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A new rapid cell-penetrating peptide based strategy to produce bacterial ghosts for plasmid delivery

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ABSTRACT

The production of bacterial ghosts involves the lysis gene E plasmid in order to lyse and empty the bacteria of their cytoplasmic contents. After lysis the ghosts can either be loaded with new desired DNA and used for delivery to mammalian cells or used in vaccination. Cell-penetrating peptides have been used as delivery vehicles of drugs and oligonucleotides. Although many of them show low toxicity they have been compared to antimicrobial peptides involved in innate immunity. Recently we showed that cell-penetrating peptides also could be antimicrobial. In this study we take advantage of the antimicrobial effect of one cell-penetrating peptide, namely MAP, which is a model amphipathic peptide and treat bacteria with the peptide to produce bacterial ghosts. This new peptide based strategy is not dependent on the lysis gene E plasmid thus; several tiresome steps are removed in the production of ghosts. In addition the ghosts can be preloaded with a desired plasmid or DNA further removing time consuming reprocessing steps. To our knowledge this is the first study that uses a cell-penetrating peptide based strategy to produce bacterial ghosts to be used in plasmid delivery.

1. Introduction

The efficiency of medical treatment would increase immensely if drugs could be targeted to specific tissues selectively. Bacterial ghosts are considered to be a new and exciting non-viral tool in gene delivery and vaccination since they efficiently target antigen-presenting cells and other eukaryotic cells. They are produced by protein E-mediated lysis of gram-negative bacteria carrying the plasmid encoding lysis gene E of bacteriophage PhiX174 [1–3]. This leads to a transmembrane tunnel in the bacteria releasing the cytoplasmic contents without altering the surface structures and antigenic features, including bioadhesive properties of the natural cell [4]. The protein E lysis is a time consuming process since the bacteria have to be loaded with the lysis plasmid and after the lysis, bacteria need to be reloaded with a new plasmid or DNA of interest.

Another vehicle in drug delivery is cell-penetrating peptides (CPPs) or protein transduction domains (PTDs). These peptides have been shown to traverse both non-mammalian and mammalian cell membranes [5,6] with or without cargo to induce biological effects [7,8]. Plasmids have been delivered to cells, however, with low efficiency [9]. Moreover, a majority of the CPPs so far have low or no tissue specificity, i.e. they can translocate almost all cell lines. It is unclear how these peptides enter cells but recent studies have suggested a direct penetration mechanism through the membrane concurrently with endocytosis [10–12]. The membrane disturbances of eukaryotic cells can be resealed by the membrane repair response (MRR), a resealing mechanism activated by calcium influx through the broken membrane. Activation of the MRR leads to mobilization of intracellular vesicles such as lysosomes to the site of disruption which donate their membrane to reseal the broken plasma membrane [13–15]. However, since bacteria do not have endocytosis, no resealing with intracellular vesicles can occur.

Recently we showed that some of these CPPs also have antimicrobial effects [5]. In this study one of those antimicrobial CPPs, MAP, was used to produce bacterial ghosts without the involvement of the plasmid coding for lysis gene E and without the need to reload the ghosts after production. Although MAP has good antimicrobial properties it cannot serve as an antibiotic due to its similar toxicity in bacteria and mammalian cells. Nevertheless it is well suited for the production of ghosts. Herein we present a quick method to produce bacterial ghosts already containing the desired plasmid without the involvement of lysis gene E plasmid or ghost reloading and show successful delivery of the ghosts into HeLa cells.

2. Materials and methods

2.1. Cell cultures

Escherichia coli (*E. coli*) with a green fluorescent protein (GFP) plasmid [16] were cultured in Luria–Bertani (LB) broth supplemented with 100 μg/mL ampicillin. HeLa cells were grown in medium 199 for incubation without CO_2 control or Dulbecco's Modified Eagle's Media

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(DMEM) F-12 with Glutamax-I (GIBCO, USA) supplemented with 10% foetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin. Cells for uptake were seeded in 24-well plates (GIBCO, USA) in 0.5 ml medium. The cells were used for experiments one day after seeding.

2.2. Peptide synthesis

MAP (KLALKLALKALKAALKLA-amide) was synthesized in a stepwise manner in a 1 mmol scale on a peptide synthesizer (Applied Biosystems, model 431A, Foster City, CA, USA) using *t*-Boc strategy of solid-phase peptide synthesis as described previously [6].

2.3. Optimization of ghost production

The antimicrobial activity was analyzed as described in [17]. Briefly, a small culture was grown overnight in LB broth at 37 °C. An aliquot of the culture was transferred to fresh culture medium and incubated for an additional 3–5 h to reach mid-logarithmic phase organisms. The culture was then diluted in LB broth to OD 0.01 and transferred to a 96-well plate together with MAP to reach a final peptide concentration of 1, 10, 20 and 50 μM. Absorbance of bacteria incubated at 37 °C for 16 h was measured at 595 nm on absorbance reader Digiscan (Labvision, Sweden). Bacteria without peptide solution or with tetracycline were used as controls.

The same procedure was performed as describe above followed by centrifugation at 1000 g for 5 min and resuspended in LB or 10 mM Na_2HPO_4 buffer (pH 7.4) and incubated with MAP at final concentrations of 1, 5, 7.5, 10 and 20 μM at an OD of 0.05 or 0.1. The bacteria were incubated at 21 °C and on ice for 1 and 2 h, and aliquots of 10 μl were transferred to a 96-well plate with 90 μl LB or spread on LB-agar plates and incubated at 37 °C for 16 h. Surviving bacteria were counted as Colony Forming Units (CFUs).

