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

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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.

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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.

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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 Pepton (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% CO₂ 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% CO₂. 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% CO₂. 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 μ mol/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 μ m 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 μ mol/kg or 2 μ mol/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 $\times$ g to obtain serum, samples were analyzed. As a parameter of kidney function, blood urea nitrogen (BUN) and creatinine levels were determined.

For liver function, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined. The blood samples were delivered to Neodin Medical Institute (Seoul, Korea) where all biological parameters were determined and analyzed.

2.9. Inhibitor of endocytosis

HeLa cells were pre-incubated for 1 h with various inhibitors of endocytosis, prior to the experiment. 25 μ M cytochalasin B (Sigma–Aldrich) was used for the disruption of microfilaments and thus inhibition of macropinocytosis. 100 mM sucrose (Sigma–Aldrich) was used for the inhibition of clathrin-mediated endocytosis. 25 μ g/ml nystatin (Sigma–Aldrich), a sterol-binding agent, was used for disruption of caveolar structure and function. The agents were present during the entire experiment and incubation was performed for 6 h, after which cells were vigorously washed 3 times with PBS and analyzed using spectrofluorometer in order to determine the intracellular penetration.

3. Results and discussion

3.1. Presence of CPP in the 30Kc19 protein

30Kc19 protein, comprised of 239 amino acids in total, has all- α helix in N-terminal domain and all- β sheet in C-terminal domain as shown in Fig. 1A [65,66]. Recently, our group has shown cell-penetrating property in various types of cells, as well as the ability to deliver cargo proteins into the cells when supplemented to culture medium. It was found to be the first cell-penetrating protein in insect hemolymph that exhibited a cell-penetration property both *in vitro* and *in vivo* [67]. In this study, we first investigated the presence of CPP within the 30Kc19 protein. To determine whether penetration of 30Kc19 is dependent on the structure of the protein itself, and to confirm the presence of CPP in the 30Kc19 protein, N- and C-terminal truncated forms of 30Kc19 protein were constructed (Fig. 1B), expressed and purified. The soluble forms of 30Kc19_{1–120} and 30Kc19_{121–239} were seen from the Western-blot analysis (Fig. 1C). However, 30Kc19_{1–120} was expressed more as soluble form and 30Kc19_{121–239} was expressed less as soluble form.

This indicates that 30Kc19_{121–239} is mostly produced as misfolded proteins; inclusion bodies and refolding into bioactive forms is necessary. Hence only soluble forms of proteins were used. HEK 293 cell was used for Western-blot analysis as in previous study to compare the cell-penetrating ability of 30Kc19_{1–120} and 30Kc19_{121–239} with the 30Kc19 protein [67]. When cells were incubated with soluble form of N-terminal truncated 30Kc19 (30Kc19_{1–120}) at both 37 °C and 4 °C, 30Kc19_{1–120} protein was found in the cell (Fig. 1D). Unlike the 30Kc19_{1–120} protein, 30Kc19_{121–239} protein was not found in the cell and further experiments were not carried out (data not shown). This raised a question of the possible presence of a cell-penetrating peptide within the 30Kc19 protein. It was likely that the N-terminal truncated domain was responsible for the cell-penetrating property of 30Kc19 protein, which meant that CPP may exist in this domain.

3.2. Computational analysis for identification of CPP

We predicted the possible location of CPP within the 30Kc19 protein. Most CPPs have common characteristics, in that they have relatively high positive charge from basic amino acids such as arginine and lysine, and also have relatively high hydrophobicity from hydrophobic residues [1,2]. Also, taking penetratin from *Antennapedia* into account, we have evaluated that CPPs are also likely to be found in the secondary structure of helix motif and surface with relatively high accessibility [68,69]. Thus, we analyzed the 30Kc19 sequence that has a high frequency of basic amino acids, as well as hydrophobic amino acids in close proximity, and we managed to identify 3 domains that have the possibility of being CPP candidates; 30Kc19_{28–39}, 30Kc19_{45–55}, and 30Kc19_{113–126} (Fig. 2A). Next, we examined whether these 3 CPP candidates fall into distinct categories using computational analyses. To determine if CPPs are located in helix motif, the secondary structure of 30Kc19 was analyzed using the CLC protein workbench program. Through this

Fig. 1. Cell-penetrating property of 30Kc19 protein and demonstration of potential presence of cell-penetrating peptide in the 30Kc19 protein. Unless indicated otherwise, 0.2 mg/ml of proteins were used in the culture medium and were incubated with HEK 293 cells for 4 h. All figures are the results from immunoblots of whole cell lysate using the anti-30Kc19 antibody. (A) 3D representation structure of 30Kc19 protein using Swiss-model program. (B) Schematic diagram of the proteins related to this work. (C) Truncated 30Kc19_{1–120} and 30Kc19_{121–239} proteins. (D) Cell-penetrating property of truncated 30Kc19 protein at low temperature. When the cells were incubated with 30Kc19_{1–120} at 4 °C as well as 37 °C, 30Kc19 protein was detected in both cases.

