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Specific interactions of the antimicrobial peptide cyclic β-sheet tachyplesin I with lipopolysaccharides

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Abstract

The cyclic β-sheet antimicrobial peptide tachyplesin I (T-SS) was found to show 280-fold higher affinity for lipopolysaccharides (LPS) compared with acidic phospholipids, whereas the linear α-helical peptide F5W-magainin 2 (MG2) could not discriminate between LPS and acidic phospholipids. The recognition site was the lipid A moiety and the cyclic structure was crucial to this specific binding. The cyclic structure also endowed the peptide with very rapid outer membrane (OM) permeabilization.

Keywords: Antimicrobial peptide; Tachyplesin I; Lipopolysaccharide; Binding isotherm; Outer membrane permeabilization

1. Introduction

Cationic antimicrobial peptides are widely recognized as important components in innate immunity of animals and insects [1–3]. These peptides have different secondary structures, such as single α-helices, helix–turn–helix hairpin motifs, β-sheets stabilized by disulfide bonds, loops, and extended structures. They also show a wide variety of mechanisms of action; some peptides kill bacteria by permeabilizing membranes, whereas others enter cells, inhibiting macromolecule synthesis. The reasons for such diversity of antimicrobial peptides and their relationships between their structures and functions are not well understood.

Tachyplesin I (T-SS) from horseshoe crab hemolymph and magainin 2 from frog skin (Fig. 1) are representative membrane-acting antimicrobial peptides with cyclic β-sheet and linear α-helical structures (in membranes), respectively [4,5]. The modes of action of these peptides are rather similar despite their different three-dimensional structures; both peptides form transient pores in acidic phospholipid membranes and translocate across the membranes upon pore disintegration [6]. However, we speculate that the cyclic β-sheet structure of T-SS should endow the peptide with some characteristics that the helical magainin peptide does not possess. In this study, we found that T-SS showed high affinity for lipopolysaccharides (LPS) compared with acidic phospholipids, whereas the magainin peptide could not discriminate between the two negatively charged lipids. Furthermore, a linear tachyplesin analog (T-Acm, Fig. 1) behaved similarly to the magainin peptide, suggesting the importance of the cyclic structure in the unique characteristics of T-SS.

2. Materials and methods

2.1. Materials

T-SS, T-Acm, and F5W-magainin 2 (MG2) were synthesized by fluoren-9-ylmethoxycarbonyl (Fmoc)-based solid phase synthesis and authenticated as described elsewhere (Fig. 1) [7,8]. LPS (smooth type and Re595) from *Salmonella minnesota*, diphosphoryl lipid A from *S. minnesota* Re595, egg yolk L-α-phosphatidylcholine (EYPC), L-α-phosphatidyl-DL-glycerol enzymatically converted from EYPC

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NPN, *N*-phenyl-1-naphthylamine; EYPC, egg yolk L-α-phosphatidylcholine; EYPG, L-α-phosphatidyl-DL-glycerol enzymatically converted from EYPC; Fmoc, fluoren-9-ylmethoxycarbonyl; LPS, lipopolysaccharides; LUVs, large unilamellar vesicles; MG2, F5W-magainin 2; MLVs, multilamellar vesicles; OM, outer membranes; SUVs, small unilamellar vesicles; T-Acm, an acyclic tachyplesin I analog with the four SH groups of tachyplesin I protected by acetamidomethyl groups; TSB, trypticase soy broth; T-SS, tachyplesin I

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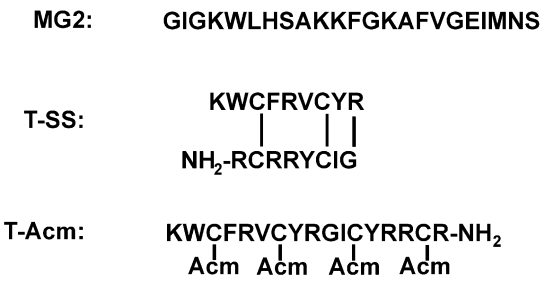

Fig. 1. Amino acid sequences of the peptides used in this study.

