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Flow cytometric analysis of the contributing factors for antimicrobial activity enhancement of cell-penetrating type peptides: Case study on engineered apidaecins

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ABSTRACT

Contributing factors for the antimicrobial activity enhancement of N-terminally engineered mutants of cell-penetrating apidaecins were analyzed based on their cell-penetration efficiency. The flow cytometric analysis of the engineered apidaecins labeled with carboxyfluorescein (FAM) revealed their enhanced cell-penetrating efficiencies into *Escherichia coli* that should be one of key factors causing the enhanced antimicrobial activity. It is noteworthy that, for one mutant, the enhancement in antimicrobial activity (18-fold higher than wild type) was greater than that of cell penetration (5.9-fold), suggesting that the N-terminal mutation may reinforce both interaction with unidentified intracellular target(s) and cell-penetration efficiency.

1. Introduction

A wide diversity of antimicrobial peptides (AMPs) has been found in various organisms ranging from prokaryotes to eukaryotes. AMPs have bactericidal and/or bacteriostatic effects against extensive strains of bacteria, and have also been shown to display antifungal activity [1,2]. AMPs are expected to be useful for food and pharmaceutical applications, because they have effectiveness against multidrug resistant bacteria but no toxicity towards eukaryotic cells. Many AMPs have been reported to kill bacteria by interacting with cell membrane through pore-forming and/or acting in a lytic manner [3]. On the other hand, proline-rich AMPs exert their antimicrobial action through interacting with intracellular targets [4]. In such a case, the antimicrobial activity of AMPs is comprised of two distinct events, (i) cell-penetration and (ii) interaction with target molecule(s). The efficiencies of both of these events are thought to contribute to the antimicrobial activity.

Apidaecin is a typical proline-rich AMP obtained from the hemolymph of honeybees immunized with bacteria. The peptide is effective against an extensive number of Gram-negative and a few Gram-positive bacteria [5,6]. The intracellular target(s) of apidaecin has not been fully identified, although the translation system [7] and chaperones [8,9] have been proposed as candidates. In our previous study, several apidaecin mutants with increased activity were acquired by region-limited mutagenesis at the N-terminal three residues [10], which had been found as a variable region based on the functional mapping [11], using the in vivo monitoring assay system [12]. For further engineering of apidaecin towards higher antimicrobial activity, elucidation of the mechanism of activity increases in terms of above-described two factors would be useful, because the information might enable the rational design of peptide based on the structure/function relationship. To this end, development of measuring method for cell-penetration efficiency of apidaecin mutants was necessary because a target interacted with apidaecin was not clearly identified.

Therefore, in this study, we attempted to quantify the cell-penetrating efficiency of the highly active apidaecin mutants by monitoring their translocation using a fluorescent tag. Fluorophore-labeled AMPs have been used for observing localization of the peptides in the cells [8,9,13]. In addition, flow cytometric analysis of the cells treated with fluorescent peptides could measure their concentration in the cells that can be an indicator of translocating efficiency of the peptides [14,15]. Here we applied this flow cytometric analysis to quantitatively compare cell-penetrating efficiencies of engineered peptides for elucidating mutational effect. In addition, connection of the cell-penetration with antimicrobial activity provided an insight on the intracellular activity of the engineered peptides.

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2. Materials and methods

2.1. Peptide labeling and fluorescence analysis

The wild-type and engineered apidaecins were manually synthesized starting with the Fmoc-Leu-Alko-PEG resin (Watanabe Chemical, Japan), as described [10]. FAM [5-(and-6)-carboxyfluorescein] was coupled by treating the resin-bound peptide (30 μmol) with a 0.8 M equivalent of FAM succinimidyl ester in 300 μL DMF (*N*,*N*-dimethylformamide) and a 1.5 M equivalent of DIPEA (*N*,*N*′-diisopropylethylamine). The crude peptide cleaved from the resin was purified by preparative HPLC and the identity of the product was confirmed by MALDI-TOF mass spectrometry using a Voyager-DE STR-H (Applied Biosystems) with α-cyano-4-hydroxycinnamic acid as a matrix. The concentrations of the peptides were determined by ninhydrin-based quantitative amino acid analysis [16] using a JLC-500V amino acid analyzer (JEOL, Japan).

