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Research paper

Antimicrobial action of histone H2B in *Escherichia coli*: Evidence for membrane translocation and DNA-binding of a histone H2B fragment after proteolytic cleavage by outer membrane proteinase T

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Abstract

Previous studies have led to the isolation of histone H2B with antibacterial properties from an extract of the skin of the Schlegel's green tree frog *Rhacophorus schlegelii* and it is now demonstrated that the intact peptide is released into norepinephrine-stimulated skin secretions. In order to investigate the mechanism of action of this peptide, a maltose-binding protein (MBP)-fused histone H2B (MBP-H2B) conjugate was prepared and subjected to antimicrobial assay. The fusion protein showed bacteriostatic activity against *Escherichia coli* strain JCM5491 with a minimum inhibitory concentration of 11 μM. The lysate prepared from JCM5491 cells was capable of fragmenting MBP-H2B within the histone H2B region, but the lysate from the outer membrane proteinase T (OmpT) gene-deleted BL21(DE3) cells was not. FITC-labeled MBP-H2B (FITC-MBP-H2B) penetrated into the bacterial cell membrane of JCM5491 and *ompT*-transformed BL21(DE3) cells, but not into *ompT*-deleted BL21(DE3) cells. Gel retardation assay using MBP-H2B-deletion mutants indicated that MBP-H2B bound to DNA at a site within the N-terminal region of histone H2B. Consequently, it is proposed that the antimicrobial action of histone H2B involves, at least in part, penetration of an OmpT-produced N-terminal histone H2B fragment into the bacterial cell membrane with subsequent inhibition of cell functions.
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Keywords: Antimicrobial peptides; Frog skin; Histone H2B; Membrane penetration; OmpT

1. Introduction

Peptides with growth inhibitory activity against a wide range of bacteria and fungi play an important role in the system of innate immunity of a wide range of vertebrate and invertebrate species [1–3]. Many such peptides have been isolated and characterized from natural sources, and the number is constantly increasing. In addition, various studies have indicated that several well characterized peptides and proteins display antimicrobial activities in addition to their accepted physiological functions: e.g., phospholipase A2 [4], α-melanocyte stimulating hormone (α-MSH) [5], a protease inhibitor of the Kunitz family [6], and ribosomal proteins [7,8].

Traditionally, histones are known as major components of the nucleosome structure in eukaryotic cells and are functionally classified into two groups: linker histones (histone H1) and core histones (histone H2A, H2B, H3, and H4) that contribute to regulation of gene transcription. Recent studies have shown that histones are present not only within the nuclear cell membrane but also in mitochondria and cytosolic granules and at the cell surface, and display biological activities different

Abbreviations: CFU, colony forming unit; DIC, differential interference contrast; IPTG, isopropyl-β-D-thiogalactopyranoside; LPS, lipopolysaccharide; MBP, maltose-binding protein; MIC, minimum inhibitory concentration; OmpT, outer membrane proteinase T.

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from those in nucleosome structures (reviewed in [9]). In particular, ‘‘extranuclear’’ histones play important roles in host defense. For example, the neutrophil extracellular traps (NETs), consisting of granule proteins and chromatin released from neutrophils, form extracellular fibers at cell surface and bind Gram-positive and Gram-negative bacteria resulting in degradation of virulence factors and bacterial cell death [10]. In addition, histones with broad-spectrum antimicrobial activity have been identified in shrimp [11], fish [8,12–15], chicken [16,17], and mammals [18–22]. As well as intact histones, histone-derived fragments with potent antimicrobial activity have also been identified. Buforin I and II, isolated from an extract of the stomach of *Bufo bufo gargarizans*, is derived from the N-terminal region of histone H2A and shows potent antimicrobial activities against fungi and Gram-positive and Gram-negative bacteria [23]. Parasin I and hipposin I from the skin mucous secretions of channel catfish and Atlantic halibut, respectively [24,25], are also histone H2A-derived antimicrobial peptides. N-terminal fragments of histone H1 and H2B with broad-spectrum antimicrobial properties have been identified in the skin mucus of the Atlantic salmon (*Salmo salar*) [26] and in human wounds [27], respectively. Buforin I corresponds to the N-terminal (1–39) domain of histone H2A and is produced from nonacetylated histone H2A by pepsin and secreted into the stomach [28,29]. However, the mechanisms of action of full-length histones have yet to be determined.