(EYPG), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and polymixin B sulfate were obtained from Sigma (St. Louis, MO). *N*-phenyl-1-naphthylamine (NPN) was purchased from Kanto Chemical (Tokyo, Japan). Spectrograde organic solvents were supplied by Dojindo (Kumamoto, Japan). All other chemicals were from Wako and were of special grade. The buffers were prepared with double-distilled water.

2.2. Binding

The binding of the peptides to various membranes was determined by direct ultracentrifugation assay. LPS and phospholipids were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol and chloroform, respectively. The concentrations of the stock solutions were determined by phosphorous analysis [9]. Negatively charged LPS, lipid A or EYPG was mixed with zwitterionic EYPC at a ratio of 1:9 (phosphorous basis) in ground glass tubes, and the solvent was removed in a rotary evaporator. After further drying under vacuum overnight, the lipid film was hydrated with 1 ml of a 20 μM peptide solution in 10 mM HEPES/150 mM NaCl/1 mM EDTA (pH 7.4) buffer and vigorously vortex-mixed to produce multilamellar vesicles (MLVs). The suspension was equilibrated at 30 °C overnight. The lipid vesicles were sedimented by ultracentrifugation at 155,000 × *g* for 1 h. The concentration of the free peptide in the supernatant was determined on the basis of Trp fluorescence in the presence of 20 mM SDS. Contamination by unsedimented lipids in the supernatant was checked by phosphorous analysis. Correction for free peptide concentration was carried out when necessary.

2.3. Outer membrane (OM) permeabilization

The OM permeabilization activity of peptides was investigated by NPN uptake assay [10]. Briefly, 1 ml of an overnight culture of *Escherichia coli* (ATCC25922) was added to 50 ml of fresh 3% (w/v) trypticase soy broth (TSB) medium and grown to an optical density at 600 nm of 0.4–0.6. The cells were washed with buffer (5 mM HEPES-NaOH/5 mM glucose/100 mM NaCl/5 μM CCCP, pH 7.4) and resuspended in the same buffer. For fluorescence measurements, 2-ml aliquots of cell suspension (optical density at 600 nm = 0.5) were prepared in cuvettes and 20 μl of 1 mM NPN solution in acetone was added followed by 20 μl of peptide aqueous solution. The fluorescence of NPN was monitored on a Shimadzu RF-5000 spectrofluorometer at an excitation wavelength of 350 nm and an emission wavelength of 420 nm at 30 °C. The maximal value of NPN uptake was determined after addition of polymixin B sulfate (0.64 mg/ml, 10 μl).

2.4. CD spectra

Small unilamellar vesicles (SUVs) for CD experiments were prepared by sonication of MLVs under a nitrogen atmosphere for 15 min (5 min × 3 times) using a probe-type sonicator. Tris buffer (10 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.4) was used to avoid absorption of UV light at shorter wavelengths. Metal debris from the titanium tip of the probe was removed by centrifugation. CD spectra were measured on a Jasco J-720 apparatus interfaced to an NEC PC9801 microcomputer, using a 1-mm path-length quartz cell to minimize the absorbance due to buffer components. The instrumental outputs were calibrated with nonhygroscopic ammonium *d*-camphor-10-sulfonate [11]. Eight scans were averaged for each sample. The averaged blank spectra (vesicle suspension or buffer) were subtracted.

3. Results

3.1. Binding

Fig. 2 shows binding isotherms of the three cationic peptides for 10% phosphorous, LPS-, diphosphoryl lipid

