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The Antimicrobial Peptide Polyphemusin Localizes to the Cytoplasm of *Escherichia coli* following Treatment

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The horseshoe crab peptide polyphemusin I possesses high antimicrobial activity, but its mechanism of action is as yet not well defined. Using a biotin-labeled polyphemusin I analogue and confocal fluorescence microscopy, we showed that the peptide accumulates in the cytoplasm of wild-type *Escherichia coli* within 30 min after addition without causing substantial membrane damage.

Polyphemusin I is a member of a family of antimicrobial peptides isolated from the hemocytes of the American horseshoe crab, *Limulus polyphemus* (3). It is 18 amino acids long and is stabilized into an amphiphilic, antiparallel β-hairpin by two disulfide bridges. It has a high affinity for lipopolysaccharide (3) and excellent antimicrobial activity against gram-negative and gram-positive bacteria, with MICs often less than 1 μg/ml, demonstrating rapid killing within 5 min of treatment (10).

Model membrane studies have shown that this peptide interacts preferentially with negatively charged membranes and induces lipid flip-flop between membrane leaflets at concentrations that show little or no disturbance to bilayer integrity (9). Polyphemusin I is able to translocate membrane bilayers and gain access to the interior of vesicles (6, 9) and has recently been shown to induce negative membrane curvature strain (5), a property that may be involved in the translocation process. Indeed, peptides from other structural classes, including buforin II (4) and pyrrhocoricin (2), have also been shown to translocate across membranes and are proposed to act on intracellular targets in eliciting their antimicrobial activity.

To further characterize the antibacterial action of the polyphemusins, it was of significant interest to determine where these peptides localize on or within the bacterium following treatment. While translocation has been inferred from model membrane assays, this finding has yet to be confirmed by whole-cell assays. To accomplish this, we synthesized a polyphemusin I analogue with a single C-terminal biotin label. This peptide, PM1-biotin, was then characterized and compared to the native polyphemusin I. To determine peptide localization, fluorescence and confocal microscopy were performed after treatment of a wild-type *Escherichia coli* strain with PM1-biotin. The data clearly indicate that polyphemusin translocated into *E. coli* with only modest cytoplasmic membrane disruption and caused disorganization of cytoplasmic structures.

Both polyphemusin I (RRWCFRVCYRGFCYRKCR-NH_2) and the free C-terminal cysteine derivative polyphemusin (PM1-Cys, RRWCFRVCYRGFCYRKCRC-NH_2) were synthesized by the *tert*-butoxycarbonyl method, folded, and purified at the Peptide Synthesis Facility, Biomedical Research Centre, University of British Columbia. Correct disulfide bond formation (between cysteine residues 4 to 17 and 8 to 13) of the purified peptides was confirmed by matrix-assisted laser desorption ionization mass spectrometry and further verified by circular dichroism (CD) spectroscopy (data not shown). PM1-Cys was labeled with biotin by use of N^{α}-(3-maleimidylpropionyl)biocytin and the method recommended by Molecular Probes (Eugene, OR). The resulting PM1-biotin was purified by reverse-phase chromatography and confirmed by matrix-assisted laser desorption ionization mass spectrometry and was found to have the expected molecular weight of 3,079 (data not shown). In addition, the biotin label did not affect the overall structure of polyphemusin I, as the CD spectra of labeled and unlabeled peptide in Tris buffer were nearly identical (data not shown).

To determine the influence of biotinylation on the antimicrobial activity of polyphemusin I, MICs were determined using the broth microdilution method with Mueller-Hinton medium (Difco Labs, Detroit, MI) (7); the MIC was defined as the lowest peptide concentration at which no growth was observed after an overnight incubation at 37°C. MIC assays were performed three separate times, and the mode values were recorded. The MIC of polyphemusin I against wild-type *E. coli* UB1005 was found to be 0.25 μM (0.6 μg/ml), which is similar to previously published values (6), and the MIC of PM1-biotin was twofold greater at 0.5 μM (1.5 μg/ml), indicating that the addition of biotin had a minimal effect on the MIC.

To further characterize the antimicrobial activity of PM1-biotin, killing curves were performed to determine the kinetics of killing. Killing curves, at peptide concentrations 10-fold higher than the MIC, were performed using *E. coli* UB1005, as previously described (10). A representative trial from three independent experiments is shown (Fig. 1). Complete killing by polyphemusin I was observed within 5 min, consistent with previous studies (10). PM1-biotin showed similar killing kinetics but incomplete killing at the concentration tested (5 μM, or approximately 10× MIC); however, a 99.9% reduction in the number of viable cells indicated that the peptide retained substantial antimicrobial activity.

