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Human Lactoferrin Induces Apoptosis-Like Cell Death in *Candida albicans*: Critical Role of K^+-Channel-Mediated K^+ Efflux[▽]

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Human lactoferrin (hLf) induced an apoptosis-like phenotype in *Candida albicans* cells, which includes phosphatidylserine externalization, nuclear chromatin condensation, DNA degradation, and increased reactive oxygen species (ROS) production. Intracellular ROS accumulation was seen to correlate with candidacidal activity in hLf-treated cells. Mitochondrial activity was involved as indicated by mitochondrial depolarization and increased hLf resistance of cells preincubated with sordarin or erythromycin, the latter of which inhibits protein synthesis in mitoribosomes. Interestingly, Cl^-- and K^+-channel blockers prevented the hLf antimicrobial activity, but only when cells were pretreated with the blocking agent (tetraethylammonium) prior to the hLf-induced K^+-release period. These results indicate for the first time that K^+-channel-mediated K^+ efflux is required for the progression of apoptosis-like process in yeast, suggesting that this essential apoptotic event of higher eukaryotes has been evolutionary conserved among species ranging from yeasts to humans.

Candida albicans is a human pathogen that causes a range of opportunistic superficial infections and life-threatening systemic infections in immunocompromised patients (37, 49). Nevertheless, in most healthy human hosts this yeast is a commensal microorganism that survives on mucosal surfaces under the concerted control of local physicochemical factors, microbiota, and the innate and adaptive defenses of the host (13). Mucosal fluids include innate defense compounds such as antimicrobial peptides to control microbial colonization and prevent mucosal tissue invasion (9, 35).

Lactoferrin is a protein present in all mammalian mucosal secretions that exhibits antifungal and antibacterial activities (12). Although the antimicrobial activity of lactoferrin on *C. albicans* cells (candidacidal effect) has been clearly demonstrated, the mechanism by which this protein causes cell death has been elusive (12). We have previously reported the influence of extracellular cation concentration and cell metabolic activity on the candidacidal effect of human lactoferrin. Moreover, cytosolic acidification, K^+ efflux, changes in cytoplasmic membrane potential, membrane integrity preservation, and the protective effect of anaerobic conditions were also observed in *C. albicans* cells exposed to lactoferrin (45, 46). All of these features are usually associated with programmed cell death in higher-eukaryote cells and fungi (7, 20, 31).

The first evidence of apoptotic-like cell death in fungi was reported by Madeo et al. (29). These authors described the presence of apoptotic markers previously observed in higher-eukaryote apoptotic cells, including phosphatidylserine externalization, accumulation of reactive oxygen species (ROS), chromatin condensation, and nuclease-mediated strand DNA breakage. Furthermore, similar apoptotic markers were observed in response to different external chemical and physical stimuli and in aging yeast cells (19, 24, 27, 31). The similarities between the apoptotic events in metazoan and yeast cells and the ability of certain proapoptotic and antiapoptotic mammalian genes to induce or inhibit this cell death process in yeast suggested the involvement of analogous apoptotic pathways in fungal and animal cells (6, 30, 47).

Although fungal apoptosis was first observed in saprophytic yeast and filamentous fungi, Phillips et al. (38) also reported this cell death process in the pathogenic yeast *C. albicans* in response to environmental stress (H_2O_2 and acetic acid) or amphotericin B exposure. The discovery that pathogenic yeast can undergo apoptosis posed the appealing prospect of augmenting the activity of antifungal agents through the induction of apoptosis-like cell death in medically relevant fungi. Moreover, it might constitute a useful way to counteract the problem of emerging resistance to conventional antifungals detected over the past two decades (36).

The similarity of certain morphological and physiological features associated with lactoferrin-induced cell death in *C. albicans* cells with others reported in apoptotic cell death prompted us to investigate whether an apoptosis-like process occurs in lactoferrin-treated *C. albicans* cells. The possible involvement of the major hallmarks of apoptosis in higher-eukaryotic cells and yeast, such as ROS production, mitochondrial changes, and the role of K^+-channel-mediated K^+ efflux, was evaluated.

MATERIALS AND METHODS

Materials. Recombinant human lactoferrin (rhLf) was obtained from Ventria Bioscience (Sacramento, CA). *N*-Acetyl-L-cysteine (NAC), 4-aminopyridine (4-AP), 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), erythromycin, gadolinium (III) chloride hexahydrate (Gd^{3+}), glusulase, lyticase, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), menadione, nystatin, propidium iodide (PI), sodium hypochlorite, sordarin, and tetraethylammonium (TEA) were obtained from Sigma-Aldrich (St. Louis, MO). Annexin V-fluorescein isothiocyanate (FITC), DAPI (4′,6′-diamidino-2-phenylindole), dihydrorhodamine (DHR-123), and rhodamine 123 (Rh-123) were purchased from Invitrogen/Molecular Probes

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(Eugene, OR). L-[4,5-^{3}H]leucine was obtained from Amersham Radiochemicals (Amersham, United Kingdom). Sodium dithionite was purchased from Merck (Darmstadt, Germany). All of the other chemicals used were of analytical grade.