Fluorescence responses of the FAM-labeled peptides were determined to calibrate the intensities of the labeled peptides. The fluorescence intensities of 3.0 nM FAM-labeled apidaecin solutions in Dulbecco's phosphate buffered saline (−) (PBS) were measured using a spectrofluorophotometer RF-1500 (Shimadzu, Japan), with excitation set at 488 nm and emission at 530 nm. The relative fluorescence responses of the engineered peptides compared to that of FAM-K-WT (1.0) were: FAM-K-(1G-17), 1.2; FAM-K-(1C-20), 0.92; FAM-K-(Ap4-18), 1.3. The standard deviation was less than 0.04 for three trials.

2.2. MIC assay

The minimum inhibitory concentration (MIC) and the sub-inhibitory concentration, the latter of which was defined as the maximum concentration of peptide allowing cells to grow, were determined using *Escherichia coli* JM109 as the test strain, as described [10].

2.3. Treatment of cells with FAM-labeled apidaecins

E. coli cells were treated with labeled peptides for confocal laser microscopy and flow cytometry as follows. A 50-μL overnight-culture was transferred into 3 mL fresh LB medium and further cultured until the OD_{600} reached 0.4. Then, 40 μL aliquot was combined with 10 μL FAM-labeled peptide (concentrations are indicated in Fig. 3). The mixture was incubated for 1 h at 30 °C. The supernatant was removed after centrifugation at 2400*g* for 10 min. After three washes with 50 μL PBS, the cells were suspended in 50 μL PBS. For microscopic observation, lipophilic styryl dye FM4-64 (Invitrogen, USA), which emits red fluorescence when inserted into the lipid membrane [17], was added at a final concentration of 25 μM. For flow cytometry, 10-fold diluted suspension was used.

2.4. Confocal laser microscopy

Confocal fluorescence images were obtained using a Zeiss LSM510 (Carl Zeiss, Germany). The microscope was equipped with an objective lens (40×), an excitation filter (488 nm, green) or (543 nm, red), a dichroic mirror (505 nm) and an emission filter (505–530 nm, green; 650 nm, red). Green and red fluorescence images of the same cell were observed sequentially to create overlay images.

2.5. Flow cytometry

A flow cytometer FACSCanto (BD, USA) with a 488-nm solid laser was used, and the signal of 515–545 nm was collected. The median fluorescence intensity was normalized by dividing by the relative fluorescence response of peptides mentioned above. The intracellular amounts of peptides are shown as relative values to that of 0.38 mM FAM-K-WT (Fig. 3).

3. Results

3.1. Synthesis of FAM-labeled apidaecins

We labeled apidaecins with FAM, which is commonly used as a fluorescent reporter in imaging studies, to monitor the cell penetration. Mutagenesis studies previously demonstrated that the C-terminal half of apidaecin was essential for antimicrobial activity [11,18], thus modification of the C-terminal likely inactivates the peptides. Additionally, N-terminally FAM-labeled pyrrhocoricin, a proline-rich AMP, in which a Lys residue was inserted as a spacer between the N-terminus of peptide and the labeled substance, retained antimicrobial activity [19]. Therefore, we used the N-terminal α-amino group of the extra Lys as a labeling site (Table 1). Two engineered apidaecins (1C-20 with the highest activity among mutants, and 1G-17 with moderate activity) were chosen for the analysis. In addition, FAM-labeled Ap4-18, the N-terminal three residues truncated form, was prepared for investigating the role of the N-terminal three residues.

The antimicrobial activities of these FAM-labeled peptides were evaluated by MIC assay (Table 1). The activities of the peptides were decreased by FAM-labeling, as observed for the case of pyrrhocoricin [10,19]. However, the two engineered apidaecins, 1G-17 and 1C-20, with FAM exhibited higher activity compared to the labeled wild-type peptide, indicating that the FAM-labeling did not impair the activity enhancement of the engineered peptides. Therefore, the FAM-labeled apidaecins were used to evaluate the effect of N-terminal mutations on the cell-penetrating potency. Ap4-18 with FAM had lowered antimicrobial activity as well as non-labeled Ap4-18 [18].

