Mol. Cells *28*, 403-406, October 31, 2009
DOI/10.1007/s10059-009-0136-6

Molecules and Cells

Candidacidal Effects of Rev (11-20) Derived from HIV-1 Rev Protein

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Rev is an essential regulatory protein for HIV-1 replication. Rev (11-20) is known as the significant region regarding the function of a nuclear entry inhibitory signal (NIS) of Rev. In this study, anticandidal effects and mechanism of action of Rev (11-20) were investigated. The result exhibited that Rev (11-20) contained candidacidal activities. To understand target site(s) of Rev (11-20), the intracellular localization of the peptide was investigated. The result showed that Rev (11-20) rapidly accumulated in the fungal cell surface. The cell wall regeneration test also indicated that Rev (11-20) exerted its anticandidal activity to fungal plasma membrane rather than cell wall. The fluorescent study using 1,6-diphenyl-1,3,5-hexatriene (DPH) further confirmed the membrane-disruption mechanism(s) of Rev (11-20). The present study suggests that Rev (11-20) possesses significant potential regarding therapeutic agents for treating fungal diseases caused by *Candida* species in humans.

INTRODUCTION

Most living organisms are frequently exposed to harmful pathogens through contact, ingestion, or inhalation (Barra and Simmaco, 1995; Hultmark, 2003). Especially, nosocomial bloodstream fungal infections remain a critical problem among hospitalised patients. *Candida* spp., including *Candida albicans,* are the fourth most common cause regarding nosocomial bloodstream infections in the USA (Edmond et al., 1999; Rangel-Frausto et al., 1999). Risk factors for such infections include solid organ and bone marrow transplantation, prolonged stays in the Intensive Care Unit (ICU), broad-spectrum antibiotics, use of invasive indwelling devices, total parenteral nutrition, and the increasing prevalence of chronically immunosuppressed patients (Bertagnolio et al., 2004; Fridkin and Jarvis, 1996). Therefore, effective and potent agents for curing human diseases, caused by not only *C. albicans* but also other human pathogenic fungi, are urgently required. Antimicrobial peptides (AMPs) are suggested as a novel model for therapeutic agents and are also known as important components of innate immunity. Their distribution throughout the animal kingdom is widespread, including bacteria, fungi, plants, insects, birds, crustaceans, amphibians, and mammals (Boman, 1996; Ganz and Lehrer, 1998; Lee et al., 2009a).

Human immunodeficiency virus type 1 (HIV-1) encodes a protein designated as Rev that binds to a cis-acting RNA target [the Rev response element (RRE)], present in all unspliced viral transcripts, and targets them for nuclear export (Pollard and Malim, 1998). Rev is constantly shuttling between cell nucleus and cytoplasm in host cells, under the control of a signal-mediated active import and export system, likely regarding the support of nuclear entry inhibitory signal (NIS) function (Kalland et al., 1994; Meyer and Malim, 1994; Richard et al., 1994; Wolff et al., 1995). Without the existence of NIS, Rev may migrate back through the nuclear pore against the well-controlled nucleo-cytoplasmic shuttling to complete its role in each compartment. Especially, residues 11-20 of the NIS (Rev (11-20), ELLKAVRLIK) comprise the conserved hydrophilic motif, which forms an amphipathic helix. Significantly, it was considered that this motif and its helical structure were important for NIS function and the HIV-1 Rev function itself (Kubota and Pomerantz, 1998). Although Rev (11-20) had potential as antifungal agents for treating the threatening *Candida* spp., anticandidal properties of this peptide had not been fully understood. In the present study, we investigated the anticandidal effects and its mechanism of Rev (11-20).

MATERIALS AND METHODS

Peptide synthesis

Peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry (Merrifield, 1986). The crude peptides were repeatedly washed with diethylether, dried in vacuum, and purified by using reversed-phase preparative HPLC on a Waters 15 μm Deltapak C_{18} column (19 × 30 cm). The purity of the peptides was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (4.6 × 25 cm; Beckman, USA). The molecular weight of the synthetic peptides was determined by using MALDI (matrix-assisted laser desorption ionization) mass spectrometer (Jungblut and Thiede, 1997).

