

Antileukoprotease: An Endogenous Protein in the Innate Mucosal Defense against Fungi

J. F. Chris Tomee,* Pieter S. Hiemstra,
Regina Heinzl-Wieland,* and Henk F. Kauffman

Department of Allergology, Groningen University Hospital, and
Department of Pulmonology, Leiden University Hospital, Netherlands;
Department of Molecular Biology, Grünenthal GmbH, Aachen, Germany

Previous studies have suggested that endogenous protease inhibitors may participate in the mucosal host defense. Antileukoprotease (ALP) is an important protease inhibitor found on various mucosal surfaces, including those of the respiratory and genital tracts. This study reports on the antimicrobial activity of recombinant (r) ALP toward the human fungal pathogens *Aspergillus fumigatus* and *Candida albicans*. rALP expressed pronounced fungicidal activity toward metabolically active *A. fumigatus* conidia and *C. albicans* yeast cells; however, metabolically quiescent *A. fumigatus* conidia were totally resistant. In contrast with the protease inhibitory activity of rALP, the fungicidal activity was localized primarily in the NH₂-terminal domain. On a molar base, the fungicidal activity of rALP was comparable with that of human defensins and lysozyme. In addition, rALP caused inhibition of *C. albicans* yeast cell growth. By exhibiting antifungal activity, ALP may play an important role in the innate mucosal defense against human pathogenic fungi.

The factors that constitute the mucosal host defense against microorganisms have been studied widely over the past decades. In addition to the various phagocyte granule-associated proteins and peptides, including defensins, lysozyme, lactoferrin, and azurocidin (reviewed in [1]), and the neutrophil cytoplasmic-associated protein calprotectin [2], protease inhibitors also may play a role in the mucosal defense in different ways. Protease inhibitors may inhibit protease-mediated degradation of opsonins or receptors involved in phagocytosis [3, 4] or may suppress airway inflammation [5]. However, some studies have suggested that protease inhibitors may act as potent antibacterial agents as well [6, 7].

Antileukoprotease (ALP), also known as secretory leukoprotease inhibitor, is widely distributed [8–13] and is a major protease inhibitor of the mucous secretions of the respiratory and genital tracts [14, 15]. ALP is an 11.7-kDa cationic nonglycosylated protein composed of two highly homologous domains. In the human lung, ALP is produced locally in the submucosal glands and lining epithelium of the bronchi; since ALP is a potent inhibitor of endogenous serine proteinase activity, it is suggested that ALP may play a role in maintaining the proteinase-antiproteinase balance in the lung [16, 17]. The ALP molecule shows sequence homology with an antimicrobial peptide isolated from equine neutrophils (eNAP-2) [18], and

recently the bactericidal activity of recombinant (r) ALP was demonstrated [19]. In the present study, the fungistatic and fungicidal activities of rALP toward the human pathogens *Aspergillus fumigatus* and *Candida albicans* are investigated.

Material and Methods

Fungal isolates. The *A. fumigatus* isolate used in this study was originally obtained from a patient with pulmonary aspergillosis. *A. fumigatus* was maintained on malt extract agar (Merck, Darmstadt, Germany) at 37°C. *C. albicans* was isolated from the blood of a patient with candidiasis and was a gift of H. G. de Vries-Hospers (Department of Medical Microbiology, Groningen University Hospital, Netherlands). The *C. albicans* isolate was maintained on Sabouraud agar (Merck) at 30°C. Conidia from the test organisms were counted in a hemacytometer and adjusted to desired concentrations.

Antimicrobial (poly)peptides. rALP was produced in *Escherichia coli* and purified as described [20, 21]. The rALP preparation was characterized by N-terminal amino acid sequencing, quantitative amino acid analysis, SDS-PAGE, and reverse-phase high-performance liquid chromatography (HPLC). Separated NH₂- and COOH-terminal domains were obtained by partial acid hydrolysis or rALP as described [19, 22]. The isolated domains were characterized by SDS-PAGE, acid urea PAGE, assessment of the elastase inhibitory activity [19], and reverse-phase HPLC. N-terminal amino acid sequencing and amino acid composition analysis further confirmed the identity of the NH₂-terminal (residues Ser-1 to Asp-49) and COOH-terminal (residues Pro-50 to Ala-107) domains. The concentration of rALP and its domains was determined by UV spectrometry based on the absorption at 276 nm. The extinction coefficient for rALP used for this calculation was determined by quantitative amino acid analysis of a highly purified sample; the coefficients for the domains were calculated from their contents of aromatic chromophores. Both the rALP and the rALP terminal domains were a gift of D. Saunders (Department of Molecular Biology, Grünenthal, Aachen, Germany).