Culture conditions. *C. albicans* ATCC 10231 was routinely cultured in Sabouraud–2% dextrose broth (SDB; Difco) at 30°C for 16 to 20 h and subcultured in SDB to mid-logarithmic growth phase at 30°C unless stated otherwise. For the expression of superoxide dismutase (SOD) genes in response to induced oxidative stress, exponentially grown cells were incubated at 30°C for 1 h with menadione as previously described (23). To determine the sordarin inhibitory effect on protein synthesis, cells were grown to stationary phase in a defined medium (pH ~6.8) supplemented with arginine at 25°C (25). To verify the erythromycin inhibitory effect on mitoribosomes, cells were cultured in SDB at stationary phase and subcultured in the same medium containing erythromycin (10 mg/ml) at 30°C for 24 h (14).

Antifungal activity. The candidacidal activity of rhLf was tested as we have described previously (45). *C. albicans* cells were harvested by centrifugation, washed in 5 mM potassium phosphate buffer (PPB; K_2HPO_4-KH_2PO_4 [pH 7.4]) and resuspended in the same buffer at a concentration of 10^5 cells/ml. For ROS inhibition assays, cell suspensions were preincubated with the antioxidant NAC at 37°C for 10 min, as described previously (33). To block ion channels, the cells were preincubated in PPB (pH 7.4) with either DIDS, Gd^{3+}, NPPB, TEA, or 4-AP at 37°C for 15 min as described by Baev et al. (1), with slight modifications. All of the above preincubated cell suspensions were then incubated with 5 μM rhLf at 37°C for 90 min and plated onto Sabouraud agar. The plates were incubated at 30°C for 24 to 48 h, and the colonies were then counted to determine the number of viable cells. The viability values were calculated as percentage of the control (untreated cells) and represent the mean ± the standard deviation of at least three independent experiments. The MICs of sordarin and erythromycin were determined by the broth microdilution procedure recommended by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (34). Sordarin stock solutions (2 mg/ml) were prepared in sterile distilled water immediately before use.

Test for apoptotic markers. DNA strand breaks in protoplasts were analyzed by the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) method. *C. albicans* cells grown to mid-logarithmic phase were collected by centrifugation and washed in 5 mM PPB (pH 7.4). The cells (10^7 cells/ml) were then suspended in sorbitol buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM K_2HPO_4, 30 mM 2-mercaptoethanol [pH 6.8]) and digested with lyticase (50 U/ml) and glusulase (5.5%) in the same buffer (30 min, 30°C) to digest the cell wall. The cells were washed twice in 5 mM PPB containing sorbitol (1.2 mM) and fixed in 3.7% (vol/vol) formaldehyde. The DNA ends were then labeled by using an in situ cell death detection kit (POD; Roche Applied Science) essentially as described by Phillips et al. (38).

Nuclear fragmentation was analyzed as described previously (30). For nuclear staining, cells were washed with PPB, resuspended in 70% (vol/vol) ethanol for fixation and permeabilization, and incubated with 1 μg of DAPI/ml for 10 min.

Annexin V-FITC labeling was performed as described previously (29, 38). Fixed cells were washed with PPB and protoplasts were obtained as described above. The protoplasts were washed in annexin binding buffer (10 mM HEPES [pH 7.4], 40 mM NaCl, 50 mM $CaCl_2$, 1.2 M sorbitol), and annexin V binding assays were performed in the same buffer by the addition of 20 μl of annexin V-FITC/ml (29, 38). The cells were incubated (20 min) with PI (20 μg/ml) and washed in annexin binding buffer.

Intracellular ROS production was determined by incubation of cells (10^5 cells/ml; 10 min) with 5 μg of DHR-123/ml before the end of each experiment. Samples were quantitatively analyzed by flow cytometry (Cytomics FC500; Beckman Coulter, Inc., California) with excitation and emission wavelengths of 488 and 525 nm, respectively. The data were recorded as histograms of fluorescence intensity versus counted events.

For quantitative assessment of stained cells (TUNEL, DAPI, annexin V, and DHR-123), at least 300 cells per sample were examined by confocal microscopy (Leica TCS SP2 AOBS; Leica Microsystems, Heidelberg, Germany), and the results were expressed as the averages (± the standard deviation) of two or more treatments.

Measurement of mitochondrial electrical potential. Mitochondrial electrical potential ($\Delta\Psi_m$) changes were assessed by using the membrane potential-dependent distributional probe Rh-123 as described previously (17, 30). Briefly, *C. albicans* cells (10^6 cells/ml) were incubated in the dark with 10 μM Rh-123 for 10 min at 37°C. After incubation, cells were centrifuged, washed in PPB, and incubated with rhLf (5 μM) in the same buffer, and the fluorescence quenching of Rh-123 was measured by flow cytometry with excitation at 480 nm and emission at 530 nm.

