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Fungicidal Effect of Antimicrobial Peptide, PMAP-23, Isolated from Porcine Myeloid against *Candida albicans*

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The antifungal activity and mechanism of a 23-mer peptide, PMAP-23, derived from pig myeloid was investigated. PMAP-23 displayed strong antifungal activity against yeast and mold. To investigate the antifungal mechanism of PMAP-23, fluorescence activated flow cytometry and confocal laser scanning microscopy were performed. *Candida albicans* treated with PMAP-23 showed higher fluorescence intensity by propidium iodide(PI) staining, which was similar to that of Melittin than untreated cells. Confocal microscopy showed that the peptide was located in the plasma membrane. The action of peptides against fungal cell membranes was examined by treating prepared protoplasts of *C. albicans* with the peptide and lipid vesicle titration test. The result showed that the peptide prevented the regeneration of fungal cell walls and induced release of the fluorescent dye trapped in the artificial membrane vesicles, indicating that the peptide exerts its antifungal activity by acting on the plasma lipid membrane.

Antimicrobial peptides have been recognized to play important roles in the innate host defense mechanisms of most living organisms including plants, insects, amphibians and mammals (1–6), and are known to possess potent antibiotic activity against bacteria, fungi, and even certain viruses (7–11).

Recently, the rapid emergence of microorganism pathogens that are resistant to currently available antibiotics has triggered considerable interest in the isolation and investigation of the mode of action of the antimicrobial peptides.

In mammalians, the cytoplasmic granules of neutrophiles are an abundant source of a number of antimicrobial peptides. cDNAs of several neutrophile-derived antimicrobial peptides have been cloned. Among these PMAP-23 peptide was identified by cDNA cloning and shown to possess potent antibacterial activity with chemically synthesized peptide (12, 13). Although PMAP-23 had already shown antibacterial activity, its antifungal effect and its mode of action are not well known.

Although, it is essential to understand their mechanisms for the development of novel bioactive peptides, the mechanism(s) of antimicrobial peptides have not been clearly elucidated. In general, antimicrobial peptides are known to act in two different ways, one by targeting the physiology of the cells, and the other by disrupting the cellular structure of the microorganism.

The mechanism of antifungal action has been investigated for several antimicrobial peptides. The major cause of the loss of fungal cell viability is believed to be the result of the formation of transmembrane channels, which increase membrane permeability and cause disruption of the microbial cell structure. This is also believed to be generally applicable to the action mechanism(s) of antifungal peptides (14–17). In contrast, some other antifungal peptides such as IB-AMPs, derived from the seeds of *Impatiens balsamina*, do not cause pore formation in the plasma membrane, and therefore, have a different antibiotic mechanism, which involves DNA synthesis inhibition by interaction with a target protein (18, 19). Therefore, the fungicidal pathway and the effect of PMAP-23 on *C. albicans* were investigated by fluorescence activated flow cytometric analysis, and confocal laser scanning microscopy. Also we will discuss which PMAP-23 is important for the phospholipid vesicle interaction.

MATERIALS AND METHODS

Peptide synthesis. PMAP-23 peptide was synthesized by the solid phase method using Fmoc(9-fluorenyl-methoxycarbonyl)-chemistry (20). Rink Amide 4-methyl benz hydrylamine (MBHA) resin (0.55 mmol/g) was used as the support to obtain a C-terminal amidate

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TABLE 1

Amino Acid Sequence of PMAP-23 and Melittin, and Molecular Weight Determination by MALDI-MS

Peptides	Sequence	Calculated value	Observed value
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR-NH_2	2962.5	2962.0
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2847.4	2850.6

peptide. Fmoc-amino acid coupling was performed with *N*-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were protected as follows: *tert*-butyl (Asp), trityl (Gln), *tert*-butyloxycarbonyl (Lys). Deprotection and cleavage from the resin were achieved with a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol, and triisopropylsilane (88:2.5:2.5:2.5:2.5:2.0, v/v) for 2 h at room temperature. The crude peptide was then repeatedly washed with diethylether, dried in vacuum, and purified by reversed-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19 $\times$ 30 cm). Peptide purity of the peptide was determined by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (Beckman, USA), 4.6 $\times$ 25 cm. Purified peptides were hydrolyzed with 6N-HCl at 110°C for 22 h, and dried in a vacuum. Residues were dissolved in 0.02 N HCl and subjected to amino acid analyzer (Hitachi Model, 8500 A, Japan). Peptide concentrations were determined by amino acid analysis. The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization (MALDI) mass spectrometer (21).