In a previous study, intact and unmodified histone H2B with growth-inhibiting activity against the Gram-negative bacterium *Escherichia coli* was isolated from an extract of the skin of Schlegel’s green tree frog *Rhacophorus schlegelii* [30]. *R. schlegelli* histone H2B contains five amino acid substitutions compared with the human orthologue. In order to investigate the mechanism of antimicrobial action of histone H2B, we prepared a recombinant *R. schlegelii* histone H2B–maltose-binding protein (MBP) fusion protein whose antimicrobial actions were analyzed using an *E. coli* strain (JCM5491) that expresses by the bacterial outer cell membrane proteinase T (OmpT) and a strain BL21(DE3) in which the gene has been deleted.

2. Materials and methods

2.1. Animals

Adult *R. schlegelii* were collected from a paddy field in Ichihara City, Chiba Prefecture, Japan. The animals were kept at 23 °C under a 12 h light–12 h dark cycle and fed crickets every other day. All experiments were approved by the Animal Research Committee for Animal Experimentation of Toho University and were carried out by authorized investigators.

2.2. Collection of skin secretions and immunological detection of histone H2B

Healthy adult *R. schlegelii* specimens ($n = 6$) were injected subcutaneously with norepinephrine (150 nmol), and placed in a beaker containing a solution (15 ml) of 25 mM NaCl/25 mM NH_3COOH, pH 7.0, to collect the skin secretions. The frogs were given two further injections of norepinephrine (150 nmol) at 5-min intervals. After the final injection, the frogs were removed and the contents in the beaker were immediately acidified with trifluoroacetic acid (0.1% final concentration) and lyophilized. The lyophilized skin secretion was redissolved in 6 M urea (500 μl) and aliquots (100 μl) were blotted onto ECL plus nitrocellulose membranes (GE Healthcare Biosciences, Piscataway, NJ, USA) with Bio-Dot Microfiltration Apparatus (BioRad, Hercules, CA, USA). The rest of the solution was separated by 12.5% acrylamide SDS-PAGE followed by electroblotting onto ECL plus nitrocellulose membranes. The sheets were briefly rinsed with PBS and preincubated with 4% skimmed milk–PBS for 1 h at room temperature for blocking, and then stained with anti-human histone H2B antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Millipore, Billerica, MA, USA). The membranes were briefly rinsed again with PBS, incubated with solutions of enhanced chemical luminescent (ECL) system (GE Healthcare Biosciences), and then exposed to X-ray film (Eastman Kodak, Rochester, NY, USA). Digital images were prepared with a computer scanner and the brightness and contrast were adjusted for publication using Adobe Photoshop (Adobe, San Jose, CA, USA).

2.3. Construction of maltose-binding protein (MBP)-tagged histone H2B (MBP-H2B)

To produce the MBP-tagged fusion protein, the coding region including the initiation codon of *R. schlegelii* cDNA was amplified by PCR from an aliquot (100 ng) of the pSTBlue–*R. schlegelii* histone H2B construct [30] using a specific forward primer with a BamHI site (5′-CCG GAT CCA TGC CTG AAC CTG CCA A-3′) and a specific reverse primer with a HindIII site (5′-CCT CTA GAC TTA CTT GGA ACT GGT G-3′). The PCR conditions were as follows: 5 min at 95 °C for the DNA denaturation followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, with a final extension step of 7 min at 72 °C. The amplified DNA was purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA), digested with BamHI and HindIII (Toyobo, Osaka, Japan) and subcloned into the pMAL-c2 plasmid vector (New England Biolabs, Ipswich, MA, USA) at the appropriate sites using Takara DNA ligation kit version 2 (Takara, Ohtsu, Japan). Aliquots of *E. coli* BL21(DE3) competent cells (Stratagene, La Jolla, CA, USA) were transformed with the constructed plasmid (pMAL-H2B) and the transformants were cultured in Luria–Bertani broth (LB) (1 l) containing ampicillin (5 μg/ml) at 37 °C until the absorbance at 600 nm (A_{600}) reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and incubated for 2 h. The cells were harvested by centrifugation at $6000 \times g$ for 10 min at 4 °C, rinsed twice with cold PBS, and resuspended in lysis buffer (10 mM Tris–HCl, pH 7.0, 200 mM NaCl, 1 mM EDTA). The suspended cells were lysed by sonication, precipitated by centrifugation, and the supernatants were recovered. MBP-tagged proteins were affinity-purified from the supernatant using maltose resin using a protein fusion and

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