Measurement of protein synthesis in *C. albicans* cells. The procedure described by Domínguez et al. (10) was followed with slight modifications. Briefly, cells grown to mid-logarithmic growth phase in defined medium (25) supplemented with arginine were harvested by centrifugation and resuspended (5×10^5 cells/ml) in the same medium without leucine. Aliquots (0.5 ml) of this cell suspension with or without sordarin (1 μg/ml) were incubated at 37°C for 30 min in the presence of 5 μCi of [^{3}H]leucine/ml. Aliquots were removed at regular intervals and pipetted into tubes containing trichloroacetic acid (15% [vol/vol]) and unlabeled leucine (50 μg/ml). The tubes were maintained for 15 min in a boiling water bath and finally collected on glass fiber filters. The filters were dried, and then the accumulated radioactivity was measured.

Cytochrome analysis. The cytochrome pattern was analyzed as described previously (14). Cells incubated in the presence or the absence of erythromycin were harvested by centrifugation in the late log phase of growth, washed with PPB (pH 7.4), and resuspended in the same buffer. The cell suspension (4×10^9 cells/ml) was divided equally, and half was oxidized by resuspension in 20 ml of 0.3% (vol/vol) sodium hypochlorite. These cells were harvested by centrifugation and resuspended in 50% (vol/vol) glycerol. The remaining sample (2×10^9 cells/ml) was resuspended in 50% glycerol containing a few crystals of sodium dithionite to reduce the cytochromes. Reduced-oxidized differential spectra were measured by using a Shimadzu (model UV-1700) double-beam spectrophotometer over 500 to 650 nm.

SOD activity assay. Exponentially grown cells, incubated at 30°C for 1 h with or without menadione, were harvested by centrifugation and washed with 50 mM potassium phosphate buffer (pH 7.8). Cell suspensions (40 ml) of the same buffer were disrupted by two passages at 20,000 lb/in^2 through a French pressure cell FA-031 (SLM Aminco, Silver Spring, MD). After cell disruption, unbroken cells and debris were removed by centrifugation at 4°C (20 min at 36,000 × *g*). Protein content of the cell extracts was determined by the method of Bradford (5) with bovine serum albumin as a standard. Aliquots of the supernatant (20 to 50 μl) were used for enzyme assay. The SOD activity was assayed with an SOD Assay Kit-WST (BioVision, Mountain View, CA) according to the instructions of the manufacturer. The assay utilizes WST-1, a tetrazolium salt, that produces a water soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related with the amount to the xanthine oxidase activity and is inhibited by SOD. The SOD activity was expressed as the percentage of inhibition of the WST-1 reduction rate.

Potassium measurement. Extracellular K^+ measurements from cell-free supernatants were determined by flame photometry, as previously described (45). Cell suspensions treated with nystatin (100 μg/ml) were used as a positive control of K^+ release. The amount of K^+ released was expressed as a percentage with respect to the total K^+ intracellular content.

Statistical analysis. The Student *t* test was used to determine statistical significance using the program GraphPad Prism v. 5.01 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant when $P < 0.05$.

RESULTS

Lactoferrin-induced apoptotic markers in *C. albicans*. Examination for phenotypic markers of apoptosis was performed with *C. albicans* cells treated with 5 μM rhLf, since this was the optimal candidacidal concentration for in vitro assays (45, 46).

The TUNEL method detects apoptotic DNA fragmentation by labeling 3′-OH termini with modified nucleotides catalyzed by terminal deoxynucleotidyltransferase. DNA strand breakage is visualized as green fluorescence-stained nuclei. Samples incubated with rhLf exhibited a TUNEL-positive phenotype in 56% ± 9% of the cells at 3 h posttreatment (Fig. 1Ab), indicating ruptured intracellular DNA strands. Fluorescence in nuclei was not observed in control cells not treated with lactoferrin (Fig. 1d). DAPI staining of rhLf-treated cells displayed the typical nuclear half-ring or tubular images (Fig. 1Af and g) corresponding to chromatin fragments. The cells not treated with rhLf (control) exhibited a single, bright, round-shaped nucleus and peripheral cell spots corresponding to stained mitochondria (Fig.1Ah). As previously reported, a DNA ladder pattern typical of mammalian apoptotic cells was not observed (data not shown) when DNA was analyzed electro-