2.4. Culture density

E. coli were centrifuged at 1000 g for 5 min and resuspended in LB broth or 10 mM Na_2HPO_4 buffer (pH 7.4) to give an absorbance of 0.05, 0.1, 0.2, 0.25, 0.4, and 0.5. Each sample was incubated with MAP (10 and 20 μM) for 1 h, at 21 °C or on ice. Aliquots of 10 μl bacteria were transferred to a 96-well plate or spread on LB-agar plates were incubated in 37 °C, 16 h. Surviving bacteria were counted as CFUs.

2.5. Time dependency and cell leakage

E. coli were centrifuged at 1000 g for 5 min and resuspended in 10 mM Na_2HPO_4 buffer (pH 7.4) and incubated with different concentrations of MAP (7.5, 10, 20 μM). Aliquots of 10 μl were taken at 0, 5, 15, 30, 60, 120 min intervals and diluted in 190 μl LB medium in a microtiter plate and incubated at 37 °C for 16 h.

E. coli were centrifuged at 1000 g for 5 min, resuspended in 10 mM Na_2HPO_4 buffer (pH 7.4) and incubated with MAP (10 μM) at 21 °C. Aliquots were taken at 0, 5, 15, 60 and 120 min and centrifuged at 6000 rpm for 3 min. The supernatants were transferred to a black 96-well plate and kept on ice until the fluorescence was measured at 490/520 nm on a Spectra Max Gemini XS fluorometer (Molecular Devices, USA).

2.6. GFP/PI-measurements

E. coli were centrifuged at 1000 g for 5 min and resuspended in 10 mM Na_2HPO_4 buffer (pH 7.4) and diluted with buffer to ~1×10^7 and ~2×10^7 bacteria. Different concentrations of MAP (1, 5, 10, 20 μM) were added to the bacteria which were stained with 10 μg/mL PI and analyzed on a Spectra Max Gemini XS fluorometer (Molecular Devices, USA). Wavelengths of GFP and PI were measured for 1 h with 12 second intervals at 21 °C.

2.7. Fluorescence microscopy

E. coli were centrifuged at 1000 g, 5 min and resuspended in 10 mM Na_2HPO_4 buffer (pH 7.4). Approximately 1×10^7 bacteria were incubated with MAP (20 μM) at 21 °C, 1 h. Bis-(1, 3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) and propidium iodide (PI) were added to the bacteria (20 μM MAP and control) to give a concentration of 1 μM and 10 μg/ml, respectively. Unstained control bacteria and MAP (20 μM) were also analyzed as a comparison. 1 μl cell sample was added to 10 μl glycerol and analyzed on a fluorescence microscope Leica DM IRE2 (Leica, Germany).

E. coli centrifuged at 1000 g for 5 min and resuspended in 10 mM Na_2HPO_4 buffer (pH 7.4). Approximately 1×10^7 bacteria were incubated with MAP (20 μM) at 21 °C for 1 h. Aliquots were taken from MAP treated and control bacteria were frozen in −70 °C. Control and MAP samples were thawed and stained with PI. Unmarked control bacteria and MAP incubated bacteria were also prepared. 1 μl cell sample was added to 10 μl glycerol and analyzed on a fluorescence microscope Leica DM IRE2 (Leica, Germany).

50,000 or 100,000 HeLa cells were seeded on cover slips one day before the experiment. The cells were washed with complete medium and incubated with ghosts (OD ~ 0.1) in complete medium for 1 h. The cells were washed with PBS two times, fixed in 4% paraformaldehyde and washed with PBS two times. Cells were mounted on cover slides in 1 μl glycerol and examined using an UltraView ERS confocal live cell imager (Perkin Elmer, Upplands Väsby, Sweden) connected to an Axiovert 200 (Zeiss, Welvin Garden City, UK).

2.8. Agarose gel of DNA

Bacteria with different OD were treated with 10 or 100 μM MAP in 10 mM Na_2HPO_4 buffer (pH 7.4) or LB for 1 h. Untreated cells were used as control. Plasmid DNA was extracted and prepared as described previously by Cheng et al. [18] and analyzed on an agarose gel.

2.9. FACS analysis

All experiments were performed with a FACScalibur flow cytometer (four-color system; Becton Dickinson) equipped with an air-cooled laser providing 15 mW at 488 nm and the standard filter setup. For acquisition and analysis of data, the CellQuest software package (Becton Dickinson) was used. The bacteria were centrifuged at 1000 g for 5 min and resuspended in 10 mM Na_2HPO_4 buffer (pH 7.4). MAP (20 μM) and controls (without peptide) were left for 1 h at 21 °C. The bacteria were then stained with PI (10 μg/mL) and DiBAC (1 μM) followed by incubation at 21 °C for 20 min prior to flow cytometric analysis.

100,000 HeLa cells were incubated with ghosts for 3 h in complete medium, washed with complete medium and incubated for an additional 45 h. Cells were centrifuged at 1000 g for 5 min, resuspended in PBS, stained with PI (10 μg/mL) for 20 min and left on ice until flow cytometry analysis.

3. Results

3.1. Optimization of ghost production

The minimal inhibitory concentration (MIC) is the concentration of an antibiotic or a peptide sufficient to inhibit bacterial growth. In a recent paper we found that several CPPs were antimicrobial against gram-negative and gram-positive bacteria. For the bacterial ghost production in this study, it was necessary to determine the LD_{100} of an *E. coli* strain loaded with a GFP plasmid. The broth microdilution method was applied to determine at what concentration the CPPs killed 100% of the bacteria [17]. Bacteria were diluted in LB and incubated with several CPPs (data not shown) at different