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Report

Relationship Between Affinity of Kn2-7 to CpG DNA and the Ability of Kn2-7 to Enhance Cellular Uptake of CpG DNA by RAW264.7 Cells

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Some α-helical antimicrobial peptides enhance the activation of immune cells induced by the recognition of DNA containing unmethylated cytosine-guanine motifs (CpG DNA). We recently found that an α-helical antimicrobial peptide FIKRIARLLRKIF, known as Kn2-7, increased CpG DNA-dependent responses in mouse macrophage-like RAW264.7 cells, and we also found that enhanced cellular uptake of CpG DNA by Kn2-7 was necessary but insufficient to augment CpG DNA-dependent responses. In this study, we clarified the relationship between the affinity of Kn2-7 to CpG DNA and the ability of Kn2-7 to enhance cellular uptake of DNA. Electrophoretic mobility analysis on a polyacrylamide gel revealed that Kn2-7 binds to CpG DNA more effectively than Kn2-7RA in which arginine residues of Kn2-7 were substituted with alanine residues, and also found that Kn2-7 binds to CpG DNA less effectively than Kn2-7KR in which lysine residues of Kn2-7 were substituted with arginine residues. The DNA-binding abilities of Kn2-7, Kn2-7RA, and Kn2-7KR correlated well with their abilities to enhance the cellular uptake of CpG DNA. In contrast, Kn2-7LA in which leucine residues of Kn2-7 were substituted with alanine residues exhibited a similar DNA-binding ability to Kn2-7, but it did not enhance cellular uptake of CpG DNA. Our results indicate that affinity to DNA is necessary for the ability of Kn2-7 to enhance cellular uptake of CpG DNA, but hydrophobicity of Kn2-7 is also necessary for the enhancement of cellular uptake.

Key words antimicrobial peptide, CpG DNA, macrophage, electrophoretic mobility analysis

INTRODUCTION

Antimicrobial peptides (AMPs) are known to play important roles in the innate host defense system.[1] AMPs are classified into several structural families, including α-helical peptides,[2,3] and contain positively charged amino acids that specifically bind to negatively charged microbial surface molecules, such as lipopolysaccharides, via electrostatic interactions. AMPs also contain hydrophobic regions that integrate the microbial membrane via hydrophobic interactions, leading to the disruption of the microbial membrane and cell death.[3] In addition, AMPs exhibit many other functions, including enhancement of the activation of immune cells induced by recognition of DNA-containing unmethylated cytosine-guanine motifs (CpG DNA).[4] Recently, we found that an α-helical antimicrobial peptide FIKRIARLLRKIF, known as Kn2-7, increased responsiveness of mouse macrophage-like RAW264.7 cells to CpG DNA, a ligand for Toll-like receptor 9 (TLR9), and we also found that enhanced cellular uptake of CpG DNA by Kn2-7 was necessary but insufficient to augment CpG DNA-dependent responses.[5] As immune responses caused by TLR9 activation have the potential to be applied to adjuvants for vaccination as well as to the treatment of immune-related diseases including autoimmune diseases and cancers,[6-8] properties of AMPs, including Kn2-7, to increase responsiveness against CpG DNA are important.

There are several AMPs known to enhance immune cell recognition of DNA through TLR9. For instance, a synthetic antimicrobial peptide KLKLLLLLKLK (herein referred to as L5), which originated from sapecin B in *Sarcophaga peregrina* (flesh fly), interacts with the TLR9 agonist oligodeoxynucleotide $d(IC)_{13}$ and associates with dendritic cells. L5 thereby promotes the uptake of $d(IC)_{13}$ and exhibits several positive effects on the adjuvant activity of $d(IC)_{13}$, while the peptide remains associated with the cell periphery.[9-11] Another peptide, human endogenous α-helical antimicrobial peptide LL-37, promotes the cellular uptake of CpG DNA and enhances the recognition of CpG DNA by dendritic cells.[12] Furthermore, LL-37 binds to endogenous DNA by electrostatic interactions and promotes the uptake of the DNA into the endosomal compartment of plasmacytoid dendritic cells, thereby enhancing TLR9 activation of dendritic cells.[13] Therefore, the immunostimulatory mechanisms of some AMPs have been reported and binding of AMPs to DNA and/or delivery of DNA into cells by AMPs are believed to be involved in the enhancement of cellular responsiveness to DNA; however, the precise relationship between the properties of AMPs and this responsiveness remain unclear.

In this study, we focused on the relationship between the CpG DNA-binding properties of Kn2-7 and enhanced cellular uptake of CpG DNA by Kn2-7. In order to analyze this relationship, we established an assay system to monitor the CpG DNA-binding abilities of the peptide and analyzed the DNA-binding abilities of Kn2-7 and its derivatives. We also com-

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pared the cellular uptake of CpG DNA in the presence or absence of the peptides.