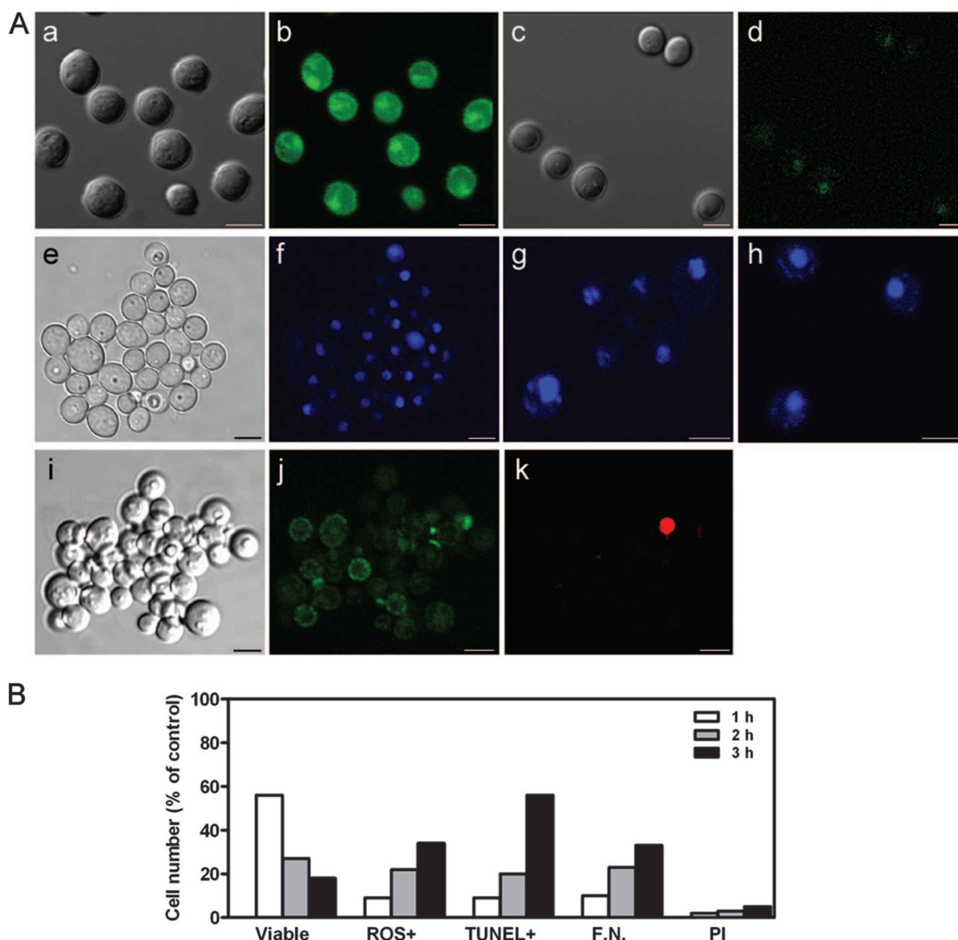

FIG. 1. Apoptotic markers induced by lactoferrin on *C. albicans* cells. (A) Representative micrographs showing cells stained with TUNEL (row 1), DAPI (row 2), and FITC-annexin V (row 3) to detect DNA strand breakage, fragmented nuclei, and phosphatidylserine externalization, respectively. Subpanels g and h show augmented micrographs displaying DAPI-stained cells with abnormal (g) and normal (h) round compact nuclei (control). Subpanel k shows PI staining. The cells were previously treated with lactoferrin (b, f, g, and j) or not treated (control) (d and h). Subpanels a, c, e, and i are phase-contrast micrographs. (B) Lactoferrin-treated cells exhibiting apoptotic markers. ROS+, TUNEL+, F.N.+, and PI+ refer to the percentage of cells (± the standard deviation) exhibiting DHR-123 fluorescence, a positive TUNEL reaction, fragmented nuclei, and PI staining, respectively. Bar, 5 μm.

phoretically, probably due to the suggested scarcity of linking DNA between nucleosomes in the yeast chromatin structure (30).

Phosphatidylserine externalization at the outer leaflet of the cytoplasmic membrane is interpreted as an early marker of apoptosis in mammalian and yeast cells. In yeast, the external exposure of phosphatidylserine is usually detected by annexin V-FITC staining upon cell wall digestion (29, 38). Cells exposed to rhLf for 3 h showed peripheral fluorescence in 23% ± 7% of the protoplasts examined indicating phosphatidylserine externalization. In this assay, apoptotic and necrotic cells were distinguished by double staining for annexin V-FITC and PI, which is a membrane-impermeant DNA fluorescent stain. Figure 1Aj shows the results of a representative assay performed in the presence of rhLf (5 μM). Annexin V-FITC-stained cells were not detected in control assays performed without rhLf (data not shown). The number of rhLf-treated and untreated cells stained with PI was similar (≤5%), which was consistent with our previously reported results showing the inability of human lactoferrin to disrupt the cytoplasmic membrane of *C. albicans* cells (45, 46).

ROS production by rhLf-treated cells was measured at 1, 2, and 3 h posttreatment, with 3 h corresponding to maximal cell death (45). The production of intracellular ROS was evaluated by using dihydrorhodamine 123, which can be oxidized by ROS to become the fluorescent chromophore Rh-123. A total of 38% (±5%) of cells treated with rhLf exhibited intense fluorescence (Fig. 1B and 2A), while controls performed without rhLf did not (data not shown). In control assays, there was a high percentage of fluorescent cells (Fig. 2A and B) when cells were treated with 3 mM H_2O_2, an inductor of apoptosis in *C. albicans* cells (38). All of these apoptotic features were increased in a time-dependent manner (Fig. 1B).