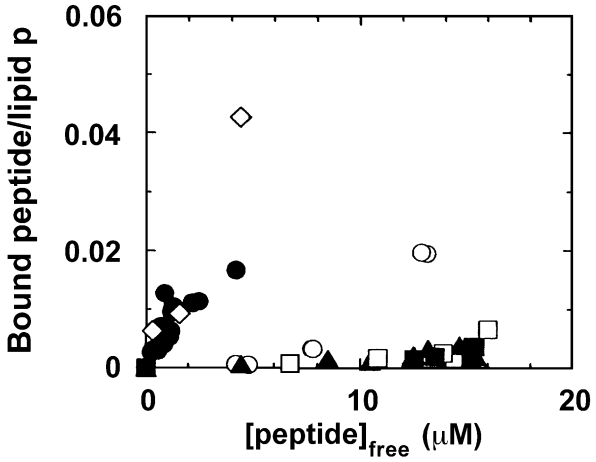

Fig. 2. Binding isotherms at 30 °C. The binding isotherms of the peptides to EYPC membranes containing 10 mol% (phosphorous basis) acidic lipids in 10 mM HEPES/150 mM NaCl/1 mM EDTA (pH 7.4) buffer were directly determined by ultracentrifugation assay. The amount of peptide bound to lipid phosphorous (mol/mol) is plotted as a function of free peptide concentration. Peptide/acidic lipid: ●, T-SS/LPS (Re595); ◇, T-SS/diphosphoryl lipid A; ○, T-SS/EYPG; ▲, T-Acm/ LPS (Re595); ■, MG2/LPS (Re595); □, MG2/EYPG.

A- or EYPG-containing EYPC membranes at 30 °C. These peptides are known to hardly bind to zwitterionic EYPC [12–14]. MG2 exhibited similar weak binding to LPS- and EYPG-loaded membranes (open versus closed squares). The initial slopes of the isotherms were about 16 M^{-1}. At higher free peptide concentrations, cooperative binding was observed, as reported previously [8]. The binding behavior of T-SS against EYPG-incorporated bilayers was similar to that of MG2, although cooperative binding started at a lower free peptide concentration (open circles). The slope of the double-logarithmic plot (not shown) was 3.3. In contrast, T-SS bound to LPS- and diphosphoryl lipid A-doped bilayers very strongly (closed circles and open diamonds). The initial slopes of the isotherms were approximately 4400 M^{-1}. On the other hand, the affinity of the linear analog T-Acm for LPS-containing membranes was as weak as that for MG2 (closed triangles). T-Acm also showed similar weak binding to EYPG-containing bilayers (data not shown).

3.2. OM permeabilization

To evaluate the membrane permeabilizing activities of these peptides, the dye leakage assay was performed using LPS-containing liposomes [15]. However, the interpretation of the results was complicated, because T-SS induced significant aggregation of vesicles (data not shown). Therefore, we examined peptide-induced perturbation of OM using the NPN assay [10], which is more biologically relevant than the liposome study. The hydrophobic dye NPN is normally excluded from OM but is partitioned into perturbed OM exhibiting increased fluorescence. In most cases, the time course of fluorescence increase was described by single exponential kinetics with a limiting value (data not shown). Therefore, the maximal limiting fluorescence (Fig. 3A) and the time ($t_{1/2}$) at which half maximal fluorescence was observed (Fig. 3B) could describe NPN uptake kinetics satisfactorily. The maximal OM perturbation was dose-dependent and in the order T-SS > MG2 ≈ T-Acm except for very low peptide concentrations, although differences were not large (Fig. 3A). The rate of OM perturbation was in the order T-SS ≈ T-Acm>MG-2 (Fig. 3B). Interestingly, the rate of T-SS-induced NPN uptake was dose-independent.