MATERIALS AND METHODS

DNAs and Peptides CpG DNA was purchased from InvivoGen (San Diego, California, USA). The sequence was 5'-TCC ATG ACG TTC CTG ATG CT-3' (ODN 1668) and all bases contained a phosphorothioate backbone. CpG DNA labeled with fluorescein at the 3' terminus (CpG DNA-FAM) was synthesized by Nippon Gene (Toyama, Japan). Peptides were commercially synthesized by Scrum Incorporated (Tokyo, Japan) and Toray Research Center (Tokyo, Japan). Amino acid sequences of the peptides were as follows: L5: KLKLLLLLKLK; L5KA: ALALLLLLALA; Kn2-7: FIKRIARLLRKIF; Kn2-7RA: FIKAIAALLAKIF; Kn2-7KR: FIRRIARLLRRIF; Kn2-7LA: FIKRIARAARKIF. C-termini of the peptides were modified with amidation.

Electrophoretic Mobility Analysis on a Polyacrylamide Gel CpG DNA (11 μg) and the indicated molar ratio of peptide were mixed in 10 mM Tris-HCl (pH 7.4) and 270 mM glycerol to a final volume of 20 μL and incubated at 37°C for 10 min. After incubation, 2 μL of 5.4 M glycerol was added to give a final concentration of 740 mM and 10 μL of samples containing 5 μg of CpG DNA were immediately loaded on a 5% (w/v) polyacrylamide gel, which was pre-run for at least for 60 min at 70 V. The samples were subjected to electrophoresis for 70 min at 70 V using 0.5 × Tris-borate-EDTA buffer (pH 8.3) (Nacalai Tesque, Kyoto, Japan). The gel was stained with 50 mL of gel fix solution [50% (v/v) methanol and 5% (v/v) acetic acid] containing 15 μL of GelRed nucleic acid gel stain (Biotium, Hayward, California, USA) for 2 h at room temperature, and was analyzed using an Atto Printgraph UV illuminator and Atto Image Saver AE-6905CF (Atto, Tokyo, Japan).

Cell Culture and Flow Cytometry Analysis The mouse macrophage-like cell line RAW264.7 (DS Pharma Biomedical, Suita, Osaka, Japan) was cultivated as described previously[5] and flow cytometry analysis was performed as described previously.[5] Cellular uptake of CpG DNA was defined by subtracting the geometric mean of fluorescence intensity (Geo MFI) of cells without CpG DNA treatment from the Geo MFI value of cells with CpG DNA treatment.

RESULTS

Establishment of an Assay System to Monitor CpG DNA-Binding Abilities of Peptides CpG DNA-binding abilities of peptides were examined by electrophoretic mobility analysis on a polyacrylamide gel. We used L5 for the establishment of an assay system to monitor CpG DNA-binding abilities of peptides, since it has been shown previously that L5 binds with oligodeoxynucleotide and this complex formation enhances cellular uptake of oligodeoxynucleotide and activation of human and mouse dendritic cells.[10] First, we confirmed enhanced cellular uptake of CpG DNA by L5 using mouse macrophage-like RAW264.7 cells. The cellular uptake of CpG DNA was increased approximately 2.7-fold in the presence of L5 compared to that in the absence of L5 (Fig. 1A). Thus, we examined the CpG DNA-binding ability of L5. CpG DNA was mixed with or without L5 and subjected to electrophoresis. A band of CpG DNA was observed under

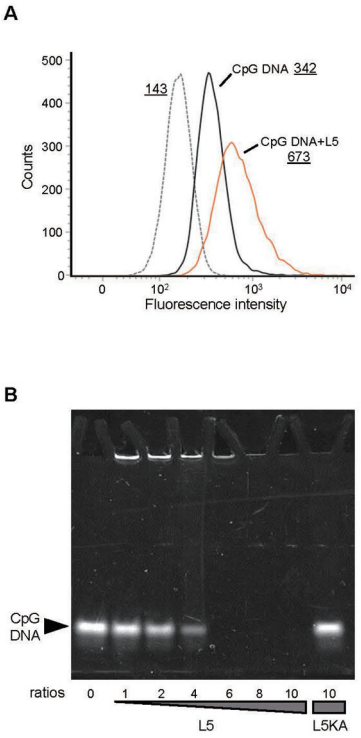

Fig. 1. L5 Binds to CpG DNA and Enhances Uptake of CpG DNA by RAW264.7

(A) CpG DNA-FAM was treated with RAW264.7 cells in the presence or absence of L5 (10 μg/mL). The fluorescence intensities of cells were analyzed by flow cytometry. Cells without treatment are indicated by the dotted gray line. The underlined values indicate Geo MFIs. (B) CpG DNA was mixed with L5 or L5KA at indicated molar ratios of peptide:CpG DNA (ratios) and subjected to electrophoresis. CpG DNA was visualized by staining. Arrowhead indicates CpG DNA bands.