3.2. Microscopic observation of cell penetration of the apidaecins

The localization of FAM-labeled apidaecin in *E. coli* cell was observed under confocal laser microscopy. Cells treated with the wild-type, and the engineered apidaecins at their MICs exhibited homogeneous green fluorescence characteristic of FAM (Fig. 1). The overlay of images verified that the FAM-labeled apidaecin had translocated into the cell interior. Thus, the FAM-labeling was suitable for measuring the amount of intracellularly translocated peptides. During the experiment, the obvious bacteriolysis that is frequently caused by membrane damage was not observed upon any of the apidaecin treatments.

3.3. Measurements of intracellular fluorescence using flow cytometry

To quantify the amount of peptide translocated into the cells, the fluorescence intensity in cells treated with labeled apidaecins was measured with flow cytometry. The fluorescence exhibited a

Table 1
Sequence and antimicrobial activity of the FAM-labeled peptides used in this study.

Peptides	Sequence	MIC (μM)[a]
FAM-K-WT (wild-type)	FAM-KGNNRPVYIPQPRPPHPRL	190–290
FAM-K-(1G-17)	FAM-KVVRRPVYIPQPRPPHPRL	50–74
FAM-K-(1C-20)	FAM-KRVRRPVYIPQPRPPHPRL	8–16
FAM-K-(Ap4-18)	FAM-KRPVYIPQPRPPHPRL	280–370

[a] MIC (minimum inhibitory concentration) was displayed with sub-inhibitory concentration (larger number).

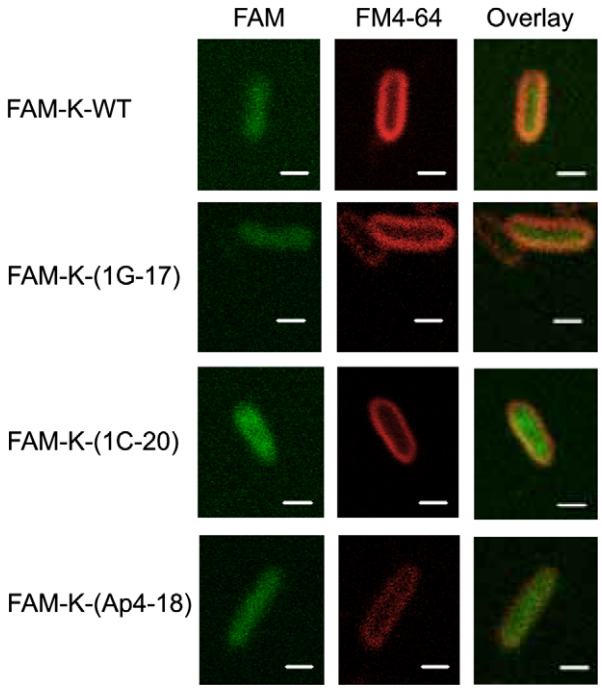

Fig. 1. Microscopic observation of *E. coli* treated with FAM-labeled apidaecins. Cells were treated with FAM-labeled wild-type and engineered apidaecins at their MIC levels, which are listed in Table 1. Bar indicates 1 μm.

homogeneous distribution within the cells, and was not decreased by three cell washes (Fig. 2), indicating that the translocation was irreversible. The result agreed with the previous result obtained with a radio-labeled wild-type apidaecin [7]. The entry of FAM alone into cells was negligible (data not shown).

