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Research paper

Cell-penetrating and cargo-delivery ability of a spider toxin-derived peptide in mammalian cells

Nisha Ponnappan, Archana Chugh *

Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, New Delhi 110016, India

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ABSTRACT

Cell-penetrating peptides are short cationic peptides with inherent ability to cross the plasma membrane barrier as well as intracellularly deliver cargo molecules conjugated to them. Venoms from snakes, scorpions and spiders are rich in membrane-active peptides. Crotamine from snake venom as well as maurocalcine and imperatoxin isolated from scorpion venoms have been reported to possess cell-penetrating property in mammalian cells. Latarcins, a group of spider venom toxins, has also been reported to possess antimicrobial property. However, cell-penetrating ability of Latarcins is still not elucidated. This is the first report where cell-penetrating ability of a peptide derived from spider toxin, Latarcin 1 has been demonstrated. Interestingly, the structurally minimized sequence of Latarcin 1 (LDP – Latarcin-derived peptide) when conjugated with nuclear localization sequence from Simian Virus T40 antigen (LDP-NLS) translocates across cell membrane in HeLa cells. The chimeric LDP-NLS peptide also did not exhibit cytotoxicity towards mammalian cells in contrast to the LDP that showed lesser uptake and higher cytotoxicity. LDP-NLS also successfully delivered macromolecular protein cargo inside the cells.

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1. Introduction

Cytolytic peptides are small molecular weight peptides that interact with lipid membranes, altering their permeability and subsequently causing cell death. Animal venoms and toxins possess a repertoire of such peptides and they are mainly used to immobilize the prey [1]. Spider venoms comprise of rich array of biologically active components including low molecular weight compounds, protein and peptide enzymes, neurotoxins as well as cytolytic peptides [2,3]. Latarcins from the spider, *Lachesana tarabaevi*, is a group of well-characterized cytolytic peptides that are 25–39 amino acids in length with abundance of lysine and arginine residues. They have been reported to interact with biological membranes by affecting their permeability [4]. These group of peptides also possess antimicrobial activity against many bacteria and fungi. Latarcins possess amphipathic structure and are structurally disordered in aqueous solution. However, they adopt an α-helical conformation in the presence of a membrane-mimicking environment [5]. We observed that their structural features along with membrane-active property make them interesting candidates for investigation as cell-penetrating peptides (CPPs).

CPPs are a small group of mostly cationic peptides with length ranging from 5 to 30 amino acids in general [6]. The distinctive feature of CPPs is the ability to traverse plasma membrane by themselves. They are also able to deliver large macromolecular cargoes inside the cells that otherwise cannot cross the plasma membrane [7–10]. Examples of cargo molecules that could be delivered inside the cells using CPPs include small molecular drugs such as doxorubicin and natamycin, oligonucleotides in the form of siRNA as well as protein and peptide cargoes [11–14]. Besides, some CPPs have been reported to possess antimicrobial property indicating that these peptides can be used as dual-purpose vectors in treating various diseases [15]. CPPs can be derived from natural sources such as Tat derived from transactivator of transcription of human immunodeficiency virus and penetratin derived from the antennapedia homeodomain of *Drosophila melanogaster* or they can be synthetic in nature such as oligoarginines and model amphipathic peptide [16–19]. CPPs have been also identified from animal toxins such as crotamine from snake venom, maurocalcine and imperatoxin from scorpion venoms [20–22]. However, spider venom peptides, despite having membrane-active properties, have not yet been characterized as potential CPPs.

Abbreviations: CD, circular dichroism; CPP, cell-penetrating peptide; FACS, fluorescence-activated cell sorter; FITC, fluorescein-isothiocyanate; LDH, lactate dehydrogenase; LDP, latarcin-derived peptide; NLS, nuclear localization sequence; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; X-gal, bromo-4-chloro-3-indolyl-D-galactopyranoside.

* Corresponding author.
E-mail address: achugh@bioschool.iitd.ac.in (A. Chugh).

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We were, therefore, interested in Latarcin group of cytolytic peptides from spider toxins because of the similar structural features to those of CPPs. However, since these peptides possess potent cytotoxic effects, minimized Latarcin peptide sequences were analyzed *in silico* and further, *in vitro* for cell penetrating ability. *In silico* analysis of the screened peptides revealed a potent 10 amino acid long peptide from Latarcin 1 (Lt1) toxin. In order to facilitate the nuclear localization of the screened peptide, it was fused with nuclear localization signal (NLS) from Simian virus T40 antigen. Interestingly, the peptide fused with NLS showed effective CPP activity *in vitro*. The peptide was further characterized for its cytotoxic activity and cargo-delivery ability.

2. Materials and methods

2.1. In silico screening of Latarcin peptides for potential cell-penetrating ability and peptide synthesis

The sequences of Latarcin group of toxins were identified from literature [4]. Since, the peptides were more than 20 amino acids long and possessed cytolytic activity, the window length of 10 amino acids sequence from each peptide were analyzed on the basis of pI, hydropathicity, hydrophilicity and amphipathicity. Prediction of potential CPPs from spider toxins was carried out using CellPPD server [23]. In addition, a well characterized nuclear localization sequence (NLS) from Simian Virus T40 antigen was attached to the C-terminus of the screened peptide to promote nuclear localization of the peptide in the cells. Specific lysine and arginine residues in the short-listed peptide were substituted with alanine residues and the resulting mutated peptide served as the negative control (Table 1).