Fig. 2. Computational analysis for the identification and selection of probable CPP candidates within the 30Kc19 protein. (A) Amino acid sequence of 30Kc19 protein, showing possible candidates of CPP; 30Kc19₂₈₋₃₉, 30Kc19₄₅₋₅₅, and 30Kc19₁₁₃₋₁₂₆. (B) Secondary structure of 30Kc19 protein for the analysis of helix motif, using CLC protein workbench program. (C) Positive surface charge analysis of 30Kc19 protein from basic amino acids, using EMBOSS program. (D) Hydrophobicity analysis of 30Kc19 protein from hydrophobic amino acids, using CLC protein workbench program. (E) Surface accessibility analysis of 30Kc19 protein, using LOOPP program.

analysis, only 30Kc19₂₈₋₃₉ and 30Kc19₄₅₋₅₅ had a high possibility of being helix motif (Fig. 2B). Then we examined the net charge of these CPP candidates using the EMBOSS program. From the analysis, it was found that 30Kc19₄₅₋₅₅ showed the greatest net positive charge of all CPP candidates (Fig. 2C). In addition, the hydrophobicity of 30Kc19 protein was predicted using the CLC protein workbench program. From the analysis, only 30Kc19₄₅₋₅₅ showed higher hydrophobicity than the other two CPP candidates (Fig. 2D). Lastly, the relative surface accessibility of 30Kc19 protein was performed using LOOPP program. From the analysis, all CPP candidates were determined to have similar surface accessibility (Fig. 2E). The results from several computational analyses; helix motif, positive surface charge, hydrophobicity, and relative surface accessibility showed that only 30Kc19₄₅₋₅₅ satisfied the characteristics and hence, it was selected as being the most probable candidate for the CPP within in the 30Kc19 protein.

3.3. Quantitative uptake of peptides for identification of CPP

In order to examine whether this region of 30Kc19 domain encompasses CPP, 17 peptides conjugated with FITC were synthesized (Fig. 3A). We extended the peptide sections starting from 41 to 57 because of hydrophobic amino acids at 41 (Val) and 42 (Ile),

and Cys at 57. We expected hydrophobic amino acid is an important factor because Antp₄₃₋₅₈ sequence (RQIKIWQNRRMKWKK) contains 6 hydrophobic amino (Ile, Ile, Trp, Phe, Met, Trp) acids in total. We also expected that cysteine may be an important factor because of previous report that it may be involved in the process of internalization *via* formation of dimer [70]. HeLa cell was used for intracellular fluorescence analysis as in previous study [67]. With the aim of narrowing down our search for cell-penetrating peptide, each FITC-linked peptide was added to culture medium and cells were later washed vigorously with PBS several times to remove any cell-bound peptides. Intracellular fluorescence from each peptide was measured using a spectrofluorometer. Out of 3 sections; 30Kc19_{x-55}, 30Kc19_{x-56}, and 30Kc19_{x-57}, each showed high fluorescence, as indicated (Fig. 3B). However, because washes in PBS may not be sufficient to remove some membrane-bound peptides, 2 peptides from each section were selected (red arrow), and thus, 6 peptides; 30Kc19₄₁₋₅₅, 30Kc19₄₂₋₅₅, 30Kc19₄₁₋₅₆, 30Kc19₄₂₋₅₆, 30Kc19₄₄₋₅₇, 30Kc19₄₅₋₅₇ were closely examined for their cell-penetrating ability by using confocal microscopy. The results showed that 2 peptides; 30Kc19₄₄₋₅₇ and 30Kc19₄₅₋₅₇, were visualized in cytoplasm and were able to penetrate the plasma membrane (Fig. 3C). The results are similar to HIV-TAT and Antp [71,72]. On the other hand, 4 peptides; 30Kc19₄₁₋₅₅, 30Kc19₄₂₋₅₅, 30Kc19₄₁₋₅₆