ROS inhibition prevents rhLf-induced cell death in *C. albicans*. To determine whether the rhLf-induced increase in intracellular ROS is essential for the rhLf-induced cell death process, superoxide anions were scavenged. By flow cytometry, rhLf-exposed cells treated with 5 mM NAC, an antioxidant

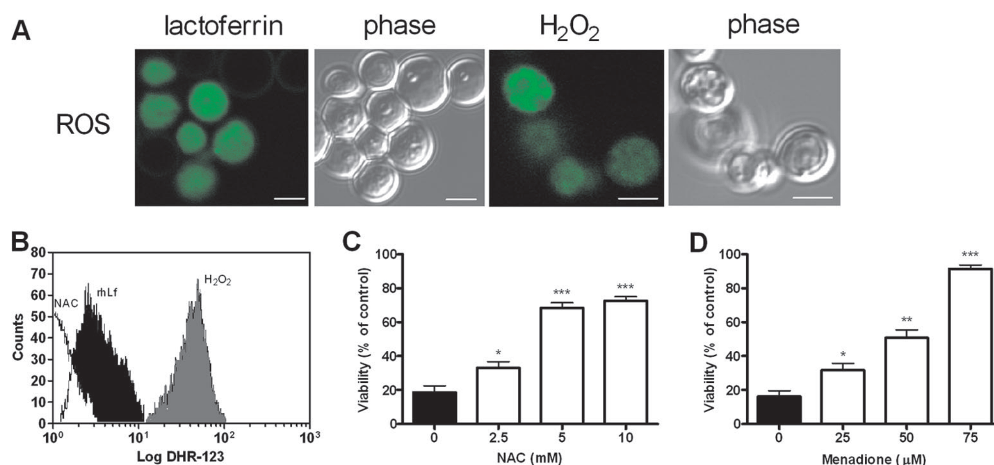

FIG. 2. ROS accumulation in lactoferrin-treated *C. albicans* cells. (A) Fluorescence and phase-contrast micrographs showing cells stained with DHR-123 previously treated with rhLf or H_2O_2 (positive control). Bar, 5 μm. (B) Flow cytometric analysis of *C. albicans* cells treated with rhLf or H_2O_2 (positive control) incubated with DHR-123. NAC, cells preincubated (10 min, 30°C) with the antioxidant agent NAC (5 mM) prior to rhLf exposure. (C and D) Percentage of viability from cell suspensions treated with rhLf (5 μM) for 3 h that were preincubated with NAC (C) or precultured (1 h, 30°C) with menadione (D). *, $P < 0.05$ versus untreated controls; **, $P < 0.01$ versus untreated controls; ***, $P < 0.001$ versus untreated controls.

known to increase the intracellular free radical scavengers, displayed low ROS levels compared to untreated cells (Fig. 2B). It was found that the decrease in intracellular ROS was associated with a decrease in the anti-*Candida* activity of 5 μM rhLf on the same inoculum of NAC-exposed cells and in a concentration-dependent manner (Fig. 2C). Furthermore, the cell viability of rhLf-treated cells was tested using *C. albicans* suspensions previously grown in the presence of low concentrations of menadione (25, 50, and 75 μM). This drug is a redox cycling agent that generates superoxide (O_2^-) in the cytosolic and mitochondrial compartments. It has been previously proven that these oxidative conditions induce the synthesis of the SODs Cu/Zn-SOD (cytosolic) and Mn-SOD (mitochondrial) that protect the yeast from oxidative stress (23). The percentage of cell viability increased in an antioxidant concentration-dependent manner in the treated cells compared to the untreated control (Fig. 2D).

To determine the toxicity of menadione and NAC on *C. albicans* cells, a range of menadione (0.025 to 2 mM) and NAC (5 to 25 mM) concentrations were tested. Menadione concentrations of ≥100 μM and NAC concentrations of ≥15 mM were cytotoxic for *C. albicans* cells in the indicated assay conditions, as determined by a colony-counting assay. The data obtained from the inhibition of WST-1 reduction rate indicated that SOD activity of cells incubated in the presence of 25, 50, or 75 μM menadione increased 18% ± 9%, 42% ± 7%, and 53% ± 8%, respectively.

Effect of lactoferrin on mitochondrial membrane potential. Changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$) have been considered as a hallmark of apoptosis (28). To determine the ability of rhLf to induce mitochondrial depolarization, the $\Delta\Psi_m$ was quantified by Rh-123 uptake, using suspensions of cells treated for different periods of time with rhLf (15, 30, 60, 120, and 180 min). As Fig. 3 illustrates, rhLf induced a decrease in cell fluorescence in a time-dependent manner. Cells exposed to rhLf (5 μM) for 15 min showed a slight loss of fluorescence intensity indicative of partial mito-