Peptides were custom synthesized by F-moc solid-phase peptide synthesis from GenPro Biotech, New Delhi, India with more than 95% purity. For assessing the cellular uptake of peptides by confocal microscopy and flow cytometry, the peptides were labelled with FITC at N-terminus.

2.2. Cell culture

HeLa cells (from National Centre for Cell Science, Pune, India) were employed for the assessment of cell-penetrating ability of the peptides. The cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (Cell Clone, Genetix Biotech Asia Pvt. Ltd., India) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (Gibco, Invitrogen, India).

2.3. Confocal microscopy

HeLa cells were seeded at a density of 300,000 cells per well onto glass cover slides in 6-well plates and cultured for 24 h. The cells were then washed with phosphate buffered saline (PBS) and incubated with 2.5–20 μM of FITC-labelled LDP-NLS and 20 μM each of LDP, NLS and Mut-LDP-NLS peptides in serum-free medium for 1 h at 37 °C in 5% CO_2. Time-dependent uptake of LDP-NLS peptide at different concentrations in HeLa cells was also assessed after incubation period of 15, 30 and 45 min. For assessing the localization of LDP-NLS peptide in nucleus and lysosomes of HeLa cells, co-localization of the peptide with Hoechst 33342 and lysotracker red DND-99 (Molecular Probes, ThermoFisher Scientific, USA), respectively, was examined. Briefly, after incubation of HeLa cells with FITC-LDP-NLS peptide for 1 h, cells were washed and treated with Hoechst 33342 dye (5 μg/ml) for 20 min at 37 °C in 5% CO_2. Further, cells were washed thoroughly with PBS. Similarly, HeLa cells were incubated with FITC-LDP-NLS (5 μM) along with lysotracker red DND-99 (50 nM) for 1 h followed by washing with PBS. To quench the extracellular fluorescence, cells were treated with trypan blue (0.05% w/v in PBS) for 5 min. Cells were subsequently washed thrice with PBS and analyzed using confocal laser scanning microscope (Olympus IX83 FV1000, Japan) at 60X magnification. 405 nm, 473 nm and 543 nm lasers were used to sequentially excite Hoechst 33342, FITC and lysotracker red DND-99 dyes.

Table 1
Sequences of Latarcin-derived peptide and analogs investigated in the study.

S. No.	Peptide name	Sequence	Molecular weight[a]
1	LDP	KWRRKLKKLR	1913.01
2	LDP-NLS	KWRRKLKKLRPKKKRKV	2634.6
3	Mut-LDP-NLS	AWRRKLKALAPAKKAKV	2437.13
4	NLS	PKKKRKV	1384.23

[a] Observed molecular weight of FITC-labelled peptides.

2.4. Flow cytometry

Comparative uptake of peptides in HeLa cells was assessed by incubating the cells with 20 μM of FITC-labelled LDP-NLS, LDP, NLS and Mut-LDP-NLS peptides in serum-free medium for 1 h at 37 °C in 5% CO_2. To assess the dose-dependent cellular uptake of LDP-NLS peptide, concentration of 2.5–20 μM was used. The cells were then washed with PBS and were further treated with trypsin (1 mg/ml) for 10 min to remove extracellular membrane-bound peptides [24]. The cells were resuspended in PBS and washed twice by centrifugation and finally suspended in PBS for quantification by flow cytometry (BD FACSAria III, Becton Dickinson, USA). A total of 10,000 events were recorded and live cells were gated by forward/side scattering. Data was obtained and analyzed using FACS Diva ver 6.0 software.

2.5. MTT assay

Effect of LDP-NLS peptide on the viability of cells was determined by MTT assay. HeLa cells were seeded into 96-well plates at a density of 10,000 cells/well and cultured for 24 h. The cells were then incubated with the peptide in serum-free medium for 24 h at 37 °C, 5% CO_2. Cells incubated in media without any peptide were used as the control and those treated with 0.1% Triton X-100 were used for toxicity comparison. After incubation, media was aspirated from the wells followed by addition of MTT (1 mg/ml) and further incubation for 4 h at 37 °C. The formazan crystals so formed were dissolved in dimethyl sulfoxide and the optical density was measured at 570 nm with a reference wavelength of 620 nm on a microplate reader (Multiskan GO microplate spectrophotometer, Thermo Scientific, USA).

2.6. Membrane integrity assay

To assess the membrane integrity of HeLa cells treated with LDP-NLS and LDP, lactate dehydrogenase (LDH)-cytotoxicity assay kit was used according to manufacturer's instructions (Biovision, USA). Briefly, the cells seeded in a 96-well plate at a density of 10,000 cells/well and cultured for 24 h were treated with different concentrations of LDP-NLS and LDP in a serum-free medium for 1 h at 37 °C. After incubation, 100 μl of supernatant from reaction media was mixed with 100 μl of assay reagent for specified period of time and absorbance was measured at 495 nm using a microplate reader (Multiskan GO microplate spectrophotometer, Thermo Scientific, USA).

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