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Identification and characterization of a novel cell-penetrating peptide of 30Kc19 protein derived from *Bombyx mori*

Hee Ho Park[a], Youngsoo Sohn[b], Ji Woo Yeo[a], Ju Hyun Park[a], Hong Jai Lee[a], Jina Ryu[b], Won Jong Rhee[c], Tai Hyun Park[a,b,d,*]

[a] The School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Republic of Korea
[b] Interdisciplinary Program for Bioengineering, Seoul National University, Seoul 151-744, Republic of Korea
[c] Division of Bioengineering, Incheon National University, Incheon 406-772, Republic of Korea
[d] Advanced Institutes of Convergence Technology, Suwon 443-270, Republic of Korea

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ABSTRACT

Cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) have attracted increasing attention due to their high potential to deliver various, otherwise impermeable, bioactive agents, such as drugs and proteins across cell membranes. A number of CPPs have been discovered since then. Recently, 30Kc19 protein has attracted attention because it was the first cell-penetrating protein that has been found in insect hemolymph. Here, we report a cell-penetrating peptide derived from 30Kc19 protein, VVNKLIRNNKMNC, which efficiently penetrates cells when supplemented to medium for mammalian cell culture. Moreover, like other CPPs, this "Pep-c19" also efficiently delivered cell-impermeable cargo proteins, such as green fluorescent protein (GFP) into cells. In addition to the *in vitro* system, Pep-c19 exhibited the cell-penetrating property *in vivo*. When Pep-c19 was intraperitoneally injected into mice, Pep-c19 successfully delivered cargo proteins into various organ tissues with higher efficiency than the 30Kc19 protein itself, and without toxicity. Our data demonstrates that Pep-c19 has a great potential as a cell-penetrating peptide that can be used as a therapeutic tool to efficiently deliver different cell-impermeable cargo molecules into the tissues of various organs.

1. Introduction

In the past two decades a new class of peptides has gained increasing attention. These so-called "cell-penetrating peptides" are usually less than 30 amino acids in length, and are comprised of cationic and/or hydrophobic residues [1,2]. These cell-penetrating peptides have the ability to penetrate rapidly into living mammalian cells, and hence, can be used to deliver various functional cargo molecules, such as proteins [3–6], small molecules [7–9], nucleic acids [10–12], antibodies [13,14], and nanoparticles [15–18]. The exact mechanism responsible for the uptake of CPPs and their cargoes has not yet been fully established; however, a number of studies are now emphasizing the role of endocytosis [19–21], and in particular macropinocytosis [22–24], direct penetration [25,26], and inverted micelle [27–29]. Proteins and peptides have been found to move across the cell membrane since the initial discovery of TAT CPP derived from HIV-1 virus in 1988 [30,31], penetratin CPP derived from Antennapedia of *Drosophila melanogaster* in 1994 [32,33], and VP22 CPP derived from herpes simplex virus in 1997 [34,35]. Although several CPPs have been identified, it remains important to find new peptides that are efficient vehicles for the delivery of cargos, and with low toxicity because some have toxic effects on membranes of cells and organelles, including toxic effects resulting from the specific interaction of CPPs with cell components [2].

30Kc19 protein is a member of the 30K protein family, a similar structured protein found in hemolymph of *Bombyx mori* [36]. These proteins have molecular weights of around 30 kDa, and 30Kc19 protein is the most abundant among 30K proteins (30Kc6, 30Kc12, 30Kc19, 30Kc21 and 30Kc23) in the hemolymph [37]. During the

Abbreviations: CPP, cell-penetrating peptide; PTD, protein transduction domain; TAT, trans-activator of transcription; GFP, green fluorescent protein; SDS, sodium dodecyl sulfate; PAGE, polyacryl amide gel electrophoresis; HRP, horseradish peroxidase; CLC, Cake-Loving Company; EMBOSS, The European Molecular Biology Open Software Suite; LOOP, Learning, Observing and Outputting Protein Patterns; DMEM, Dulbecco's modified Eagle medium; BUN, blood urea nitrogen; ALT, aspartate aminotransferase; AST, alanine aminotransferase.

* Corresponding author at: The School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Republic of Korea. Tel.: +82 2 880 8020; fax: +82 2 875 9348.
E-mail address: thpark@snu.ac.kr (T.H. Park).