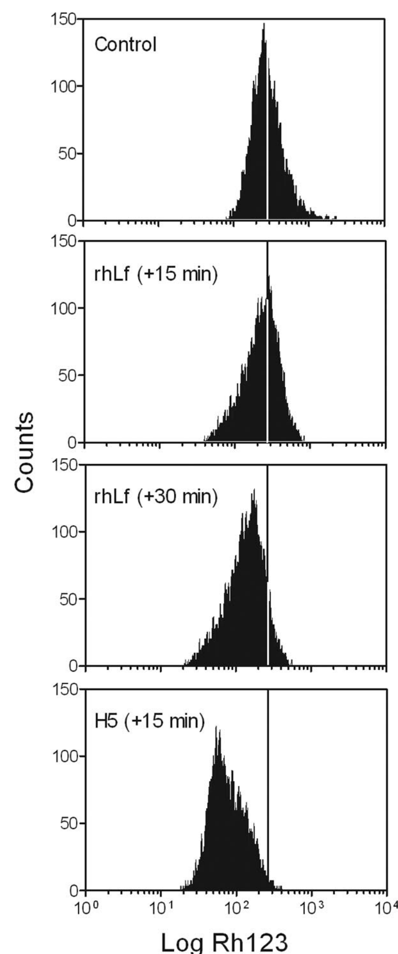

FIG. 3. Effect of lactoferrin on mitochondrial transmembrane potential. *C. albicans* cells (10^6 cells/ml) resuspended in 5 mM PPB were treated with 5 μM lactoferrin (rhLf) or 25 μM histatin 5 (positive control). The mitochondrial transmembrane potential was then measured at different times using 10 μM Rh-123; Rh-123 fluorescence quenching was measured by flow cytometry with excitation at 480 nm and emission at 530 nm.

chondrial depolarization, which increased after ≥30 min (Fig. 3). In control assays, cells exposed to histatin 5 (25 μM), a candidacidal human salivary peptide that induces mitochondrial depolarization (17, 18) displayed a rapid (~15-min) loss of $\Delta\Psi_m$ (Fig. 3). These results were confirmed by confocal fluorescence microscopy, where cells exposed to a candidacidal concentration of rhLf (5 μM) exhibited a visible loss of fluorescence intensity (data not shown).

Attenuation of rhLf activity by protein synthesis inhibitors. Another specific indicator used to distinguish apoptosis from necrosis in eukaryotes and yeast (i.e., *Saccharomyces cerevisiae*) is the prevention of cell death by protein synthesis inhibitors (27, 30, 42). Sordarin and erythromycin, the latter an inhibitor of yeast mitoribosomes (14), were used to determine the effect of protein synthesis on rhLf-induced cell death. Figure 4 shows how the candidacidal activity of different rhLf concentrations substantially decreased when the killing assays were performed with cells cultured for 30 min or 24 h in the presence of subinhibitory concentrations of sordarin (1 μg/ml) or erythromycin (10 mg/ml), respectively. The data from colony-counting assays indicated that concentrations of sordarin of ≥2 μg/ml were cytotoxic at the indicated incubation time (30 min). The cell viability was unchanged after incubation (24 h) with erythromycin (10 mg/ml). The MIC values for sordarin and erythromycin were 8 and ≥15 mg/ml, respectively.

Protein synthesis was inhibited by sordarin, an antifungal that specifically blocks protein synthesis (10). The incorporation of [^{3}H]leucine into cells incubated with 1 μg of sordarin/ml (10, 20, and 30 min) was completely prevented (data not shown). Erythromycin (10 mg/ml) was able to inhibit the mitoribosomal protein synthesis of *C. albicans* cells, as indicated by the analysis of the cytochrome profile (Fig. 4C). Alteration of this profile, particularly the disruption of the cytochrome aa_3 peak, was more evident in cells cultured in the presence of erythromycin, as described previously (14).

Role of K^+ channels in rhLf-induced *C. albicans* cell death. To determine the role of ion channels in rhLf-induced cell death, killing assays in the presence of either K^+ or Cl^- channel blockers were performed (Fig. 5). The K^+ channel blockers TEA and 4-AP and the stretch-activated cation channel blocker Gd^{3+} prevented rhLf-induced cell death in concentration-dependent manners (Fig. 5A and B). Remarkably, *C. albicans* cells treated with 10 mM TEA, a K^+ channel blocker, were less susceptible to rhLf (5 μM) than cells treated with rhLf but not TEA (89% ± 8% versus 19% ± 7%, respectively) and were also almost totally protected by 1 mM DIDS, a broad-specificity Cl^- channel blocking drug (81% ± 6% versus 21% ± 4%). However, NPPB was unable to inhibit the candidacidal activity of rhLf, and concentrations of >0.5 mM were cytotoxic. Extracellular K^+ measurements (Fig. 5C) did reveal an almost total inhibition of K^+ release in rhLf-treated cells preincubated with TEA with respect to control cells (4% ± 3% versus 23% ± 6%, respectively), indicating that all extracellular K^+ was effluxed through K^+ channels. The percentage of K^+ released from cell suspensions treated with nystatin was 94% ± 7% as determined by flame photometry.

In agreement with previous reports, showing a rapid K^+ release (~20 min) in rhLf-treated *C. albicans* cells (45, 46), the protective effect of TEA was not observed when this blocking agent was added 5 or 15 min after the addition of rhLf (Fig. 5D).