Fungal strains. *Saccharomyces cerevisiae* (KCTC 7296), *Trichophyton rubrum* (KCTC 6345), and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB) (Taejon, Korea). *Candida albicans* (TIMM 1768) was obtained from the Center for Academic Societies (Osaka, Japan).

Antifungal activity. Fungal strains were grown at 28°C in Potato Dextrose Broth (PDB) medium. The fungal cells were seeded in the well of a 96-microtiter plate of PDB media at a density of 1×10^3 cells (per 100 μl well). Ten microliters of the serially diluted-peptide solution was added to each well, and the cell suspension incubated for 24 h at 28°C. Ten microliters of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution [5 mg/ml MTT in phosphate-buffered saline (PBS), pH 7.4] was added to each of the wells, and the plates incubated at 37°C for 4 h. Thirty microliters of 20% (w/v) SDS solution containing 0.02 M HCl was then added, and the plates further incubated at 37°C for 16 h to dissolve the formed formazan crystals (22). The turbidity of each well was measured at 570 nm using a microtiter ELISA reader (Molecular Devices Emax, CA).

FACScan analysis. To determine membrane integrity after peptide treatment, fungal cells (4×10^5 cells in PDB media) were harvested at the log growth phase and mixed with peptides to a final concentration of 5 μM. They were then incubated for a further 30 min at 28°C with constant shaking (140 rpm). Melittin was used in this study as a positive control which has a well known to strong antimicrobial effect. After incubation, cells were harvested by centrifugation and washed three times with PBS. Permeabilization of the cell membrane was detected by incubating peptide treated cells in propidium iodide (PI, 50 μg/ml final concentration) for 30 min at 4°C followed by removal of unbound dye by excessive washing with PBS. Flow cytometry was performed using a FACScan (Becton Dickinson, San Jose, CA).

Confocal laser scanning microscopy (CLSM). The intracellular distribution of PMAP-23 peptide was analyzed by CLSM. Cells treated with fluorescein isothiocyanate(FITC) labelled PMAP-23 were incubated for 30 min at 28°C. After incubation, cells were harvested by centrifugation at 10,000 rpm for 5 min and washed with PBS. Visualization and localization of the labeled peptide was performed using Leica TCS 4D connected to a Leica DAS upright microscope (Leica Lasertech. GmbH, Heidelberg, Germany).

Cell wall regeneration of protoplast. To prepare *C. albicans* protoplasts, cells (1×10^6) were digested with 10 mM phosphate buffer (pH 6.0) containing 0.8 M NaCl, Novozyme 234 (Sigma, St. Louis, MO) (5 mg/ml) and Cellulase (Sigma, St. Louis, MO) (5 mg/ml) for 3 h at 30°C by gentle agitation. The protoplasts so obtained were gently centrifuged at 700*g* and then suspended in 50 mM Tris–HCl buffer (pH 7.5) containing 0.8 M NaCl, and 10 mM $CaCl_2$. The peptide solutions (10 μg/ml) were added to the protoplasts and incubated for 3 h at 28°C. Protoplasts treated with peptide were then transferred into Potato Dextrose soft-agar solutions containing 0.8 M NaCl and 0.5% agar, and then spread on agar plates of Potato Dextrose medium containing 0.8 M NaCl and 2% agar. Colonies regenerated were counted after plates had been incubated at 28°C for 5 days.

Carboxyfluorescein leakage measurement. Carboxyfluorescein (CF)-encapsulated large unilamellar vesicles (LUV) composed of phosphatidylcholine (PC) and phsphatidylserine (PS) (3:1, w/w) were prepared by reverse-phase ether evaporation methods (23) using 100 mM CF in phosphate-buffered saline, pH 7.4. The initially formed vesicles were extruded through Nucleopore filter of 0.1 μm. To remove free CF dye, the vesicles were passed through a Bio-Gel A 0.5 m (Bio-Rad, Richmond, USA) column (1.5 $\times$ 30 cm) usign the eluting buffer. The separated LUV fractions, after appropriate dilution to final concentration of 6.36 μM phosphate, were mixed with PMAP-23 and filled up with buffer to 2 ml in a cuvette at 25°C. The time-course leakages of CF from LUV were monitored by measuring fluorescence intensity at 520 nm excited at 490 nm on a Shimadzu RF-5000 spectrofluorometer (Tokyo, Japan). The apparent percent leakage value at a fluorescence intensity, F, was calculated by the following equation:

$$\% \text{ leakage (apparent)} = 100 \times (F - F_0)/(F_t - F_0)$$

F_t denotes the fluorescence intensity corresponding to 100% leakage after the addition of 20 μl of 10% Triton X-100. Represents the fluorescence of the intact vesicle.