the conditions in which L5 was mixed at L5:CpG DNA molar ratios of 1, 2, and 4, but not at the molar ratios of 6, 8, and 10 (Fig. 1B). These observations indicated that L5 neutralizes positive charges of CpG DNA at the molar ratio of more than 6 to form L5-CpG DNA complexes that were not observed on the polyacrylamide gel. The L5-CpG DNA complex may be too large to migrate on a polyacrylamide gel due to fluorescence observed at the top of the gel, but its exact location is unknown. In contrast, CpG DNA mixed with L5KA, in which lysine residues of L5 were substituted with alanine residues, at a molar ratio of 10, exhibited a distinct CpG DNA band indicating that L5KA did not neutralize positive charges of CpG DNA. These observations, taken together, indicate that charge-dependent binding of L5 with CpG DNA is monitored by this assay system.

CpG DNA-Binding Ability of Kn2-7 Correlates Well with the Ability of Kn2-7 to Enhance Cellular Uptake of CpG DNA The CpG DNA-binding ability of Kn2-7 was examined by the electrophoretic mobility analysis established above. A band of CpG DNA was observed under the conditions in which Kn2-7 was mixed at the molar ratios of 1, 2, and 4, but not at the molar ratios of 6, 8, and 10 (Fig. 2A), suggesting that the DNA-binding ability of Kn2-7 was similar to that of L5. In order to analyze whether the ability of Kn2-7 to bind to CpG DNA correlates with that of Kn2-7 to enhance cellular uptake of CpG DNA by RAW264.7 cells, we examined the DNA-binding abilities of the Kn2-7 derivatives Kn2-7RA and Kn2-7KR. In the former, arginine residues were

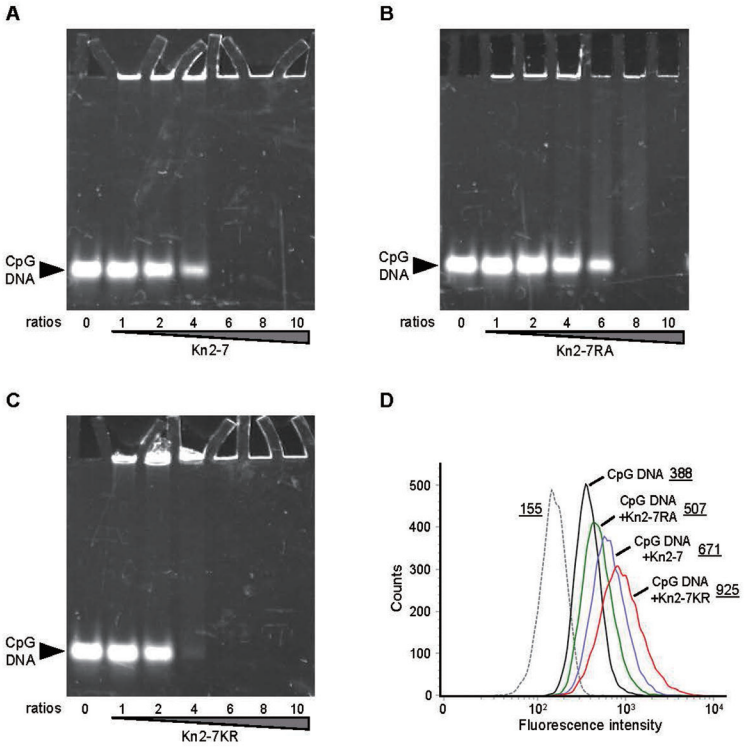

Fig. 2. CpG DNA-Binding Ability of Kn2-7 is Related to Enhance Cellular Uptake of CpG DNA

(A to C) CpG DNA was mixed with Kn2-7 (A), Kn2-7RA (B), or Kn2-7KR (C) at the indicated molar ratios of peptide:CpG DNA (ratios) and subjected to electrophoresis. CpG DNA was visualized by staining. Arrowheads indicate CpG DNA bands. (D) CpG DNA-FAM was treated with RAW264.7 cells in the presence or absence of 12.5 μg/mL Kn2-7, 12.5 μg/mL Kn2-7RA, or 12.5 μg/mL Kn2-7KR. The fluorescence intensities of cells were analyzed by flow cytometry. Cells without treatment are indicated by the dotted gray line. The underlined values indicate Geo MFIs.