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5th instar larva to early pupa stage, these 30K proteins are synthesized in fat body cells and accumulate in the hemolymph [38,39]. They are then transferred from the hemolymph to fat body cells during metamorphosis from larva to pupa, and are deposited there until later use [40,41]. Although the biological functions of the 30K proteins in silkworms have not been fully determined, several studies have recently examined their functional properties for 30Kc6 and 30Kc19 [41,42]. In previous studies, we have demonstrated that gene expression or addition of recombinant 30K proteins to culture medium produced from *Escherichia coli* (*E. coli*) exhibited anti-apoptotic effects in various cells [43–55]. 30K proteins also enhanced productions of recombinant erythropoietin, interferon-β, and monoclonal antibody, as well as increasing glycosylation, cell growth, and viability in various cells, and also had enzyme-stabilizing effects [56–62]. A recent study has shown that 30Kc19 protein has a cell-penetrating property when supplemented to the culture medium [63]. Therefore, 30Kc19 protein is a very unique multi-functional protein, and can be applied for the delivery of therapeutic proteins, including enzymes, as it can penetrate cell membrane as well as stabilizing cargo proteins. However, for the practical use in delivery of cell-impermeable cargo molecules, it is necessary to find a cell-penetrating domain like other cell-penetrating proteins that can efficiently deliver cargo molecules into cells.

Here, we report a cell-penetrating peptide of 30Kc19 protein (Pep-c19), originating from the silkworm. Through computational analyses, we managed to identify a peptide that has a cell-penetrating property and investigated the efficiency and toxicity of this "Pep-c19" in comparison with its original protein, 30Kc19, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Total RNA was isolated from *Bombyx mori* silkworm at the fifth-instar larval stage using RNeasy (Qiagen, Valencia, CA, USA). The 30Kc19 cDNA was obtained by RT-PCR, and the 30Kc19 gene was then amplified using PCR. This DNA fragment was then inserted into a pET-23a expression vector (Novagen, Madison, WI, USA) with a T7 tag at the N-terminus and a 6-His tag at C-terminus. Then, truncated forms of 30Kc19; $30Kc19_{1–120}$ and $30Kc19_{121–239}$ were also constructed. For GFP-30Kc19, ORFs of GFP were cloned from pCMV-AC-GFP vector (Origene, Rockville, MD, USA) to N-terminal of 30Kc19 in pET-23a vector. The GFP-30Kc19 contained two amino acids (Glu, Phe) derived from the EcoR I sequence (GAATTC) between GFP and 30Kc19. GFP-Pep-c19; $30Kc19_{42–57}$ sequence at the C-terminus of GFP was constructed to pET-23a vector. Constructed vectors were then transformed to *E. coli* BL21 (DE3, Novagen) and cells were grown in LB-ampicillin medium at 37 °C. Isopropyl 1-thio-β-D-galactopyranoside (IPTG, 1 mM) was used for the induction. *E. coli* were then further incubated at 37 °C for the production of protein, except for GFP-30Kc19, for which 30 °C was selected as the induction temperature. After centrifugation, cells were harvested and disrupted by sonication. The purified proteins, including the 30Kc19, were then obtained as described previously [64]. Briefly, following the lysis of cells, all recombinant proteins were purified from the supernatant using a HisTrap HP column (GE Healthcare, Uppsala, Sweden) and was dialyzed against 20 mM Tris–HCl buffer (pH 8.0) using HiTrap Desalting (GE Healthcare) to eliminate the lipopolysaccharide (LPS) endotoxins. The purity was higher than 90% (data not shown), and was then stored at −70 °C until use. The quantitative analysis of each protein was performed using a Micro BCA kit (Thermo Scientific Inc., Rockford, IL, USA). N-terminal FITC-linked CPP candidates and Pep-c19 with purity of 90% were ordered from Peptron (Daejeon, Korea), and were diluted and stored at −70 °C until use.

2.2. Cell culture

HEK 293 and HeLa cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C in DMEM (Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 10% (w/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin streptomycin (PS, Gibco). For the 4 °C experiment, cells were pre-incubated at 4 °C for 1 h before proteins were added. After the addition of proteins to the culture medium, cells were incubated at either 37 °C or 4 °C for 4 h, unless otherwise indicated.

2.3. Immunoblot analysis

HEK 293 cells were treated with trypsin-EDTA (Sigma–Aldrich, St. Louis, MO, USA) and then washed with PBS three times for strict distinction between intracellular and membrane-bound proteins. Cell extracts were collected with RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, proteases inhibitor cocktail) at 4 °C for 1 h followed by centrifugation. Each cell extract containing an equal amount of total protein was resolved by PAGE and examined by immunoblot analysis. For preparation of the anti-30Kc19 rabbit antibody, 30Kc19 was first purified from the silkworm hemolymph using a two-step chromatography purification method (size exclusion, ion exchange). Anti-30Kc19 polyclonal rabbit antibody was produced by immunizing a rabbit with the purified 30Kc19 protein, which was subsequently purified by Protein G chromatography (AbFrontier, Seoul, Korea). 30Kc19 was detected using this anti-30Kc19 antibody, followed by HRP-conjugated anti-rabbit antibody (Invitrogen).