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Reprints or correspondence: Dr. H. F. Kauffman, Dept. of Allergology, Clinic for Internal Medicine, University Hospital Groningen, Hanzeplein 1, 9713 GZ Groningen, Netherlands.

* Present affiliations: Department of Pulmonology, Groningen University Hospital, Netherlands (J.F.C.T.); Fachhochschule Darmstadt, Fachbereich Chemische Technologie, Darmstadt, Germany (R.H.-W.).

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rALP and the rALP domains were dialyzed for 24 h at 4°C in 0.01% acetic acid, using dialysis tubes with a 6- to 8-kDa (rALP) or 3.5 kDa (rALP domains) molecular cutoff (Spectra/Por; Spectrum Medical Industries, Houston) and subsequently stored at -20°C. Before and after dialysis, the protein concentration was determined by the method of Lowry et al. [23], using bovine serum albumin as a standard, and the results were used to calculate the dilution caused by the dialysis procedure. This information was used in combination with the UV spectrometry findings to calculate the concentrations of rALP and its domains after dialysis. Furthermore, the rALP concentration was confirmed using a secretory leukoprotease inhibitor (SLPI) immunoassay (R&D Systems, Minneapolis) and an ELISA for quantification of rALP described by Kramps et al. [8], both recognizing natural and *E. coli*-expressed recombinant SLPI (and ALP).

A mixture of the human neutrophil defensins HNP-1, HNP-2, and HNP-3 (HNP1-3) was prepared from purulent sputum of patients with chronic bronchitis and was characterized as described [19, 24]. Lyophilized lysozyme (from human milk) was obtained from Sigma (St. Louis), and the protein content was determined by the method of Lowry et al. [23]. The purity of the lysozyme and defensin preparations was verified by SDS-PAGE analysis. All antimicrobial (poly)peptides were dissolved in 0.01% acetic acid and stored at -20°C.

Conidia from *A. fumigatus*. *A. fumigatus* conidia were harvested by mild tapping of 10-day old malt extract agar cultures. To obtain single cell suspensions, fungal conidia were placed in 10 mL of sterile distilled water (~1.10⁸ conidia/mL) containing 0.5% (vol/vol) Tween 20 (Merck) and shaken vigorously for at least 2 h at room temperature. Two different types of conidia were prepared. The dormant (metabolically quiescent) type of conidia was prepared by diluting the freshly prepared conidia suspensions in liquid Sabouraud medium (Merck) containing 50 µg/mL gentamicin sulfate (SG medium; Centrafarm Services, Etten-Leur, Netherlands) to 4.10⁶-7.10⁶ conidia/mL. The swollen (metabolically active) type of conidia was prepared by incubating the dormant type for 19 h at 25°C in SG medium (under stationary conditions) followed by an additional incubation for 1-2 h at 37°C. By then, microscopic observations revealed cells that had approximately doubled in size (became "swollen") compared with the dormant type.

Yeast cells from *C. albicans*. Approximately 3.10⁸ yeast cells, obtained from *C. albicans* precultures on SG medium, were seeded in 200 mL of SG medium, followed by culturing for 5 days at 30°C under mild shaking conditions (150 rpm). By then, the yeast cell cultures had reached 80%-85% of their maximum growth density.

Quantification of fungicidal activity. *A. fumigatus* conidia (1000/mL) were incubated with rALP or the rALP domains in 1 mL of 10 mM sodium phosphate buffer (pH 7.4) for 2.5 h at 37°C; control samples lacked rALP or rALP domains. Next, 300-µL samples were spread in triplicate on YM agar (Difco, Detroit) plates (140 mm diameter; Phoenix Biomedical Products, Mississauga, Canada) containing gentamicin sulfate (50 µg/mL). After 24 h at 33°C, the colony forming units (cfu) were enumerated visually. For comparison, incubations were done with lysozyme or HNP1-3. The fungicidal activity toward *C. albicans* was studied by incubating yeast cells with the antimicrobial proteins for 5 h at 30°C in 10 mM sodium phosphate buffer. In some experiments,

C. albicans samples were incubated with rALP for various time periods to assess survival of the remaining organisms over time. The fungicidal activity was measured as the reduction of inoculum cfu and calculated by the formula $[(N_{\text{control}} - N_{\text{exp}})/N_{\text{control}}] \times 100$, in which N_{exp} and N_{control} represent the number of cfu obtained after incubation in the presence (N_{exp}) or absence (N_{control}) of the antimicrobial (poly)peptides.