Next, we investigated the cell-penetrating efficiency of the peptides by measuring the cells treated at various peptide concentrations. The result in Fig. 3 indicated that the amount of intracellular peptide linearly increased with the increase in concentration of the extracellularly applied peptide. The slopes of the linearly plotted

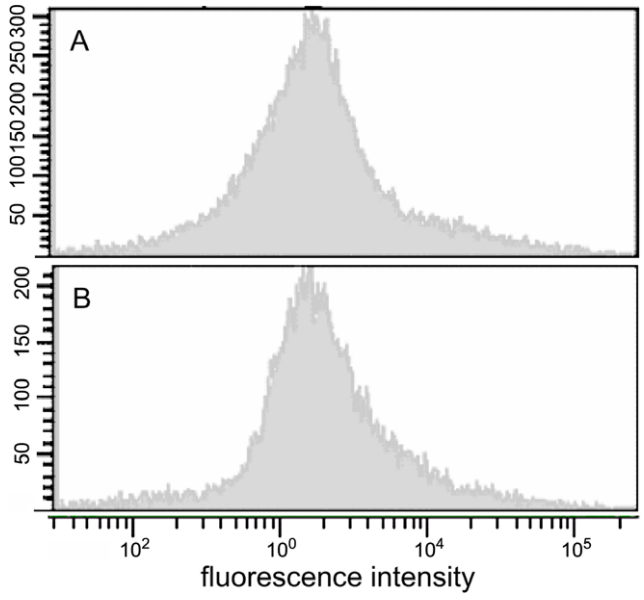

Fig. 2. Flow cytometric analysis of *E. coli* treated with FAM-labeled wild-type apidaecin. (A) Cells without wash. (B) Cells after three washes with PBS buffer.

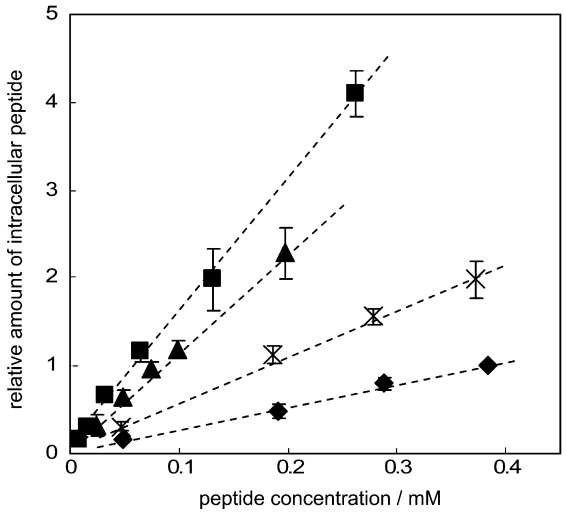

Fig. 3. Relative fluorescence intensity of *E. coli* treated with FAM-labeled apidaecins. Fluorescence was measured by flow cytometry. The data is the average of at least three trials and displayed as a relative value to that of cells treated with 0.38 mM FAM-K-WT: FAM-K-WT (diamond), FAM-K-(1C-20) (square), FAM-K-(1G-17) (triangle), FAM-K-(Ap4-18) (cross).

curves indicate the cell-penetrating efficiencies of these peptides. The slopes of 1G-17, 1C-20 and Ap4-18 were 4.5, 5.9 and 2.1-fold higher than that of the wild-type peptide, respectively. Thus, the mutated peptides possessed enhanced cell-penetrating efficiency than the wild type. Furthermore, the result indicated the importance of N-terminal region of apidaecin for cell-penetration, being consistent with the previous result that truncated apidaecin fragment with lacking eight N-terminal residues entered *E. coli* cells very inefficiently [13].

4. Discussion

It has been reported that the synthetic apidaecin with exclusively D-amino acid substituents (D-apidaecin) was temporarily associated with cells, but was then disassociated by washes [7]. Based on this finding, the cell-penetrating action of apidaecin was thought to consist of at least two steps: a physicochemical interaction with the cell membrane and a stereo-specific interaction with membrane-bound proteins. Regarding the cell-penetrating mechanism, it should be noted that the amount of translocated apidaecins (wild-type and mutants) into *E. coli* was proportional to the concentration of extracellularly applied peptides (Fig. 3). A similar phenomenon was observed by measuring the uptake of wild-type apidaecin into a model membrane using a quartz crystal microbalance (QCM) [20]. The result in the native bacterial membrane was mimicked in the model membrane, suggesting that the physicochemical effect was a dominant factor in the efficiency of the transmembrane action of apidaecin. Therefore, the interaction with the phospholipid membrane, rather than membrane-bound proteins, is likely to contribute to the enhanced apidaecin uptake. In fact, the engineered peptides (1G-17 and 1C-20) gained cationic and hydrophobic residues, such as Arg and Val, at the N-terminal. Generally, the cationic and amphipathic character of the N-terminus tends to enable the peptides to associate with and insert into the negatively-charged microbial membrane, leading to the higher activity. Indeed, many rationally designed artificial peptides have been created based on a knowledge of the biophysical properties of AMPs (for review, see [21]). Thus, rational modification of the N-terminal sequence, such as by increasing its charge and/or amphipathicity, may further enhance the antimicrobial activity of apidaecin.