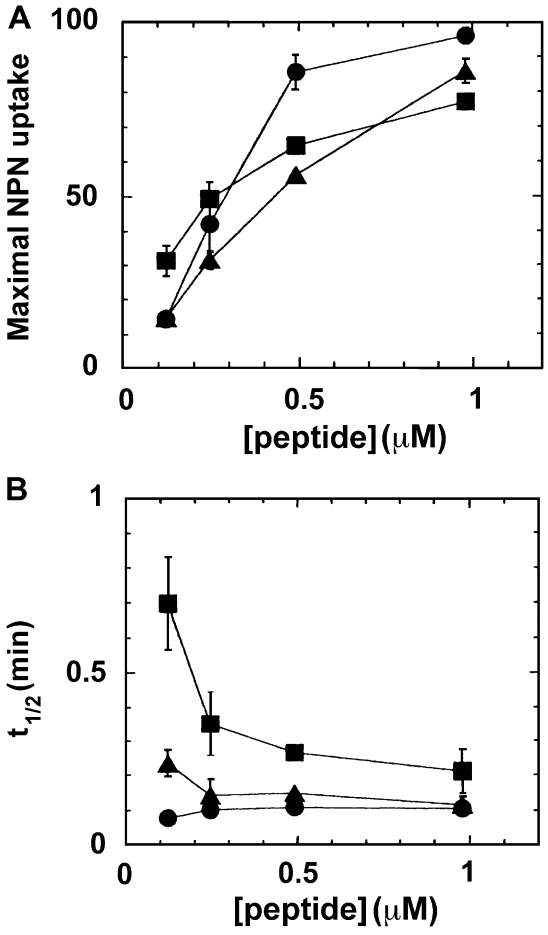

Fig. 3. Peptide-induced OM permeabilization. Permeabilization of *E. coli* OM was estimated by NPN assay [10]. The NPN uptake kinetics are expressed by (A) maximal NPN fluorescence and (B) the time ($t_{1/2}$) at which half maximal fluorescence was observed. Peptide: ●, T-SS; ▲, T-Acm; ■, MG2.

3.3. CD spectra

Secondary structures of the tachyplesin peptides were estimated by CD spectrometry (Fig. 4). T-SS in aqueous solution adopts a conformation consisting of an antiparallel β-sheet and a type II β-turn with flexible N- and C-termini [16]. The CD spectrum of T-SS in buffer (broken trace in Fig. 4A) exhibited two positive bands (230 and 198 nm) and one negative band (207 nm), as reported previously [17,18]. The peak at 198 nm is characteristic of antiparallel β-sheet structures, whereas the other two bands are assignable to turn structures [19]. The presence of EYPG/EYPC bilayers markedly changed the CD spectrum (dotted trace in Fig. 4A). The positive band at 230 nm was shifted by 3 nm and enhanced in intensity. A strong negative band and a shoulder were observed at 203 and 214 nm, reminiscent of turn and antiparallel β-sheet structures, respectively [19]. The sheet and turn structures as well as the flexible termini might be more stabilized in membranes, resulting in strong CD signals. Our infrared study indicated that T-SS mainly forms an antiparallel β-sheet that lies on the surface of an acidic phospholipid-containing membrane [13]. In the presence of LPS-containing vesicles (solid trace in Fig. 4A), the CD spectrum was further intensified.

In contrast to T-SS, T-Acm lacking S–S bridges assumed a random structure in solution (broken trace in Fig. 4B), similarly to a linear T-SS analog with the four Cys residues replaced by Ala [18]. The spectrum in the coexistence of LPS-containing vesicles showed a shallow minimum at 217 nm (solid trace in Fig. 4B), indicating an induction of a β-sheet structure [19]. All spectra in the presence of vesicles