In vivo, fungal growth may be limited by several factors, such as nutrient limitations, the concerted actions of fungicidal proteins (e.g., lysozyme) and phagocytic cells (e.g., macrophages), or suboptimal growth temperatures. Since temperatures for growth of *A. fumigatus* are optimal between 37 and 39°C, we studied the effect of suboptimal temperatures on the fungicidal activity of rALP by incubating *A. fumigatus* conidia with rALP at 37°C for 2.5 h, followed by incubation of the agar plates at a lower temperature (33°C) overnight. Results were compared with control plates incubated at 37°C.

Quantification of fungistatic activity towards *C. albicans* yeast cells. *C. albicans* yeast cells obtained from 5-day 30°C precultures were centrifuged for 5 min at 200 g and resuspended in prewarmed 10 mM sodium phosphate buffer (pH 7.4) containing 1% SG medium. Yeast cells (1.0 × 10⁶ in 75 µL) were seeded in microwells of sterile 96-well microplates (Costar, Cambridge, MA) and incubated with rALP for 2.5 h at 30°C. Plates were shaken mildly. Control samples lacked antimicrobial proteins. Next, 75 µL of double-concentrated SG medium was added, and the increase in cell density was evaluated at 37°C for 21 h by reading the absorbance at 630 nm every 10 min with a computerized plate reader (Thermomax; Sopar-biochem, Nieuwegein, Netherlands). From the sigmoidal growth curves, the maximum rate of increase of cell density (V_{max}) and the lag time before V_{max} was reached (T_{lag}) were calculated. The fungistatic activity is defined as the increase in T_{lag} (ΔT_{lag}) compared with the T_{lag} of control samples.

Statistics. All data are presented as mean ± SE. Comparisons were made using the 2-tailed Student's *t* test. Differences were considered significant if $P < .05$. Values are from four independent experiments unless indicated otherwise. The 50% fungicidal concentration (FC₅₀) was calculated from the linear regression of the dose-response curves.

Results

Fungicidal activity of the antimicrobial proteins toward *A. fumigatus* conidia: the effect of temperature. Figure 1 shows dose-response curves for the fungicidal activity of rALP toward *A. fumigatus* conidia. The fungicidal activity toward swollen *A. fumigatus* conidia was dose-dependent and reached statistical significance at 2 µM rALP (12.5% ± 5.4%; $P = .05$; $n = 9$; figure 1, solid circles). At 15 µM, the fungicidal activity was 86.2% ± 2.6%. The FC₅₀ calculated from the individual dose-response curves was 5.6 ± 0.7 µM. The dormant *A. fumigatus* conidia were not killed in the dose ranges tested (figure 1, open circles).

When the fungicidal activities of rALP were compared with those of HNP1-3 and lysozyme (table 1), rALP and the HNP1-3 reached approximately the same maximum level of fungicidal activity (71.7% ± 4.0% and 71.2% ± 3.5%, respec-

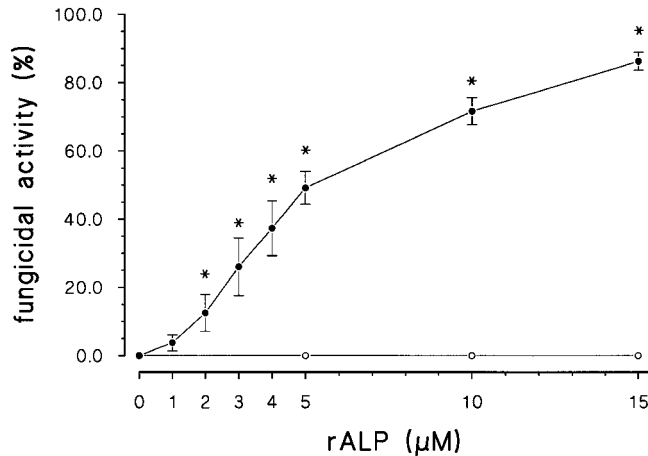


Figure 1. Recombinant (r) ALP-mediated killing of *Aspergillus fumigatus* conidia. *A. fumigatus* conidia in 10 mM phosphate buffer (pH 7.4) were exposed to increasing concentrations of rALP for 2.5 h and the no. of cfu was evaluated after overnight incubations at 33°C on YM agar. Shown are results found with swollen type (●) and dormant type (○) of *A. fumigatus* conidia. Comparisons were made using 2-tailed Student's *t* test. * Statistically significant fungicidal activity.