Fluorescence microscopy was performed to determine the effects of polyphemusin on bacterial cells (Fig. 2). *E. coli* UB1005 was grown to mid-log phase in LB broth (optical density at 600 nm of 0.3 to 0.5) and used to inoculate 10 mM

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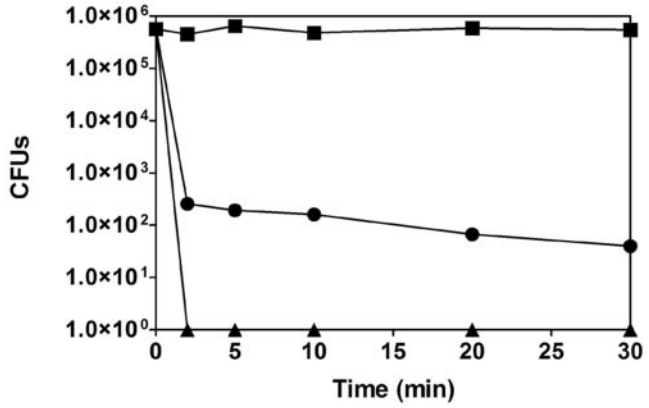

FIG. 1. Killing of *E. coli* UB1005 by polyphemusin I (triangles) and PM1-biotin (circles). Killing curves were performed at 10× peptide MIC and represent results from one experiment of three that demonstrated similar trends. A non-peptide-treated control experiment (squares) was also performed.

Tris, 100 mM NaCl, pH 7.4 (5×10^6 CFU/ml), preincubated at 4°C or 37°C with or without 0.25 μM and 2 μM PM1-biotin. The solutions were incubated with shaking for 30 min at their respective temperatures, and cells were pelleted, maintaining temperature, and resuspended in 5% glutaraldehyde (Canemco, Montreal, Quebec, Canada) in phosphate-buffered saline (PBS) at room temperature for 10 min. The cells were washed with PBS and treated with 0.2% Triton X-100 in PBS for 2 min (a non-Triton-treated control experiment was also performed by incubation with PBS alone). The cells were pelleted, suspended in a solution of 20 μg/ml streptavidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR), and incubated at room temperature for 30 min. The cells were washed twice with PBS, resuspended in Vectashield with DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride) (Vector Labs, Burlingame, CA), and used to prepare slides for visualization by fluorescence and confocal microscopy. Fluorescence microscopy was performed with a Zeiss Axioskop fluorescence microscope with a 100× oil immersion lens. Confocal microscopy was performed with a Bio-Rad Radiance Plus inverted confocal microscope with a 100× oil immersion lens. Confocal stacks were processed with ImageJ (1).

E. coli cells treated with PM1-biotin at 37°C appeared as green rods, with fluorescence throughout the cell, indicating the presence of the peptide in the cytoplasm (Fig. 2E and F). Treatment with 0.25 μM PM1-biotin (one-half MIC) did not appear to affect membrane integrity on a macroscopic level in wild-type *E. coli* (Fig. 2E). Clear, defined membranes were observed and cell lysis was not apparent, as the DAPI-stained DNA was present inside each cell in a condensed state similar to that with the untreated samples (Fig. 2A and D). Further increasing the peptide concentration to 2 μM (4× MIC) did not have any increased effect on macroscopic membrane integrity (Fig. 2F). Interestingly, the DAPI-stained DNA in the peptide-treated samples appeared less condensed than that in untreated, control cells (Fig. 2A and D, respectively) and was observed predominantly at the edges of the cytoplasm. Indeed, the related horseshoe crab peptide tachyplesin I has been shown to bind the minor groove of DNA (8) and the less condensed nature observed here may indicate direct PM1-biotin/DNA binding.

As a control, *E. coli* cells were treated with PM1-biotin at 4°C. At this temperature, membrane translocation was prevented due to the rigid state of the lipids in both inner and outer membrane bilayers. Figures 2B and C confirmed this choice as a control, as intracellular peptide fluorescence was not observed and a clear delineation of the bacterium due to membrane-bound peptide was observed. These observations agreed with previous studies and supported the view that