RESULTS

Peptide Synthesis and Antifungal Activities

The amino acid sequences of PMAP-23 and a positive control, Melittin used in this study are summarized in Table 1. The synthetic peptides were purified by reverse-phase HPLC and quantitated by amino acid analysis (data not shown). The accurate molecular weight of the synthetic peptides were confirmed by MALDI mass spectrometry (Table 1).

PMAP-23 has been shown to strongly inhibit the growth of Gram-positive and Gram-negative bacteria (13). Melittin, a honeybee venom toxin, has been reported to have a high antifungal activity (16). On the

TABLE 2

Antifungal Activities of PMAP-23

	MIC: μM		
Peptides	*C. albicans*	*S. cerevisiae*	*T. beigelii*
PMAP-23	2.5–5	5	2.5

other hand, the antifungal activity and mechanism of PMAP-23 have not been previously reported.

Therefore, the antifungal activity of PMAP-23 peptide, was measured against yeast by the MTT assay (24), and expressed as MIC (Minimal Inhibitory Concentration) (Table 2). The results demonstrate that PMAP-23 has remarkable antifungal activity. In order to visualize its antifungal effect, the pathogenic filamentous fungus, *Trichophyton rubrum* was treated with the peptide using the agar diffuse method. As shown in Fig. 1, the peptide inhibited the apical growth of the filamentous fungus.

Peptide Interaction upon the Fungal Cell Membrane

Although the overall antibiotic mechanisms of amphipathic antibacterial peptides having α-helical structure, have not been clearly elucidated, disruption of the cell structure by pore formation (14–17) or ion channel generation seems to be the most likely mechanism (25–27). In order to investigate whether the antifungal effect of the PMAP-23 is caused by either damaging the plasma membrane or by influencing cell physiology, cells were incubated with propidium iodide (PI) (28). The predicted amphipathic nature of PMAP-23 suggested that its activity might be related to cell membrane damage. The result indicated that while untreated normal cells showed no PI fluorescence activity signal, the fluorescence histogram of cells treated with PMAP-23 and Melittin, which has a known potent antifungal effect, at a concentration of 5 μM for 30 min on ice, showed a total shift to the right (Fig. 2).

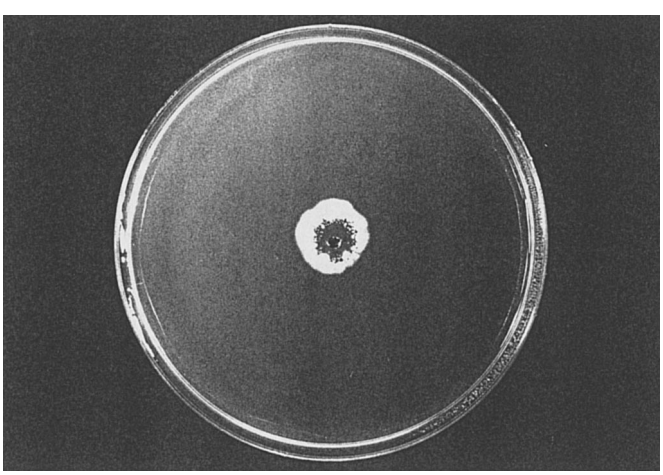

FIG. 1. Agar hole assay for antifungal effect of PMAP-23 against *T. ruburum*. PMAP-23 (5 μM/10 μl) was located at the center of a *T. ruburum* mycelium disk on the 1% PDB agarose plate. The plate was incubated for 3 days at 28°C.