tively). At low concentrations ($\leq 0.5 \mu\text{M}$), only lysozyme was fungicidal; the fungicidal activity of lysozyme reached statistical significance at $0.5 \mu\text{M}$ ($25.9\% \pm 4.9\%$; $P < .02$). At higher concentrations, lysozyme tended to express a lower fungicidal activity ($55.8\% \pm 6.9\%$ and $50.8\% \pm 7.2\%$ at 5 and 10 μM , respectively) compared with rALP and HNP1-3 tested at the same concentrations; however, these differences were not statistically significant ($P > .06$). In comparing the FC_{50} values from these experiments, those of rALP ($\text{FC}_{50} = 5.8 \pm 1.2 \mu\text{M}$) and HNP1-3 ($\text{FC}_{50} = 6.0 \pm 0.4 \mu\text{M}$) tended to be higher than that of lysozyme ($\text{FC}_{50} = 4.9 \pm 2.1 \mu\text{M}$), although differences were not statistically significant ($P > .4$).

Figure 2 shows the results of a lower temperature for overnight incubation of the agar plates on the fungicidal activity of rALP toward *A. fumigatus*. Fungicidal activity was significantly

Table 1. Fungicidal activity of recombinant (r) ALP, human neutrophil defensins HNP-1, HNP-2, and HNP-3 (HNP1-3), and lysozyme toward swollen *Aspergillus fumigatus* conidia.

Conidia (μM)	rALP	HNP1-3	Lysozyme
0.1	0.0 \pm 0.0	0.0 \pm 0.0	6.7 \pm 3.4
0.5	0.0 \pm 0.0	0.0 \pm 0.0	25.9 \pm 4.9*
2	12.5 \pm 5.4*	3.4 \pm 2.2	55.0 \pm 8.4*
5	49.2 \pm 4.9*	44.6 \pm 4.7*	55.8 \pm 6.9*
10	71.7 \pm 4.0*	71.2 \pm 3.5*	50.8 \pm 7.2*
FC_{50}^{\dagger} (μM)	5.8 \pm 1.2	6.0 \pm 0.4	4.9 \pm 2.1

NOTE. FC_{50} , 50% fungicidal concentration. Data \pm SE are from 5 independent experiments.

* Statistically significant fungicidal activity, Student's *t* test.

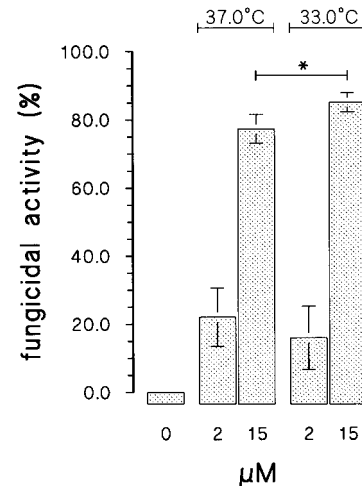


Figure 2. Effect of incubation temperature on fungicidal activity of recombinant (r) ALP toward *Aspergillus fumigatus* swollen conidia. *A. fumigatus* swollen conidia in 10 mM phosphate buffer (pH 7.4) were exposed to rALP (2 or 15 μM) for 2.5 h, followed by subculturing on YM agar overnight at 37 or 33°C. Comparisons were made using 2-tailed Student's *t* test. * Statistically significant fungicidal activity.

increased with 15 μM rALP at 33°C compared with 37°C ($85.1\% \pm 2.8\%$ and $77.3\% \pm 4.2\%$, respectively; $P < .04$; $n = 5$; figure 2). No such difference was found with a lower rALP concentration (2 μM rALP; $P > .3$; $n = 5$; figure 2). On the basis of these findings, the overnight incubations of *A. fumigatus* spread on agar plates were done at 33°C in all experiments.

It is difficult to completely rule out the contribution of spore clumping in our observations on the fungicidal activity of rALP. However, we have done control experiments in which we prepared swollen *A. fumigatus* conidia suspensions at high concentrations (5×10^6) and monitored the effect of addition of rALP (30 μM) for 2.5 h at 37°C. The number and sizes of the spore clumps involved were evaluated by light microscopy. Although spore clumping was significant with *A. fumigatus* ($\sim 25\%$) under these conditions, there tended to be a decrease in spore clumping in the 30 μM rALP-treated samples (mean, 23.2; SE, 1.1%; $n = 4$) compared with the untreated controls (mean, 30.2; SE, 2.2%; $n = 4$). There was no difference in the number of spores in the clumps. Comparable control experiments were performed with *C. albicans*; there was much less spore clumping than with *A. fumigatus* and no differences were found between 30 μM rALP-treated samples (6%; $n = 1$) and untreated controls (7%; $n = 1$).