substituted with alanine residues, while in the latter, lysine residues were substituted with arginine residues. CpG DNA mixed with or without Kn2-7RA or Kn2-7KR was subjected to electrophoresis. A band of CpG DNA was observed under the conditions in which Kn2-7RA was mixed at the molar ratios of 1, 2, 4, and 6, but not at the molar ratios of 8 and 10 (Fig. 2B), and a band of CpG DNA was observed under the conditions in which Kn2-7KR was mixed at the molar ratios of 1 and 2 but not at the molar ratios of 4, 6, 8, and 10 (Fig. 2C). These results, taken together, indicate that the CpG DNA-binding ability of Kn2-7 was greater than Kn2-7RA and was less than Kn2-7KR. As the degree to which these peptides enhanced the cellular uptake of CpG DNA correlated well with their abilities to bind to DNA (Fig. 2D),[5] the ability of Kn2-7 to bind to CpG DNA was related to the enhanced cellular uptake of CpG DNA by RAW264.7 cells.

Kn2-7LA Binds to CpG DNA, but Does Not Enhance Cellular Uptake of CpG DNA To further investigate the relationship between the DNA-binding ability of Kn2-7 and enhanced cellular uptake of CpG DNA by Kn2-7, we examined the properties of Kn2-7LA, in which leucine residues of Kn2-7 were substituted with alanine residues. A band of CpG DNA was observed under the conditions in which Kn2-7LA was mixed at the molar ratios of 1, 2, and 4, but not at the molar ratios of 6, 8, and 10 (Fig. 3A), indicating that the CpG DNA-binding ability of Kn2-7LA is similar to that of Kn2-7. Thus, we examined the effect of Kn2-7LA on the cellular uptake of CpG DNA by RAW264.7 cells. As shown in Fig. 3B, Kn2-7LA did not enhance the cellular uptake of CpG DNA, indicating that the hydrophobic amino acid residues of Kn2-

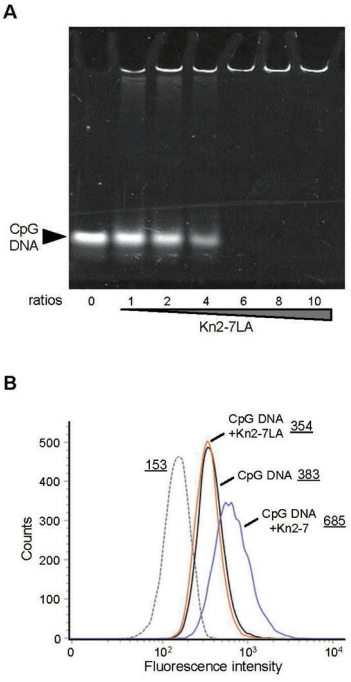

Fig. 3. Kn2-7LA Does Not Enhance Cellular Uptake of CpG DNA, Although it Does Bind to CpG DNA

(A) CpG DNA was mixed with Kn2-7LA at indicated molar ratios of peptide:CpG DNA (ratios) and subjected to electrophoresis. CpG DNA was visualized by staining. Arrowhead indicates CpG DNA bands. (B) CpG DNA-FAM was treated with RAW264.7 cells in the presence or absence of 12.5 μg/mL Kn2-7LA or 12.5 μg/mL Kn2-7. The fluorescence intensities of cells were analyzed by flow cytometry. Cells without treatment are indicated by the dotted gray line. The underlined values indicate Geo MFIs.

7 were important for the ability to enhance cellular uptake of CpG DNA.

DISCUSSION

In this study, we established an assay system to monitor the CpG DNA-binding ability of peptides, and we showed that an affinity to CpG DNA was necessary for Kn2-7 to enhance cellular uptake of CpG DNA by RAW264.7 cells. However, the enhanced uptake can be attributed not only to the affinity to CpG DNA, but also to the hydrophobicity of Kn2-7.

Some AMPs are known to act as cell-penetrating peptides (CPPs).[14-16] CPPs are short peptides of less than 20 amino acids that can traverse the plasma membranes of mammalian cells and deliver biologically active molecules such as oligonucleotides, proteins, and nanoparticles.[17] Although CPPs are derived from a variety of sources including AMPs, they have some common features: an α-helical structure, positively charged amino acid-rich motifs, and hydrophobic regions.[18] While AMPs disrupt cell membranes through pore formation using hydrophobic regions,[3] it has become increasingly clear that the CPPs enter cells by endocytosis including macropinocytosis.[19,20] Although Kn2-7 has not been demonstrated to be a CPP, this study showed the importance of charged and hydrophobic amino acids in enhancing cellular uptake of CpG DNA. In order to use AMPs, including Kn2-7, in immunomodulatory applications such as vaccine adjuvants, enhancements to the effectiveness of ligand binding, such as to CpG DNA, as well as cellular penetration, are crucial. Our findings in this study should be useful for these future applications.

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Conflict of interest The authors declare no conflict of interest.

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