Anticandidal activity assay

C. albicans (TIMM 1768) was obtained from the Center for

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Received August 26, 2009; accepted September 1, 2009; published online September 30, 2009

Keywords: antifungal peptide, *Candida* spp., membrane-disruption mechanism, Rev (11-20)

Table 1. Amino acid sequence and molecular mass determined by MALDI-MS

Peptide	Sequence	Calculated values	Observed values
Rev (11-20)	ELLKAVRLIK	1182.5	1183.2
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2847.4	2850.6

Table 2. Anticandidal and candidacidal activity of Rev (11-20)

	C. albicans (TIMM 1768)		*C. albicans* (ATCC 90028)		*C. parapsilosis* (ATCC 22019)	
	MIC (μM)	MFC	MIC	MFC	MIC	MFC
Rev (11-20)	10	20	20	80	10-20	80
Melittin	2.5	5	5	20	5	20
Fluconazole	20	80	20	40	20	40

Academic Societies (Japan). *C. albicans* (ATCC 90028) and *C. parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, USA). Fungal cells were cultured in YPD broth (Difco) with aeration at 28°C. Fungal cells (2×10^6 cells/ml) were inoculated into YPD broth and then 0.1 ml/well was dispensed into microtiter plates. MIC (minimum inhibitory concentration) was determined by twofold serial dilution of peptides or fluconazole (Pfizer), as a positive control compound, following the micro-dilution method and MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay (Sung et al., 2007). After 48 h of incubation at 28°C, the minimal concentration level to prevent the growth of the fungal strains was determined and defined as MIC. Growth was assayed with microtiter ELISA Reader by monitoring absorption at 580 nm. After MICs were read, the entire contents of each well with peptides or fluconazole concentrations above the MIC were plated onto Sabouraud dextrose agar (SDA) plates. Aliquots were placed in a single spot on the agar plate and, after drying, the cells were dispersed by streaking. Plates were incubated at 35°C for 48 h. MFC (minimum fungicidal concentration) was defined as the lowest peptides or fluconazole concentration that resulted in a 99.9% reduction in the starting inoculum. All assays were performed in duplicate and repeated at least twice (Cantón et al., 2004; Sóczó et al., 2007).

Dimorphic transition in *C. albicans*

C. albicans (ATCC 90028) cells by periodic subculturing were maintained in liquid YPD media. To induce and to form mycelia, cultures were directly supplemented with 20% fetal bovine serum (FBS) (McLain et al., 2000). The dimorphic transition of *C. albicans* was investigated from cultures containing 20 μM of Rev (11-20), which were incubated at 37°C for 48 h. The dimorphic transition was detected by phase contrast light microscopy (NIKON, ECLIPSE TE300, Japan) (Jung et al., 2006).

Confocal laser scanning microscopy (CLSM)

The cellular distribution of Rev (11-20) in *C. albicans* (ATCC 90028) cells was analyzed by CLSM. Cells were treated with fluorescein isothiocyanate (FITC)-labeled Rev (11-20) and incubated for 2 min at 28°C. After incubation, cells were harvested by centrifugation at 12,000 rpm for 5 min and washed with ice-cold phosphate buffered saline (PBS, pH 7.4). Visualization and localization of the peptide were performed by Laser Scanning Spectral Confocal Microscope (ZEISS LSM 5 EXCITER, Carl Zeiss, Germany).

Cell wall regeneration of fungal protoplasts

C. albicans (ATCC 90028) protoplasts were prepared by treatment with 10 mM phosphate buffer (pH 6.0), containing 0.8 M NaCl, lysing enzyme (Sigma) (20 mg/ml) and cellulase (Sigma) (20 mg/ml) for 4 h at 28°C. The protoplasts were centrifuged at 700 g for 10 min and then suspended in 50 mM Tris-HCl buffer (pH 7.5), containing 0.8 M NaCl, and 10 mM $CaCl_2$. The protoplasts were detected through phase contrast light microscopy (NIKON, ECLIPSE TE200, Japan). Peptides (at the MIC) were added to the protoplasts (2×10^4 cells), and incubated for 3 h at 28°C. The protoplasts treated with the peptides were transferred to YPD soft-agar solutions, containing 1 M sorbitol and 0.5% agar, and then spread on agar plates of YPD media containing 1 M sorbitol and 2.0% agar. The regenerated colonies were counted following incubation of the plates at 28°C for 2 d (Sung et al., 2008).