Fungicidal activity of the rALP domains toward A. fumigatus swollen conidia. Both the NH_2 - and COOH -terminal domains of rALP were fungicidal toward swollen *A. fumigatus* conidia in a dose-dependent fashion (figure 3). However, the fungicidal activity of the NH_2 -terminal domain was higher than that of the COOH -terminal domain. The FC_{50} for the NH_2 -terminal

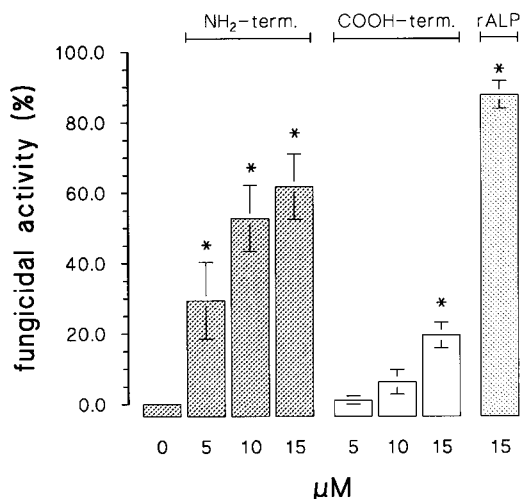


Figure 3. Dose-dependent killing of swollen *Aspergillus fumigatus* conidia by NH₂- and COOH-terminal domains and intact recombinant (r) ALP molecule. *A. fumigatus* conidia in 10 mM phosphate buffer (pH 7.4) were exposed to rALP or increasing concentrations of rALP domains for 2.5 h, and no. of cfu was evaluated after overnight incubations at 33°C on YM agar. Comparisons were made using 2-tailed Student's *t* test. * Statistically significant fungicidal activity.

domain was $9.1 \pm 2.9 \mu\text{M}$; FC₅₀ for the COOH-terminal domain could not be calculated due to low fungicidal activity of this domain. With the protein concentrations tested, the fungicidal activity was statistically significant for the NH₂-terminal domain at $5 \mu\text{M}$ ($29.4\% \pm 10.9\%$; $P < .04$) and for the COOH-terminal domain at $15 \mu\text{M}$ ($19.6\% \pm 3.6\%$; $P < .002$). Compared with the fungicidal activity of the intact rALP molecule on a molar base, however, that of the NH₂-terminal domain was lower. In one experiment, we compared the fungicidal activity of the individual domains with a combination of both domains and intact rALP (all tested at $15 \mu\text{M}$) and found that the fungicidal activity using a combination of both domains (63.1%) approximated the sum of the fungicidal activities that were found with the individual domains (NH₂ domain, 45.0%; COOH domain, 11.9%). However, the fungicidal activity found with the combination of the domains was lower compared with that found with the intact rALP molecule (71.4%, not shown).

Fungicidal activity of rALP toward *C. albicans* yeast cells.

Figure 4 shows the dose-response curve for the fungicidal activity of rALP toward *C. albicans* cells. The fungicidal activity was dose-dependent, reaching statistical significance at $1 \mu\text{M}$ rALP ($9.7\% \pm 1.2\%$; $P < .005$). At $15 \mu\text{M}$ rALP, the fungicidal activity was $59.9\% \pm 4.6\%$. The FC₅₀ calculated from these experiments was $10.0 \pm 1.5 \mu\text{M}$.

In some experiments, we tested the fungicidal activity of rALP toward *A. fumigatus* and *C. albicans* and varied the temperature (range, 30–37°C) and duration (0–24 h) of the incubations with rALP. Although the fungicidal activities were somewhat affected, killing of both *A. fumigatus* and *C. albicans* conidia remained evident. Figure 5 demonstrates the effect of

