concomitant expression and availability of conventional and alternative, cyanide-insensitive, respiratory pathways in *Candida albicans*. Mitochondrion **5:**200–211. http://dx.doi.org/10.1016/j.mito.2005.04.001.

31. **Niimi M, Kamiyama A, Tokunaga M.** 1988. Respiration of medically important *Candida* species and *Saccharomyces cerevisiae* in relation to glucose effect. J Med Vet Mycol **26:**195–198. http://dx.doi.org/10.1080/02681218880000271.
32. **De Deken RH.** 1966. The Crabtree effect: a regulatory system in yeast. J Gen Microbiol **44:**149–156.
33. **Pereira C, Silva RD, Saraiva L, Johansson B, Sousa MJ, Côrte-Real M.** 2008. Mitochondria-dependent apoptosis in yeast. Biochim Biophys Acta **1783:**1286–1302. http://dx.doi.org/10.1016/j.bbamcr.2008.03.010.
34. **Matsuyama S, Xu Q, Velours J, Reed JC.** 1998. The mitochondrial F_0F_1-ATPase proton pump is required for function of the proapoptotic protein Bax in yeast and mammalian cells. Mol Cell **1:**327–336. http://dx.doi.org/10.1016/S1097-2765(00)80033-7.
35. **Matsuyama S, Reed JC.** 2000. Mitochondria-dependent apoptosis and cellular pH regulation. Cell Death Diff **7:**1155–1165. http://dx.doi.org/10.1038/sj.cdd.4400779.
36. **Bowman EJ, Siebers A, Altendorf K.** 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells and plant cells. Proc Natl Acad Sci U S A **85:**7972–7976. http://dx.doi.org/10.1073/pnas.85.21.7972.
37. **Northrop FD, Ljubojevic S, Davies JM.** 1997. Influence of Na^+ and anions on the dimorphic transition of *Candida albicans*. Microbiology **143:**3757–3765. http://dx.doi.org/10.1099/00221287-143-12-3757.
38. **Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P.** 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med **185:**1481–1486. http://dx.doi.org/10.1084/jem.185.8.1481.
39. **Zamaraeva RZ, Sabirov E, Maeno Y, Ando-Akatsuka SV, Bessonova SV, Okada Y.** 2005. Cells die with increased cytosolic ATP during apoptosis: a bioluminescence study with intracellular luciferase. Cell Death Diff **12:**1390–1397. http://dx.doi.org/10.1038/sj.cdd.4401661.
40. **Olsen LF, Andersen AZ, Lunding A, Brasen JC, Poulsen AK.** 2009. Regulation of glycolytic oscillations by mitochondrial and plasma membrane H^+-ATPases. Biophys J **96:**3850–3861. http://dx.doi.org/10.1016/j.bpj.2009.02.026.
41. **Morsomme P, Slayman CW, Goffeau A.** 2000. Mutagenic study of the structure, function and biogenesis of the yeast plasma membrane H^+-ATPase. Biochim Biophys Acta **1469:**133–157. http://dx.doi.org/10.1016/S0304-4157(00)00015-0.
42. **Acosta-Zaldívar M, Andrés MT, Rego A, Pereira CS, Fierro JF, Côrte-Real M.** 2016. Human lactoferrin triggers a mitochondrial- and caspase-dependent regulated cell death in *Saccharomyces cerevisiae*. Apoptosis **21:**163–173. http://dx.doi.org/10.1007/s10495-015-1199-9.
43. **Villalobo A, Briquet M, Goffeau A.** 1981. Electrogenic proton ejection coupled to electron transport through the energy-conserving site 2 and K^+/H^+ exchange in yeast mitochondria. Biochim Biophys Acta **637:**124–129. http://dx.doi.org/10.1016/0005-2728(81)90217-6.
44. **Viejo-Díaz M, Andrés MT, Pérez-Gil J, Sánchez M, Fierro JF.** 2003. Potassium efflux induced by a new lactoferrin-derived peptide mimicking the effect of native human lactoferrin on the bacterial cytoplasmic membrane. Biochemistry (Moscow) **68:**217–227. http://dx.doi.org/10.1023/A:1022657630698.
45. **Viejo-Díaz M, Andrés MT, Fierro JF.** 2005. Different anti-*Candida* activities of two human lactoferrin-derived peptides, Lfpep and kaliocin-1. Antimicrob Agents Chemother **49:**2583–2588. http://dx.doi.org/10.1128/AAC.49.7.2583-2588.2005.
46. **Yount NY, Andrés MT, Fierro JF, Yeaman MR.** 2007. The gamma-core motif correlates with antimicrobial activity in cysteine-containing kaliocin-1 originating from transferrins. Biochim Biophys Acta **1768:**2862–2872. http://dx.doi.org/10.1016/j.bbamem.2007.07.024.
47. **Yount NY, Yeaman MR.** 2004. Multidimensional signatures in antimicrobial peptides. Proc Natl Acad Sci U S A **101:**7363–7368. http://dx.doi.org/10.1073/pnas.0401567101.
48. **Madeo F, Herker E, Wissing S, Jungwirth H, Eisenberg T, Fröhlich KU.** 2004. Apoptosis in yeast. Curr Opin Microbiol **7:**655–660. http://dx.doi.org/10.1016/j.mib.2004.10.012.