1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence analysis

Fluorescent intensities from the plasma membrane of exponential phased *C. albicans* (ATCC 90028) cells, labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, USA), were assessed to monitor changes induced by the peptides in fungal membrane dynamics. Specifically, 0.6 mM DPH was added to a final concentration of 120 μM in PBS. The cells (2×10^6 cells/ml) were treated with the peptides in the concentration range between 0 and 20 μM and incubated at 28°C for 1 h. Samples of the fungal cultures were fixed by 0.37% formaldehyde, collected and washed with PBS. The cells were frozen by using liquid nitrogen. For DPH labeling purposes, cells were thawed and resuspended in PBS. The suspended mixture was incubated with DPH solution for 45 min at 28°C, followed by washing with PBS. The fluorescent intensity was measured by using RF-5301PC spectrofluorophotometer (Shimadzu, Japan) at the wavelengths (λ_{ex} = 350 nm, λ_{em} = 425 nm) (Lee et al., 2002; 2009b).

RESULTS AND DISCUSSION

Candidacidal activity of Rev (11-20)

Peptides were chemically synthesized (Table 1), and anticandidal effects and mechanism of action of Rev (11-20) were investigated. Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), derived from the venom of the European honey bee *Apis mellifera*, was used as a positive control in this study. Melittin is one of the most representative membrane-active antimicrobial peptides, containing powerful lytic activity against both bacterial and eukaryotic cells (Blondelle and Houghten, 1991). This peptide has also been employed as a model membrane-seeking peptide to obtain energetics of phospholipids/protein interactions (Ladokhin and White, 1999).

According to the MIC test, Rev (11-20), with MIC values being measured in the 10-20 μM range, showed anticandidal

Fig. 1. The effect of Rev (11-20) on the dimorphic transition of *C. albicans*. (A) Yeast control; (B) Cells treated with only FBS; (C) Cells treated with FBS and Rev (11-20).

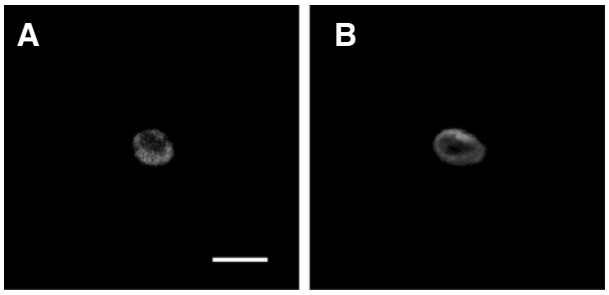

Fig. 2. Confocal laser scanning microscopy (CLSM) of *C. albicans* cells treated with the peptides. (A) Cells treated with FITC-labeled melittin; (B) Cells treated with FITC-labeled Rev (11-20). The bar corresponds to 5 μm.

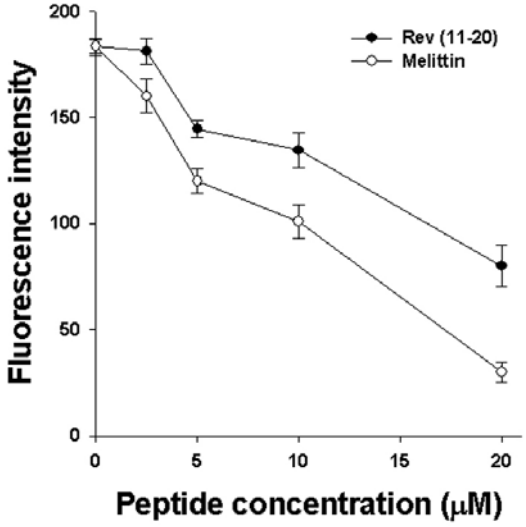

Fig. 3. DPH fluorescence intensity after the peptide treatment. Subcultured *C. albicans* cells containing peptides of various concentrations (2.5, 5, 10 and 20 μM) were incubated at 28°C for 1 h. The error bars represent the standard deviation (SD) values for three independent experiments, performed in triplicate.

activities (Table 2). Moreover, Rev (11-20), with MFC values being determined in the 20-80 μM range, had candidacidal activities. Although melittin showed much more potent anticandidal activities, Rev (11-20) exhibited more potent activities than fluconazole, a conventional antifungal agent. Rev (11-20) also exerted antifungal activities against *Aspergillus flavus* and *A. parasiticus*, with MIC values of 40 μM or the values over 40 μM (data not shown). These results suggested that Rev (11-20) had significant potential for treating human diseases caused by not only yeast but also filamentous fungi.