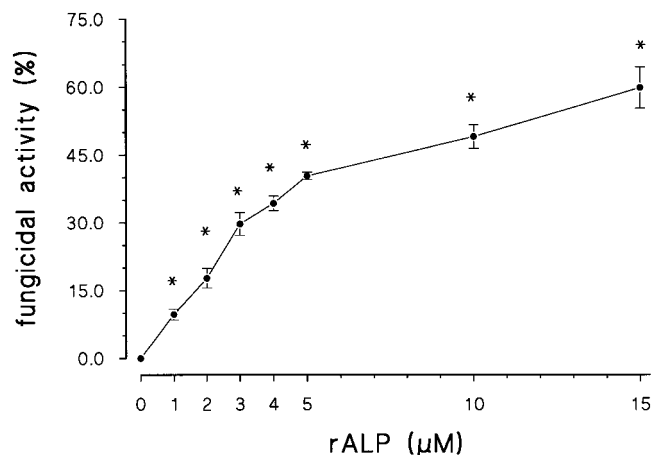


Figure 4. Recombinant (r) ALP-mediated killing of *Candida albicans*. *C. albicans* pseudoconidia in 10 mM phosphate buffer (pH 7.4) were exposed to increasing concentrations of rALP for 5 h. No. of cfu was evaluated after overnight incubations at 30°C on YM agar. Comparisons were made using 2-tailed Student's *t* test. * Statistically significant fungicidal activity.

varied incubation periods on the fungicidal activity of $10 \mu\text{M}$ rALP toward *C. albicans* pseudoconidia. The fungicidal activity was time-dependent, reaching 92.7% after 10 h. With longer incubation periods (up to 24 h), the fungicidal activity reached 98.2%. However, when the assays were performed with increasing ionic strength of the buffer solutions, rALP lost its fungicidal activity toward *C. albicans* more quickly than did human defensins and lysozyme (figure 6). Using YM agar plates without gentamicin sulfate, Sabouraud agar, or malt extract agar plates for the quantification of cfu, comparable results

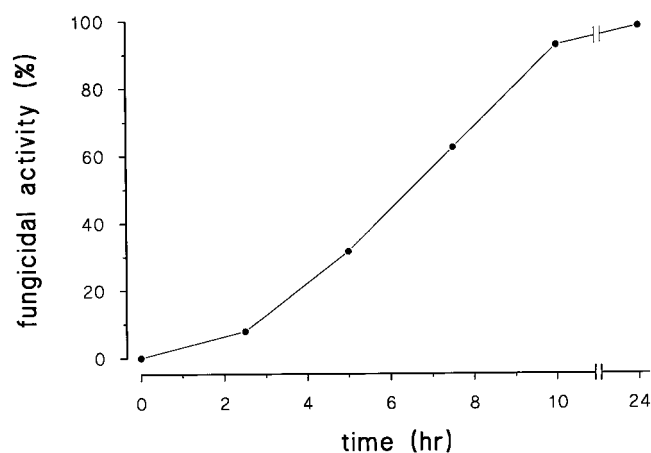


Figure 5. Kinetics of fungicidal activity. *Candida albicans* pseudoconidia in 10 mM phosphate buffer (pH 7.4) supplemented with 0.05% GS medium were exposed to 10 mM sodium phosphate buffer (control) or $10 \mu\text{M}$ recombinant (r) ALP for 0–24 h. No. of cfu of control and rALP-treated samples was evaluated after >16 h of incubation at 30°C on YM agar plates. Results shown are from 1 experiment.

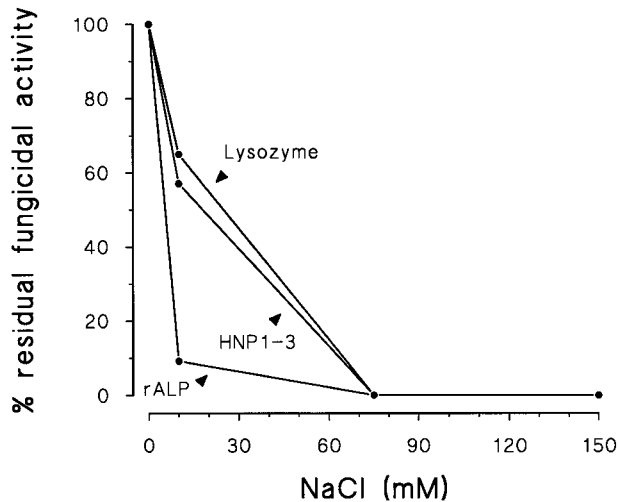


Figure 6. Effect of ionic strength on fungicidal activity of recombinant (r) ALP, human neutrophil defensins HNP-1, -2, and -3 (HNP1-3), and lysozyme. *Candida albicans* pseudoconidia were exposed to rALP (10 μ M), HNP1-3 (6 μ M), or human lysozyme (5 μ M) for 5 h in 10 mM phosphate buffer with increasing saline concentrations. No. of cfu of control and rALP-treated samples was evaluated after >16 h of incubation at 30°C on YM agar plates. Fungicidal activity of each protein tested at 0 M NaCl was arbitrarily set at 100%. Results show are from 1 experiment.

were obtained, indicating that the decrease in cfu count following the incubation with rALP was not due to substrate inhibition phenomena. Longer incubation periods (>24 h) of the YM agar plates did not result in a substantial increase in the residual cfu, indicating that the effect was, in fact, fungicidal and not merely fungistatic.