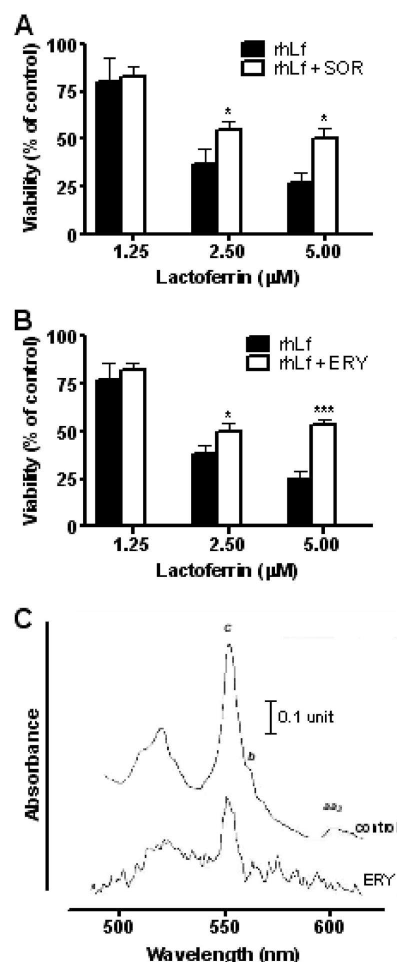

FIG. 4. Effect of protein synthesis inhibitors on lactoferrin activity. *C. albicans* cells were precultured with (A) 1 μg of sordarin (SOR)/ml or (B) 10 mg of erythromycin (ERY)/ml for 30 min and 24 h, respectively. Cell suspensions (10^5 cells/ml) in 5 mM PPB were then treated with 1.25, 2.5, or 5 μM rhLf for 3 h, and appropriate dilutions were plated onto SDB plates. After incubation at 30°C for 24 to 48 h, the colonies were counted to determine the number of viable cells on each plate. (C) Cytochrome spectra of cells treated with erythromycin (ERY) or not treated (control). The absorption maxima for cytochromes $a+a_3$, b, and c are 602, 564, and 550 nm, respectively. The data are expressed as means ± the standard deviation of three independent assays. *, $P < 0.05$ versus untreated controls; ***, $P < 0.001$ versus untreated controls.

DISCUSSION

C. albicans cells exposed to lactoferrin exhibited the main phenotypic hallmarks of an apoptosis-like process such as phosphatidylserine exposure, nuclear chromatin condensation, DNA degradation, and increase of intracellular ROS levels. These apoptototic markers have been described previously during apoptosis of this human pathogen and other eukaryotic cells (20, 38).

Although ROS accumulation has been proven to play a key role in programmed cell death of metazoa and yeast (30, 43),

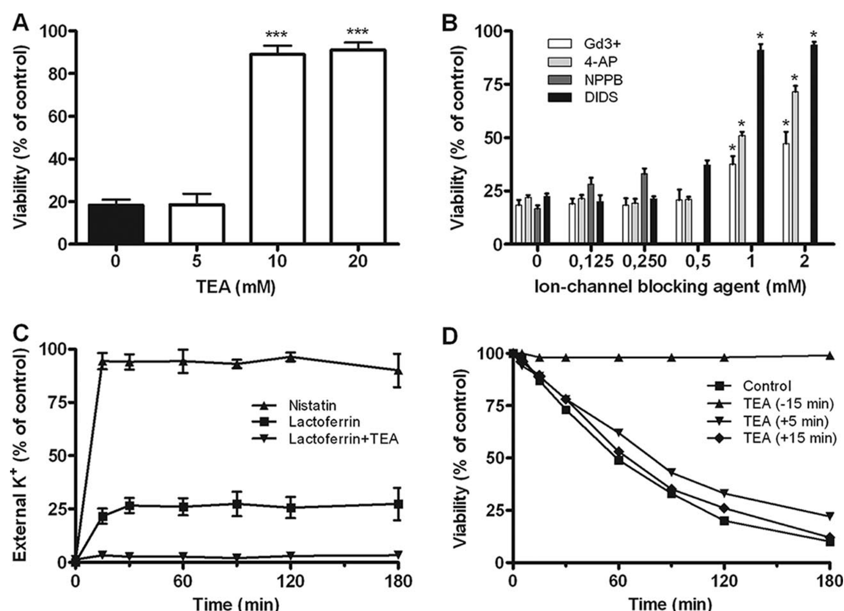

FIG. 5. Effect of ion channel blocking agents on lactoferrin activity. (A and B) Cytoprotective effect on *C. albicans* cells preincubated with TEA (A) and other K^+ or Cl^- channel blocking agents (B) before treatment with 5 μM lactoferrin. (C) Time course of K^+ efflux from lactoferrin-treated cells preincubated (10 min) with or without 10 mM TEA. Nystatin (100 μg/ml) was used as a positive control of K^+ release. (D) Time-kill curve of lactoferrin-treated cells. The K^+ channel blocking agent TEA (10 mM) was added before (−15 min) or after (+5 and +15 min) lactoferrin addition. The data are expressed as means ± the standard deviation of three independent assays. *, $P < 0.05$ versus untreated controls; ***, $P < 0.001$ versus untreated controls.