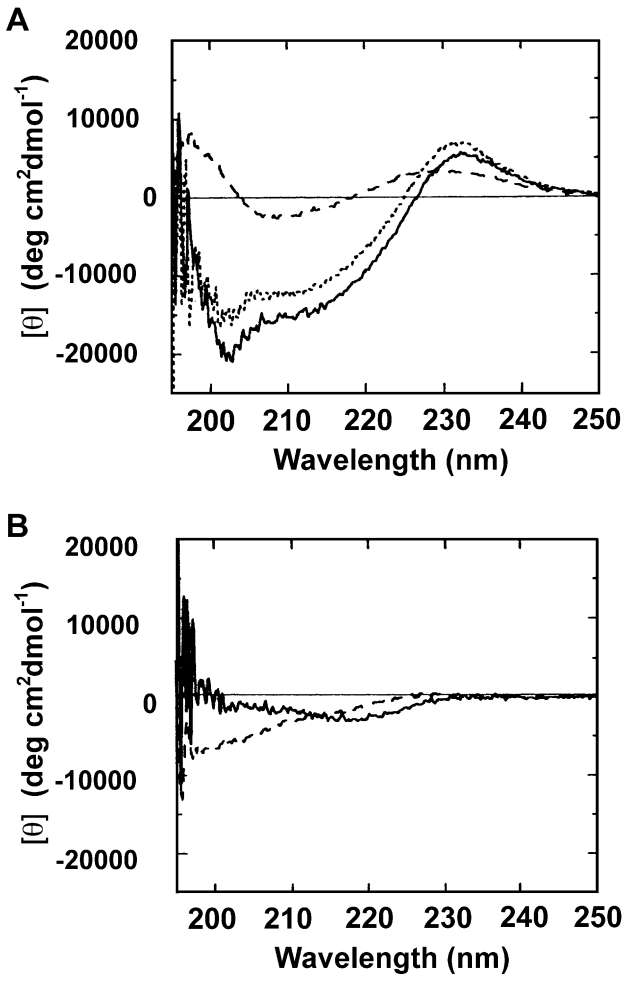

Fig. 4. Secondary structure. CD spectra of 20 μM T-SS (A) and T-Acm (B) were obtained in 10 mM Tris/150 mM NaCl/1 mM EDTA (pH 7.4) buffer (broken trace) and in the presence of 1 mM LPS (Re595)/EYPG (1/9, solid trace) and EYPG/EYPC (1/9, dotted trace) SUVs at 30 °C.

were those of membrane-bound forms, because further addition of lipids did not markedly alter the spectra.

4. Discussion

Elucidation of interactions between antimicrobial peptides and LPS are important not only for understanding the mechanism of killing of gram-negative bacteria but also for development of anti-endotoxin agents. T-SS and magainins are known to bind to LPS [4,15,20] similarly to other antimicrobial peptides [10,21]. These peptides also show affinity for acidic phospholipids [8,12,13]. Magainin–acidic phospholipid interactions have been extensively investigated [22] and the acidic phospholipid selectivity of the peptide can be explained by simple electrostatic interactions [23]. However, it is not clear whether interactions between the peptides and LPS are specific or merely electrostatic interactions as in the case of magainin–acidic phospholipid. EYPC bilayers doped with LPS or EYPG are a suitable system in which to address this question. We used LPS from *S. minnesota* because their chemical structures have been extensively studied [24–27] and they are readily available commercially. We also tried to determine binding isotherms using smooth-type LPS. However, experimental problems such as incomplete free/bound separation hampered precise determination.

Fig. 2 clearly shows that T-SS discriminates between LPS and EYPG and that the lipid A moiety of the LPS constitutes the binding site, although the secondary structures were similar upon binding to both types of lipid (Fig. 4A). The 280-fold difference in partition coefficient between LPS and EYPG corresponds to a Gibbs free energy difference of 14 kJ/mol. The cyclic structure of T-SS was crucial for this specific binding because T-Acm did not show high affinity for LPS. The LPS-bound conformation of T-Acm was also rather different from that of T-SS (Fig. 4). Polymyxin B, a strong LPS binder, also has a cyclic structure and recognizes lipid A [28]. The LPS binding activity of cyclic bactenecin was reported to be significantly reduced upon linearization [29]. In contrast to T-SS, the affinity of MG2 for LPS was comparable to that for EYPG. That is, magainin's preference of LPS over EYPC is a result of simple electrostatic interactions. T-SS and MG2 exhibited cooperative binding to EYPG-containing bilayers, in keeping with oligomeric pore formation in this type of membrane [8,30,31].

The first step to antimicrobial action against gram-negative bacteria is OM permeabilization, which was estimated by the NPN assay. The minimum inhibitory concentration against *E. coli* is in the order T-SS (0.5 μM) < T-Acm (2 μM) < MG2 (20 μM) [32]. In contrast, the extent of NPN uptake for T-SS was the largest, yet not markedly different from those of the other peptides (Fig. 3A). T-SS also more strongly permeabilizes negatively charged phospholipid membranes mimicking inner membranes than T-Acm [13] and MG2 [32], suggesting that permeabilization of both outer and inner membranes contributes to overall antimicrobial activity. It should be noted that in the OM permeabilization experiments, peptide binding was much more complete than in the binding experiments (Fig. 2) using EYPC-based membranes containing only 10% LPS. The NPN assay also revealed unique properties of T-SS: the uptake rate of T-SS was very rapid (several seconds) and dose-independent (Fig. 3B). The latter observation suggested that the mode of OM permeabilization by T-SS is an "all-or-none" process. That is, some bacterial cells undergo rapid and complete OM permeabilization, whereas the rest remain intact.

In conclusion, the cyclic β-sheet structure of T-SS endows the peptide with LPS recognition ability and fast OM permeabilization activity. Thus, this study provides an example of a relationship between structural diversity and functional variety of antimicrobial peptides.

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