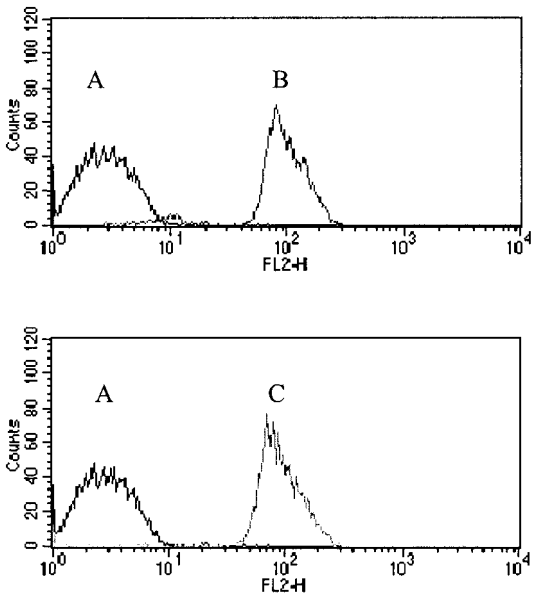

FIG. 2. FACScan analysis of propidium iodide staining in *C. albicans*. Histograms show the fluorescence intensity of internalized PI after peptide treatment of *C. albicans*. (A) Control staining without any peptide treatment; (B) PI staining of PMAP-23 cells treated; (C) staining of Melittin treated cells.

In order to follow the fate of PMAP-23 after treatment, the peptide was labeled with FITC, and locatized by confocal laser scanning microscopy. As shown in Fig. 3, the peptide was found to be distributed throughout the plasma membrane.

The Effect of the PMAP-23 on the Regeneration of C. albicans Protoplast and Interaction between PMAP-23 and Artificial Membrane Vesicle (Phospholipid Vesicle)

To confirm whether the peptide, which showed enhanced antifungal activity, exert its antifungal effect by damaging the plasma membrane of fungal cells, protoplasts of *C. albicans* were prepared. After treatment of the protoplasts with PMAP-23, cell wall regeneration was measured. As shown in Table 3, cell wall regeneration of the PMAP-23 peptide treated protoplasts was extremely low compared to the control (no peptide treatment). We also examined whether the PMAP-23 showed the antifungal activity through pore-formation on the plasma membrane of fingi by investigating the interaction of PMAP-23 against negative charged artificial membrane liposome (PC:PS = 3:1), which mimicked the biological membrane, and monitoring the leakage of carboxyfluorescein(CF) dye

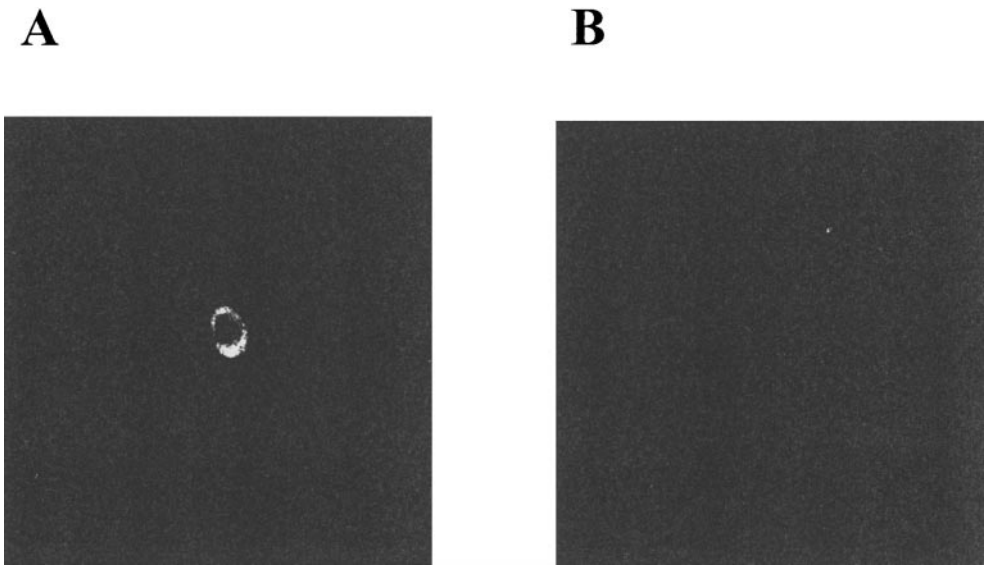

FIG. 3. Distribution of PMAP-23 in *C. albicans* as visualized by confocal laser scanning microscopy. (A) *C. albicans* cells incubated with FITC labelled PMAP-23. (B) *C. albicans* cells incubated without peptide.

trapped in liposome during incubation with PMAP-23. The result shows that CF was released from LUV (Large Unilamellar Vesicle) after treatment of 1.25 and 20 μM in a dose-dependent manner (Fig. 4).