very few ROS-independent apoptotic processes have been described in fungi (3, 7, 39, 44). These previous reports prompted us to determine whether the observed ROS production induced by rhLf was the causal effect of cell death or a side effect of other changes accompanying the killing process. In yeast, ROS are considered crucial elements for cell death since the depletion of ROS or hypoxia can prevent many apoptotic processes (30, 38). In agreement with these earlier reports, *C. albicans* cells grown in the presence of an inducer of intracellular ROS-detoxifying enzymes (menadione) or preincubated with an antioxidant agent (NAC) were less susceptible to rhLf. The protective effect of these different agents was proportional to their concentration, as expected if ROS accumulation was the cause of rhLf-induced cell death. Furthermore, our previous data showing the absence of candidacidal activity of rhLf under anaerobic conditions emphasize the importance of ROS in this cell death process (45). Although more work is necessary to confirm the role of intracellular ROS induced by lactoferrin, these results are compatible with a ROS-dependent apoptotic process.

We have previously suggested the involvement of mitochondria in the candidacidal activity of both lactoferrin and its derived antimicrobial peptide kaliocin-1, which includes the structural γ-core motif characteristic of antimicrobial Cys-peptides (45, 50). Accordingly, the ROS production associated with the presence of apoptotic markers in rhLf-treated *C. albicans* cells supports the central role of mitochondria previously demonstrated in the apoptosis of yeast and higher-eukaryote cells (11, 16, 18, 32, 40, 43). Moreover, the dissipation of the mitochondrial transmembrane potential observed in rhLf-treated cells is very similar to the mitochondrial dysfunction demonstrated during the apoptosis of yeast and other eukaryotic cells (11, 22, 28). Additional evidence of the participation of mitochondria in the apoptosis-like induced by rhLf was the observed protective effect of erythromycin, a mitoribosomal inhibitor in yeast (14), suggesting that the candidacidal activity of rhLf depends in part on mitochondrial protein biosynthesis. Sordarin also increased the viability of rhLf-treated cells, in agreement with the previously suggested essential role for protein synthesis in apoptosis-like cell death in yeast (27, 30). These findings support the notion that mitochondrial function is involved in the apoptosis-like cell death response induced by lactoferrin in *C. albicans*.

It has been previously demonstrated that K^+ efflux is one of the earliest events of the apoptotic process in metazoan cells and is presumed to be necessary for activation of the biochemical apoptotic pathways (4, 8, 15, 21, 41, 48). However, a similar role for the intracytoplasmic loss of potassium ions has not been reported in fungi. We previously demonstrated that lactoferrin causes a rapid release of K^+ from *C. albicans* cells (45, 46). In addition, the cell death induced by rhLf was inhibited by high extracellular K^+, as has also been shown in a number of diverse apoptotic model systems (4, 45). Since membrane integrity is maintained in lactoferrin-treated cells (45, 46), the majority of the K^+ released could arise from K^+ movement through K^+ channels identified previously in this yeast (26); some of them appear to be involved in the candidacidal activity of other antimicrobial peptides such as histatin 5 (2, 26). It is known that K^+ efflux is concomitant to a Cl^- efflux to maintain intracellular electroneutrality, and the intracellular decrease of both ions contributes to cell shrinkage favoring apoptotic cell death (8, 41). Here we present evidence that blocking of K^+ or Cl^- channels with different chemical agents caused a significant attenuation of the rhLf-induced apoptosis, indicating that

the cytoplasmic ionic imbalance irreversibly triggered this cell death process. We have shown previously that lactoferrin alone stimulate the K^+ release (~27%) during the first 17 min after lactoferrin addition (46). However, K^+ release from cells preincubated with TEA before addition of rhLf was not observed, and these cells were protected from the rhLf activity. The protective effect of TEA was not observed when the blocking agent was added 15 min after to the addition of rhLf, suggesting that K^+-channel-mediated K^+ efflux activated the cell death process. Furthermore, flow cytometry of rhLf-treated *C. albicans* cells revealed a substantial reduction of cell volume that was attenuated by DIDS, a blocker of volume-regulating Cl^- channels (data not shown), supporting the idea that a perturbation in ion homeostasis promoted the apoptosis-like process induced by lactoferrin.

Taken together, these data indicate that K^+-channel-mediated K^+ efflux is the first checkpoint for the progress of rhLf-induced apoptosis in *C. albicans* cells and suggests that this early event could be as essential to triggering apoptosis in unicellular eukaryotes (i.e., yeast) as it is in cells of multicellular organisms, including human cells.

In summary, the presence of phenotypic markers and sequenced cellular events, usually associated with the apoptotic process of eukaryotic cells lend support to the notion that lactoferrin triggers apoptosis-like cell death in *C. albicans* cells. Demonstration, for the first time, that K^+-channel-mediated K^+ efflux is involved in this process provides an additional link between the apoptotic cell death pathways of yeast and higher-eukaryote cells and suggests that this critical event developed before the evolutionary separation between fungi and metazoans. The lactoferrin-induced cell death of *C. albicans* cells may be an attractive model to study other intracellular events of the apoptosis-like process in yeast, leading to a more comprehensive view of host-microbe interactions useful in the design of novel therapeutic strategies.

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