Fungistatic activity of rALP toward *C. albicans* yeast cells. Within the dose range of rALP used in this study, the fungicidal activity toward *C. albicans* (and *A. fumigatus*) never reached 100%. The residual colonies in the rALP-treated *C. albicans* samples, however, were markedly smaller compared with those of the untreated samples. Since this most likely reflects a process of cell-growth inhibition following the treatment with rALP, we initially tried to quantify growth inhibition by measuring the maximum rates of increase of cell density of *C. albicans* (V_{max}) in GS medium in the presence of various concentrations of rALP. However, when the V_{max} readings were evaluated over longer incubation periods in GS medium (>16 h), we found that, although it was somewhat delayed, eventually the same V_{max} was reached in the rALP-treated samples as in the control (rALP-free) samples. This indicates that the initial growth inhibition is not maintained over longer periods of time, possibly as a result of functional inactivation of the rALP. However, the time of onset of cell growth and, with that, the time before V_{max} was reached (T_{lag}) were increased markedly in the samples treated with the antimicrobial proteins. Therefore, we calculated the increase in T_{lag} (ΔT_{lag}) to quantify the cell growth inhibition. As shown in table 2, rALP inhibited

cell growth of *C. albicans* in a dose-dependent manner, reaching statistical significance at 10 μ M rALP and a ΔT_{lag} of 2 h at 30 μ M rALP.

Discussion

This report describes the fungicidal and fungistatic activity of rALP toward *A. fumigatus* and *C. albicans*. ALP is not glycosylated; therefore, rALP that is synthesized in *E. coli* is identical to ALP in terms of structure and function [13, 25]. The fungicidal activity of rALP was dose-dependent and comparable to that of human defensins and human lysozyme. The fungicidal activity of rALP was statistically significant with an rALP concentration >1 μ M (*C. albicans*) or 2 μ M (*A. fumigatus*), although the antimicrobial activity of rALP, like that of other cationic antimicrobial polypeptides [1, 26], is limited to conditions of low ionic strength (this study, [19]). The possible relevance of this restriction was underlined recently by Smith et al. [27], who described that the airway surface fluid of cultured epithelial cells from cystic fibrosis patients essentially lacks antibacterial activity due to its high NaCl concentration. Data on the local ionic strength at sites in the lung where ALP is present are lacking.

The concentration at which ALP displays antifungal activity in vitro (>2 μ M) appears to be relevant to the in vivo situation in the lung [8, 28, 29], since the ALP concentration in the epithelial lining fluid of the central airways was estimated to be 8.7 μ M [30]. However, one might argue that, at the site of ALP production, serous cells in the submucosal glands and Clara and goblet cells of the bronchiolar and bronchial lining epithelium [31], the local concentration may be even higher. Therefore, in the lung, ALP may contribute to host defense against infection by providing the epithelial cells from the central airways with a protective antimicrobial shield. Because of its presence at other mucosal surfaces, including the genital tract [14, 15], this role is likely not to be restricted to the lung. However, the contribution of ALP to antimicrobial defense relative to that of other endogenous epithelial antibiotics remains to be determined.

Table 2. Fungistatic activity of recombinant (r) ALP toward *Candida albicans* yeast cells.

rALP (μ M)	ΔT_{lag} (min)
2	1.0 \pm 8.2
5	14.5 \pm 6.2
10	32.6 \pm 9.1*
15	54.0 \pm 11.0*
30	120.8 \pm 17.2*

NOTE. Data are increase in lag time ($\Delta T_{lag} \pm$ SE) before maximum rate of increase of cell density was reached. Data are from 6 experiments.

* Statistically significant increase, 2-tailed Student's *t* test.