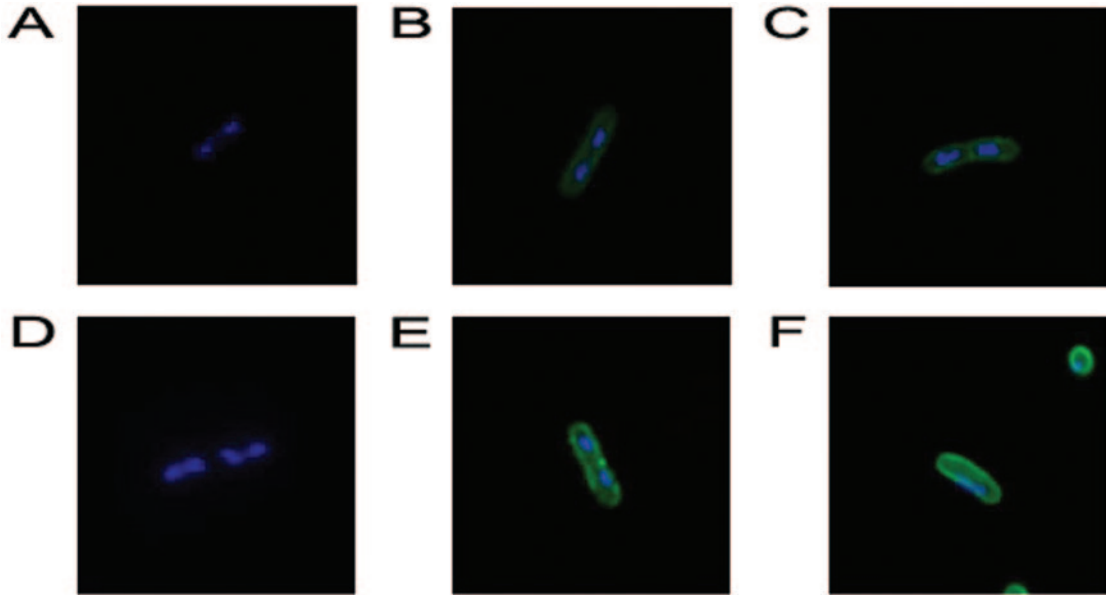

FIG. 2. Fluorescence microscopy of *E. coli* UB1005 treated with PM1-biotin. Bacteria were incubated at 4°C (top panels) or 37°C (bottom panels) without peptide (A and D) and at peptide concentrations of one-half MIC (B and E) and 4× MIC (C and F) for 30 min. Blue fluorescence staining represents intracellular DAPI-stained DNA, while green fluorescence staining represents the Alexa Fluor-labeled peptide.

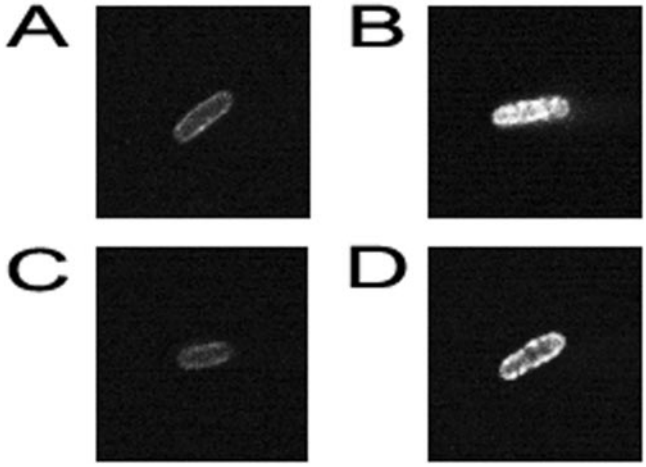

FIG. 3. Confocal microscopy of *E. coli* UB1005 treated with PM1-biotin. Bacteria were incubated with peptide at 4°C (A and C) or 37°C (B and D) at one-half MIC for 30 min. Prior to fluorescence labeling, cells were treated (A and B) or not treated (C and D) with 0.2% Triton X-100.

polyphemusin I does not cause pore formation or significant membrane damage (9, 10).

To more precisely determine the localization of polyphemusin, confocal microscopy was performed with the same *E. coli* samples treated at one-half-MIC PM1-biotin, as described above (Fig. 3). Control samples incubated with peptide at 4°C (Fig. 3A) appeared as hollow rods, with fluorescence clearly defining the bacterial surface membranes. Intracellular fluorescence was not observed, indicating that peptide membrane translocation did not occur. Conversely, *E. coli* samples treated with peptide at 37°C (Fig. 3B) appeared as solid fluorescent rods, indicating the presence of peptide within the cytoplasm. This is consistent with studies demonstrating that polyphemusin I can translocate across liposome membranes and even at twofold MIC is only able to depolarize by 50% the *E. coli* cytoplasmic membrane (i.e., make it partly leaky to protons) (6).

For these studies, to permit visualization of the peptide, treatment with Triton X-100 was performed after fixation with glutaraldehyde so as to permeabilize the bacteria and allow fluorescent-conjugated streptavidin to access intracellular PM1-biotin. As an additional control in the confocal experiments, peptide-treated *E. coli* samples were not treated with Triton X-100 (Fig. 3C and D). By eliminating this treatment, membrane integrity was retained. Thus, bacteria appeared as hollow rods, indicating that PM1-biotin did not greatly affect cytoplasmic membrane integrity and indeed did not permeabilize membranes to fluorescent-conjugated streptavidin, since fluorescence staining was not observed within these cells.

The data presented here demonstrate for the first time membrane translocation of a polyphemusin I analogue, PM1-biotin, in intact bacterial cells. This finding agrees with previously published translocation studies using model membranes (6, 9). In addition, the absence of cytoplasmic fluorescence in cells treated with peptide but not permeabilized with Triton X-100 indicated that PM1-biotin did not induce significant membrane damage or pore formation. These findings confirmed our hypothesis that polyphemusin is capable of translocating membranes and does not cause major membrane damage allowing the entry or leakage of molecules into or out of cells.

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