DISCUSSION

A peptide corresponding to PMAP-23 was chemically synthesized, purified to homogeneity and shown to be correct by mass determination (2962.0 Da compared to the calculated mass of 2962.5 Da). PMAP-23 showed strong antifungal activity to yeast and filamentous fungus (Table 1, Fig. 1). In our previous study, the peptide was shown to possess potent antibacterial activity against Gram-positive and negative strains but no cytotoxic activity against human red blood cells (13). These results suggested that the antimicrobial peptide, PMAP-23, has a broad spectrum of activity to bacteria, yeast and mold but does not effect eukaryotic cells.

Growth measurement is commonly used to determine antifungal activity, but this method yield little information uipon the fungicidal mechanism. To obtain further information upon the antifungal cytotoxic mechanism of the peptide, FACScan analysis was performed after PI staining peptide treated cells.

Should the plasma membrane be damaged by ion channel or pore formation, the fungal cell membrane would be permeabilized and allow free diffusion of PI into the cytoplasm. Detection of infleux PI was analyzed on single cells by FACScan analysis. The results indicated that PMAP-23 powerfully permeabilized cells compared to the control (no peptide treated) (Fig. 2). The fluorescence signal was comparable to that of

TABLE 3

Effects of the PMAP-23 Peptide on the Regeneration of Protoplast of *C. albicans*

Peptides	F.R.[b]
Control[a]	76.2
PMAP-23	5.9×10^{-1}

[a] Control indicates no peptide treatment.

[b] Frequency of regeneration (F.R.) values were calculated by using the formula FR(%) = [(number of colonies on plate)/(number of protoplasts used)] $\times$ 100.

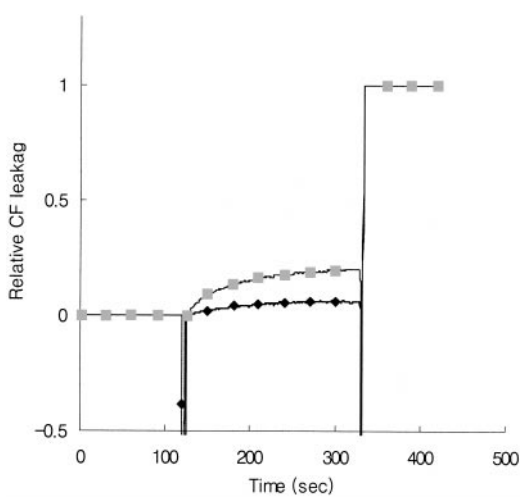

FIG. 4. Interaction of PMAP-23 with large unilamellar vesicles (LUV). The PC/PS (3:1) vesicle containing CF was incubated with PMAP-23 in the phosphate-buffered saline (pH 7.4). The CF release were monitored after the addition of PMAP-23. Twenty micromolars of PMAP-23 (■), 1.25 μM of PMAP-23 (◆).

Melittin which is a well known and potent antimicrobial peptide, and therefore, proved the antifungal effect of PMAP-23. As shown in Fig. 3, the peptide was distributed throughout the cell membrane.

The results in Table 3 show that the cell wall regeneration of the peptide treated protoplasts was extremely low compared to the control (no peptide treated) and indicate that the peptide acts on the fungal plasma membranes. Furthermore, Fig. 4 suggests that the interaction of PMAP-23 with negatively charged phospholipid vesicle is necessary for the peptide-membrane interaction event. This confirms previous data which suggested that the peptide might act on the intracellular region including the plasma membrane of *C. albicans* instead of the cell wall structure.

These results suggest that the peptide, PMAP-23 exerts its antifungal activity by causing pore formation in the cell membranes, rather than of the cell wall, by some detergent-like disruption mechanism.

The peptide, PMAP-23 has a potent antimicrobial activity but no hemolytic activity, which could make it useful as a topical antimicrobial agent, and a model peptide for studying the relationships between structure and antimicrobial activity.

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