Fig. 3. Identification of Pep-c19. (A) FITC-linked peptides were selected based on the computational results from the most probable CPP candidate; 30Kc19₄₅₋₅₅, for the cell-penetrating ability. (B) Intracellular fluorescence of 30Kc19_{x-y} CPP candidates was measured using spectrofluorometer (ex. 485 nm/em. 535 nm). * $p < 0.001$, compared with the FITC-treated group ($n = 6$). Error bars represent standard deviation. Arrows indicate the peptides selected for confocal microscopy analysis. (C) Confocal microscopy image of the cell internalization of 30Kc19_{x-y} CPP candidates in living cells. In all cases, HeLa cells were incubated for 4 h with the same molar concentration of 30Kc19_{x-y} peptides (10 μ M), and were washed 3 times with PBS. The cell internalization of 30Kc19_{x-y} peptides were visualized by FITC fluorescence (Green) and nuclei were visualized by Hoechst 33342 (Blue). x and y denote the amino acid number of 30Kc19 protein.

and 30Kc19₄₂₋₅₆, were unable to penetrate the cell, and some actually formed membrane-bound aggregates (data not shown), which could explain the fluorescence given out in Fig. 3B. The formation of aggregates could have risen from the hydrophobic amino acids; Val-41 and Ile-42, where the number of hydrophobic amino acids in those 4 peptides was too many to allow for stability and solubility in the culture medium. 30Kc19₄₅₋₅₇ is shorter in length than 30Kc19₄₄₋₅₇, and thus, 30Kc19₄₅₋₅₇ was chosen as the CPP of 30Kc19 protein. Thus, 30Kc19₄₅₋₅₇, which will be named “Pep-c19”, was found to be the cell-penetrating peptide from 30Kc19 protein that efficiently penetrated cells when supplemented into medium for mammalian cell culture.

3.4. Cellular protein delivery of protein-conjugated Pep-c19

In order to examine the ability of Pep-c19 to deliver foreign proteins into the cell as well as its cell-penetrating efficiency,

a GFP-Pep-c19 as well as GFP and GFP-30Kc19 was expressed in *E. coli* and purified (Fig. 4A). HeLa cell was used for fluorescence analysis as in previous study to compare the cargo-delivering ability of Pep-c19 with the 30Kc19 protein [67]. Each protein was added to culture medium of HeLa cells and the increase in intracellular GFP-Pep-c19 was determined to be dependent on the concentration of the protein in the culture medium. The efficiency of the Pep-c19 was higher than the GFP-30Kc19, even after vigorous washing with PBS for the removal of any cell-bound proteins (Fig. 4B). The increase in the intracellular penetrated GFP-Pep-c19 was dependent on the time of the protein in the culture medium, and the efficiency of the Pep-c19 was also higher than the GFP-30Kc19, after vigorous washing with PBS (Fig. 4C). Then, each protein was added to the culture medium of HeLa cells and immunocytochemistry was performed. Similar to GFP-30Kc19, GFP-Pep-c19 also penetrated into the cells. Live cell images were taken under confocal microscope after vigorous washing with

Fig. 4. Delivery of GFP into the cells by Pep-c19. (A) SDS-PAGE result of GFP, GFP-30Kc19, and GFP-Pep-c19 proteins. (B) Intracellular fluorescence of GFP-Pep-c19 in dose-dependent (4 h fixed incubation time) and (C) time-dependent (10 μ M fixed concentration) manner was measured using spectrofluorometer (ex. 488 nm/em. 51 nm) ($n = 6$). (D) Immunocytochemistry image of the cell internalization of GFP-Pep-c19 using confocal microscopy. HeLa cells were fixed with paraformaldehyde. The GFP-Pep-c19 was visualized by GFP fluorescence (Green) and Rhodopsin-conjugated anti-T7 tag antibody (Red), and nuclei were visualized by Hoechst 33342 (Blue). (E) Confocal microscopy image of the cell internalization of GFP-Pep-c19 in living cells. The GFP-Pep-c19 was visualized by GFP fluorescence (Green) and nuclei were visualized by Hoechst 33342 (Blue). In all cases, HeLa cells were incubated for 4 h with the same molar concentration of proteins (10 μ M) and were washed 3 times with PBS. (F) Intracellular fluorescence of GFP-Pep-c19 (10 μ M, 6 h) was measured after treatment with trypsin-EDTA for removal of membrane-bound proteins using spectrofluorometer (ex. 488 nm/em. 51 nm) ($n = 3$). Error bars represent standard deviation.