To further elucidate the anticandidal activity of Rev (11-20), we examined the effect on the dimorphic transition of *C. albicans*. With respect to *C. albicans*, the dimorphism plays a crucial role in pathogenesis, with mycelial shapes being predominantly found during a host tissue invasion (Alvarez-Peral et al., 2000). The result showed that Rev (11-20) not only inhibited but also destroyed the mycelial forms of *C. albicans* cells (Fig. 1). This could uphold the result of MIC test and also confirmed the antifungal activity of Rev (11-20).

Intracellular localization of Rev (11-20) in *C. albicans*

Several pathways regarding the mechanism of AMPs have been suggested, including inhibition of synthesis of specific membrane proteins, synthesis of stress proteins, arrest of DNA synthesis, breakage of single-strand DNA, interaction with DNA (without arrest of synthesis), or production of hydrogen peroxide. However, studies on both live organisms and model membranes have indicated that most AMPs provoke an increase of the permeability in cell plasma membrane. Actually, a direct correlation between antibiotic effects and permeabilization ability has been found for several antimicrobial peptides like defensins, magainins, cecropins, bactenecins, or dermaseptins (Andreu and Rivas, 1998).

In order to provide information about the mechanism of anticandidal action of Rev (11-20), *C. albicans* (ATCC 90028) was selected, which causes a variety of superficial and deep-seated mycoses, such as candidiasis. This fungus is an important pathogen regarding opportunistic infections caused by fungi, and is becoming of increasing importance in patients who are immunocompromised due to cancer chemotherapy, organ or bone marrow transplantation, or HIV infection (Hoffman et al., 2000).

To investigate the target site(s) of Rev (11-20) in *C. albicans*, CLSM was conducted. The result showed that both FITC-labeled melittin and FITC-labeled Rev (11-20) promptly interacted with and accumulated in the fungal cell surface after the peptides treatment (Figs. 2A and 2B). This indicated that the target site(s) of Rev (11-20) could be the cell surface including the cell plasma membrane or cell wall. Also, it was presumed that Rev (11-20) could contain the membrane-active mechanism like well-known antimicrobial peptides.

Membrane-disruption mechanism(s) of Rev (11-20)

To clarify the exact target site of Rev (11-20), we conducted the cell wall regeneration test with protoplasts of *C. albicans*. This test was applied as an index to assess the rate of the membrane damage by antimicrobial activity of peptides (Jung et al., 2007). The result showed that the frequency of regeneration of both melittin and Rev (11-20) was much lower than the negative control (Table 3). This indicated that Rev (11-20) exerted its antifungal activity on the cell plasma membrane, rather than the cell wall.

Subsequently, to confirm the membrane-disruptive action of Rev (11-20), changes caused by Rev (11-20) in fungal membrane dynamics were investigated by using DPH as a fluorescent probe. DPH is a hydrophobic molecule and this property makes it possible to associate with the hydrocarbon tail region of phospholipids, within the cytoplasmic membrane, without

Table 3. The effect of Rev (11-20) on the regeneration of *C. albicans* protoplasts.

Peptide	F.R.*
Control†	73
Rev (11-20)	8.2
Melittin	3.2

*Frequency of regeneration (F.R.) values were calculated by the formula: F.R.(%) = [(number of colonies on plate)/(number of protoplasts used)] × 100.
†Control indicated no peptide treatment.

disturbing the structure of the lipid bilayer (Barrow and Lentz, 1985; Vincent et al., 2004). The decrease of fluorescence intensity by Rev (11-20) indicated that the cell plasma membrane of *C. albicans* was disrupted when exposed to the peptide (Fig. 3). This result also suggested that the major site of action of Rev (11-20) was the fungal plasma membrane.

CONCLUSION

Recently, the antimicrobial peptide is one of the fastest growing models for novel therapeutic agents in the field of pharmacology and medical science generally. The motivation of our study was to suggest the novel AMP as an alternative of conventional antibiotics. In this study, Rev (11-20) exhibited the candidacidal activity with the membrane-disruption mechanism. Although the exact mechanism of Rev (11-20) must be further clarified, the current study suggested that Rev (11-20) had a momentous potential as a new therapeutic model regarding human fungal diseases.

ACKNOWLEDGMENT

This work was supported by a grant (Code 20080401-034-017) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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