A role for human serine proteinase inhibitors in the host defense against infections has been suggested previously. First, proteinase inhibitors may inhibit the degradation of opsonins and receptors involved in phagocytosis mediated by endogenous protease activity [3, 4]. In addition, in a previous study, we have shown that rALP may also inhibit exogenous proteases from microorganisms such as *A. fumigatus*, thereby partially protecting airway epithelial cells against cell detachment and inhibiting the production of proinflammatory cytokines by these cells [32]. Second, protease inhibitors may display antibacterial activity toward gram-positive and -negative bacteria, as has been described for aprotinin [6] and rALP [19]. A synthetic peptide mimicking the cysteine proteinase inhibitory site of human cystatin C was found to kill group A streptococci [7], while eNAP-2, a cationic proteinase inhibitor purified from equine neutrophils, killed various bacteria, including *E. coli* [18]. An anti-human immunodeficiency virus type 1 activity for ALP was recently suggested by McNeely et al. [33], although this was not confirmed by others [34]. With respect to the fungicidal activity of proteinase inhibitors, however, data are limited. Recent studies by Lorito et al. [35] have shown that proteinase inhibitors from plants can suppress spore germination and germ tube elongation of two species of phytopathogenic fungi, causing leakage of the intracellular contents from these plant pathogens.

The mechanisms by which cationic proteins, especially defensins, kill microorganisms have been studied for many years. Although the mechanisms involved are not yet fully understood, the bactericidal activity of defensins may be related to pore formation phenomena in the outer membrane of bacteria (reviewed in [36]), resulting in loss of cell homeostasis. Comparable mechanisms may also pertain to the fungicidal activity of cationic proteins such as ALP, and in fact, binding of cationic antimicrobial proteins, a prerequisite for pore formation, to the fungal cell wall chitin has been demonstrated [37]. An alternative mechanism for the fungicidal activity of bacteria has been suggested by studies with *E. coli*, showing the binding of ALP to bacterial DNA and its interfering with bacterial RNases [38], although such a mechanism alone probably does not explain the observed antibacterial activity [19].

Previous studies with *A. fumigatus* have shown that, unlike metabolically active (swollen) conidia, metabolically quiescent conidia are highly resistant to both oxidative and nonoxidative phagocyte-mediated fungicidal activity mechanisms [39]. Studies with the rabbit defensin NP-1 showed that binding of this cationic peptide to metabolically quiescent conidia is restricted to the outer layer and may therefore not lead to actual cell wall penetration [40]. Comparable mechanisms probably also explain the low susceptibility of metabolically quiescent *A. fumigatus* conidia to rALP in our study. However, in order to be able to germinate, the metabolically quiescent fungal conidia will eventually become metabolically active, rendering the fungal cell susceptible to fungicidal products such as ALP.

ALP is composed of two highly homologous domains [13]. The COOH-terminal domain contains the proteinase inhibitory site, but the function of the NH₂-terminal domain is largely unknown [22, 41]. Comparisons of the antifungal activities of both rALP domains showed that the fungicidal activity was localized primarily in the NH₂-terminal domain. As discussed by Hiemstra et al. [19], this may be explained by a higher degree of cationicity of the NH₂-terminal domain and by a clustering of positively charged amino acid residues in the NH₂-terminal domain. The antifungal activity of the individual rALP domains, either alone or in combination, was lower than that of the intact rALP molecule on a molar base. Conformational changes coinciding with diminished exposure of cationic sites in the individual domains may explain these differences. Alternatively, the COOH-terminal domain may contribute to the fungicidal activity of the NH₂-terminal domain only in the intact molecule.

In addition to being fungicidal, rALP was fungistatic for *C. albicans* as well. Growth inhibition may contribute substantially to the mucosal host defense, since the ability to limit the proliferation of fast-growing fungi such as *C. albicans* could limit their spread on mucocutaneous surfaces and may buy time for the cell-dependent and -independent host defense mechanisms to clear the fungus from the mucosa and host tissues.

Over recent years, interest in the discovery and development of new antimicrobial agents has grown rapidly. The widespread use of immunosuppressive agents and the AIDS pandemic has led to a dramatic increase of mycoses [42–46]. In addition, the treatment of mycoses with most of the antifungal drugs currently available is hampered by various adverse effects, including toxicity [47–50]. Of major concern, however, is the vast emerging resistance to antibiotic treatment of clinically important human pathogens, including fungi [51–53]. Our results indicate that rALP has pronounced antifungal activity, although its activity is limited to conditions of low ionic strength. Given its broad spectrum of antimicrobial activity, however, rALP may prove to be a promising agent in the prevention and treatment of microbial infections in humans.

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