PBS to exempt membrane-bound proteins and possible artifacts from fixation process of immunocytochemistry, where cell surface-bound proteins could be moved into cells [73]. The confocal images indicated that GFP-Pep-c19 successfully penetrated inside the living cells (Fig. 4E). Similar to Fig. 3C, it was worthwhile to notice that GFP-Pep-c19 was visualized in a punctate form. It was shown that internalized foreign proteins fused with the cell-penetrating peptide were observed as punctate forms [72,74]. Quantification of the intracellular fluorescence from the cell lysate was performed to fully exclude the possible membrane-bound proteins. Protein-treated cells were treated with trypsin-EDTA for removal of membrane-bound proteins and then fluorescence of cell lysate was measured (Fig. 4F). The result showed that both GFP-30Kc19 and GFP-Pep-c19 proteins were located in the cell lysate, but more GFP-Pep-c19 was found inside the cell. These results demonstrated that Pep-c19 can, not only penetrates itself, but it is also able to deliver impermeable cargo proteins, such as GFP into the cell with higher efficiency than the whole 30Kc19 protein.

3.5. *In vivo* protein delivery of protein-conjugated Pep-c19

Previously, CPPs have been used to successfully deliver cell-impermeable cargos such as proteins [3–6], small molecules [7–9], nucleic acids [10–12], antibodies [13,14], and nanoparticles [15–18] both *in vitro* and *in vivo*. To investigate *in vivo* penetration and efficiency in comparison with the whole 30Kc19 protein, we intraperitoneally injected each protein to 5-week-old ICR mice, and the organs, including brain, heart, lung, kidney, and liver, were isolated 12 h after injection [75–77]. In order to avoid an artifact and to address the potential issue caused by fixation process from immunohistochemistry, fluorescence images were taken without the fixation process, and thus, the penetration of GFP-Pep-c19 into tissues was analyzed by the fluorescence from the GFP protein. No fluorescence was detected for the native GFP in tissues (Fig. 5, top panel). Fluorescence was detected in all the tissues isolated from the mouse that were injected with GFP-30Kc19, although the fluorescence intensities varied among tissues (Fig. 5, middle panel).

Fig. 5. *In vivo* delivery of GFP into various tissues by Pep-c19. GFP, GFP-30Kc19, and GFP-Pep-c19 were dissolved in PBS with 10 mg/ml, 20 mg/mg, 10 mg/ml, respectively, and were intraperitoneally injected into female 5-week-old ICR mice weighing about 25 g on average. Finally, the same molar amount of GFP, GFP-30Kc19, and GFP-Pep-c19 were injected into mice (3.5 μ mol/kg; 100 mg/kg mouse for GFP, 200 mg/kg mouse for GFP-30Kc19, 100 mg/kg mouse for GFP-Pep-c19). As a control experiment, the same volume of PBS was injected into mice. After 12 h of injection, the organs; brain, heart, lung, kidney, and liver were collected, and tissues were sectioned to 10 μ m width using a microtome-cryostat. The penetrated GFP-Pep-c19 was visualized by GFP fluorescence (Green), and nuclei were visualized by Hoechst 33342 (Blue). Confocal microscopy images of the tissue internalization of GFP-Pep-c19 in living tissues of brain, heart, lung, kidney, and liver are presented in (A), (B), (C) and (D), respectively.

On the contrary, for GFP-Pep-c19, we observed that fluorescence intensity was much higher than that from GFP-30Kc19 in tissues (Fig. 5, bottom panel). This indicates that Pep-c19 penetrated and delivered cargo into various tissues across the blood vessel barriers with a high efficiency.

3.6. Toxicity test

We have previously shown that 30K proteins do not show any toxic effect *in vitro* cell cultures and *in vivo* study [45,48,51,52,67]. To examine *in vivo* toxicity of Pep-c19, we measured the toxicity parameters that represent toxicity in kidney and liver. Toxicity in kidney is shown by increase in the levels of blood urea nitrogen (BUN) and creatinine, whereas toxicity on liver is shown by increase in the levels of aspartate aminotransferase (ALT) and alanine aminotransferase (AST) [78,79]. The level of BUN, creatinine, ALT and AST from the blood serum of Pep-c19-injected mice were analyzed by Neodin Institute. We injected 3 different mice with 0.2 μ mol/kg and 2 μ mol/kg of each protein, every day, for

14 days, and evaluated the toxicity. After 2 weeks of injection, no apparent differences in the body weights and behaviors of mice were observed in any of the groups (data not shown). Toxicity results showed that high dose and long-term administration of Pep-c19 did not cause statistically significant or meaningful differences between 30Kc19 protein and Pep-c19 from all four toxicity parameters (Fig. 6). In all tests, the p-values between the control group and the 30Kc19-injected group and Pep-c19-injected group were mostly higher than 0.1. These results indicate that long-term administration of Pep-c19 did not cause significant toxicity *in vivo*. Previously, it was reported that Tat and VP22 are non-toxic both *in vitro* [80–82] and Tat is non-toxic *in vivo* [75]. In another group, toxicity was evaluated *in vivo* by applying peptides to the cornea 4 times daily for 7 days. At very high concentrations, the Antennapedia peptide showed no toxicity, whereas Tat caused some mild eyelid swelling [83]. The results in Fig. 6 show that Pep-c19 has similar non-toxic property. Therefore, it is anticipated that Pep-c19 could be utilized as an efficient and nontoxic carrier for the delivery of biological molecules into tissues *in vivo*. Other than fusing the CPP