2.4. Computational analysis

For the identification and selection of probable CPP candidates within the 30Kc19 protein, computational analyses were performed for helix motif region, positive amino acid region, hydrophobic amino acid region, and relative surface accessibility region of 30Kc19 protein. Whole amino acid sequence from 1-239 of the 30Kc19 protein was put in for the following analyses. For the helix and hydrophobic amino acid analyses, CLC protein workbench program was used (Insilicogen, Suwon, Korea). For the amino acid charge and surface accessibility analyses, EMBOSS and LOOPP programs (Cornell University, Ithaca, NY, USA) were used, respectively.

2.5. Quantitative internalization analysis of Pep-c19

Internalization of FITC-linked Pep-c19 and GFP-Pep-c19 protein was measured by fluorescence intensity using a microplate reader (Tecan GENios Pro, Tecan, Durham, NC, USA). HeLa cells were seeded on 96-well plate (Nunc Lab-Tek, Thermo Scientific) and incubated overnight. FITC-linked peptides or protein were added to the culture medium and were incubated in 37 °C in humidified atmosphere of 5% CO_2. Unless indicated otherwise, after incubation, cells were washed vigorously three times with PBS to minimize the possible presence of membrane-bound peptides and fluorescence was measured with excitation at 485 nm (20 nm bandwidth) and emission at 535 nm (25 nm bandwidth) with a gain of 60. If indicated, protein-treated cells were washed vigorously with PBS three times and treated with trypsin-EDTA for removal of membrane-bound proteins, then fluorescence of cell lysate was measured.

2.6. Fluorescence microscopy

For live cell analysis, cell penetration was visualized using confocal laser microscopy (EZ-C1, Nikon, Japan). HeLa cells were seeded on 8 well chamber slide (Nunc Lab-Tek, Thermo Scientific) and were incubated overnight. FITC-linked peptide or protein was added to the culture medium and was incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. Nuclei of cells were then stained with Hoechst 33342 for 10 min. Cells were washed vigorously with PBS three times to minimize the possible presence of membrane-bound peptides and then live cell intracellular fluorescence images were taken by the manufacture's software (Nikon, Japan).

For immunocytochemistry, HeLa cells were incubated with protein for 4 h and were then washed vigorously with PBS three times. Fixation was carried out with 4% paraformaldehyde for 20 min, followed by 10 min incubation with 0.25% Triton X-100 in PBS for permeabilization. The fixed cells were blocked with 3% BSA in 0.1% PBS-T for 1 h. The cells were then incubated with the anti-T7 tag rabbit antibody (Abcam, Cambridge, UK) and Rhodopsin-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA). Nuclei of cells were stained with Hoechst 33342 for 10 min. A confocal laser microscope was used to observe intracellular fluorescence and images were taken by the manufacture's software (Nikon, Japan).

2.7. In vivo penetration of Pep-c19

To investigate the *in vivo* penetration of Pep-c19, GFP-30Kc19 and GFP-Pep-c19 proteins were each dissolved in PBS and intraperitoneally injected to 5-week-old female ICR mice with an average weight of about 25 g (3.5 μmol/kg). Following 12 h incubation time, mice were euthanized and organs were collected. Then, the organs were frozen with optimal cutting temperature (OCT, Miles Laboratories, Elkhart, IN, USA) compound and tissues were sectioned at a thickness of 10 μm using microtome-cryostat (Microm, Walldorf, Germany) and were stored at −70 °C until further analysis for confocal microscopy.

2.8. In vivo toxicity analysis

To investigate the *in vivo* toxicity of Pep-c19, serum biological parameters were determined. 30Kc19 protein and Pep-c19 were dissolved in PBS and were intraperitoneally injected to 5-week-old female ICR mice with an average weight of about 25 g (0.2 μmol/kg or 2 μmol/kg). Mice were euthanized after 14 days, and blood samples were collected by heart-puncture method, and were maintained in serum separating tube (SST) at room temperature for 30 min. Following centrifugation for 10 min at 300 × *g* to obtain serum, samples were analyzed. As a parameter of kidney function, blood urea nitrogen (BUN) and creatinine levels were determined.