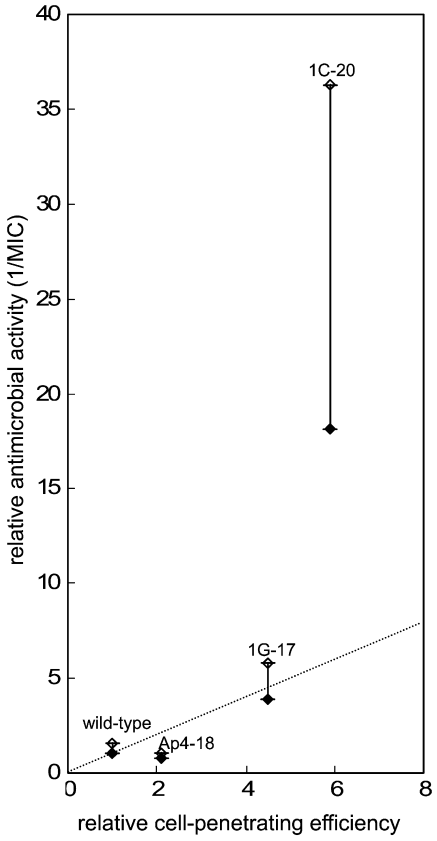

Fig. 4. Plots of cell-penetrating efficiency of FAM-labeled peptides versus their antimicrobial activity. Data was presented as relative value to those of the wild-type peptide. Cell-penetrating efficiencies were determined based on the slopes of plots in Fig. 3. The antimicrobial activities were presented by the range of inverse numbers of MIC (black symbol) and sub-inhibitory concentration (white symbol). Dashed line indicates the line of $y = x$: x (relative cell-penetrating efficiency), y (relative antimicrobial activity).

The combination of flow cytometry-based analysis of cell-penetrating efficiency and MIC assay consequently provided a useful insight on the effects of mutations on intracellular activity, namely strength of interaction with intracellular target molecule(s). Fig. 4 showed the plot of relative cell-penetrating efficiency versus relative antimicrobial activity. There were different patterns for activity increases. In the case of the 1G-17 mutant, the increase in cell-penetrating efficiency (4.5-fold) was near the enhancement in antimicrobial activity (4.0-fold), suggesting that higher cell-penetrating efficiency was a major factor for enhanced antimicrobial activity. In contrast, the activity increase of 1C-20 mutant (18-fold) was greater than its enhancement in the cell penetration (5.9-fold). This could suggest that the mutant gained higher intracellular activity than the wild type. On the other hand, the lowered activity (0.8-fold) of the Ap4-18 mutant was accounted for by a remarkable decrease in intracellular activity than slight increase in the cell-penetration efficiency (2.1-fold). These results suggested that the N-terminal region of apidaecin contributed to both the cell-penetration and inhibition of target molecule(s). In fact, the N-terminus of apidaecin has been proposed as an inhibitor domain interacting with intracellular targets by analogy with pyrrhocoricin, in which the C-terminally truncated peptide interacted with its target, DnaK [8,19]. The results in this study supported this hypothesis.

In this work, we have demonstrated that enhancements in the antimicrobial activities of apidaecin mutants can be tracked by flow cytometric analysis in terms of two factors, cell-penetrating efficiency and interaction to intracellular targets. To date, there is no report on connecting the flow cytometric monitoring of the membrane translocation of AMPs to their antimicrobial activities. The flow cytometry-based method described here was convenient tool for characterizing AMPs since it is applicable even if target molecule(s) were not identified.

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