Fig. 6. Toxicity test for the long-term administration of Pep-c19. 30Kc19 and Pep-c19 were injected to mice daily (0.2 $\mu\text{mol/kg}$ and 2 $\mu\text{mol/kg}$) for 14 days. For the control experiment, the same volume of PBS was injected. 3 different mice were used for each experiment ($n = 3$). To evaluate serum biological parameters, blood samples were collected from mice and serum were obtained by centrifugation using a serum separating tube. (A), (B) To analyze the kidney function, blood urea nitrogen (BUN) and creatinine levels were determined. (C), (D) As a parameter of the liver function, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined. The p -values on each bar were evaluated by comparing with the control group (PBS-treated). Error bars represent the standard deviation.

with the cargo, the non-conjugation approach may also be adapted for the delivery of cell-impermeable cargos; for instance siRNA, by mixing the CPP and siRNA and forming CPP/siRNA complex.

3.7. Intracellular penetration Pep-c19 CPP in the presence of inhibitors of endocytosis

It was notable that Pep-c19 successfully penetrates into cells and tissues upon addition to culture medium and intraperitoneal injection to mice, respectively. However, the mode of intracellular penetration was still unknown. Hence, inhibitors of endocytosis were used to find out how the Pep-c19 penetrates into cells. Prior to the treatment of FITC-Pep-c19 peptide, HeLa cells were treated with inhibitors of endocytosis; cytochalasin B, sucrose, and nystatin, for the disruption of microfilaments/inhibition of macropinocytosis, inhibition of clathrin-mediated endocytosis, and disruption of caveolar structure and function, respectively (Fig. 7) [9]. Sucrose was treated to give cells a hyperosmolar condition, but no markedly difference in the penetration ability of the Pep-c19 was seen. This showed that Pep-c19 does not penetrate by clathrin-mediated endocytosis. However, when cytochalasin B or nystatin was treated to cells, slightly lowered cell-penetrating ability of the Pep-c19 was seen, which demonstrates that it penetrates cells by macropinocytosis and caveolin-mediated endocytosis. Others reported that cytochalasin B reduced the cellular uptake of CPP by half [84], and nystatin reduced the CPP reporter β -gal activity by 50% in various cells [85]. The mechanism of entry of Pep-c19

is similar to other CPPs, but Pep-c19 involves 2 uptake pathways. In our current studies, we are undergoing molecular mechanism study of Pep-c19 for clarification of endosomal escape property.

3.8. Comparison of Pep-c19 with other cell-penetrating peptides

Well-known cell-penetrating peptides derived from protein transduction domains are Tat from human immunodeficiency virus-1 (HIV-1) [30,31], VP22 from herpes simplex virus-1 (HSV-1) [34,35], and Antp (also known as Penetratin) from Antennapedia homeodomain [32,33]. In this research, we have found a new cell-penetrating peptide from the third helix of *Bombyx mori* silkworm hemolymph. When we compare Pep-c19 with other CPPs in terms of the primary structure, Pep-c19 CPP contains less positive amino acids (3 in the sequence) than other CPPs (TAT has 8, VP22 has 9, Antp; has 7) (Table 1). Instead, Pep-c19 has more hydrophobic acids (6 in the sequence) than the most of other CPPs (TAT has 0, VP22 has 1, Antp has 6). With the secondary structure, Antp's homeodomain is comprised of 60 residues and it is consisted of 3 alpha-helices. Third alpha-helix is responsible for penetration (Antp), which is similar to our Pep-c19, because it is also from the third alpha helix of the 30Kc19 protein. HSV-1 protein 22 has size that is a little bigger than the 30Kc19 protein, 38 kDa, and VP22 is located at the very last 34 residues. These CPPs have been used to transduce proteins into cells and tissues, and for some (Tat and Antp) even across blood brain barrier but efficiency various depending on the cargo that is attached to the CPPs [86]. Though advantages and disadvantages

Fig. 7. Intracellular penetration in the presence of inhibitors of endocytosis. HeLa cells were pre-incubated for 1 h with various endocytosis inhibitors; cytochalasin B (25 μ M) for the disruption of microfilaments/inhibition of macropinocytosis, sucrose (100 mM) for the inhibition of clathrin-mediated endocytosis, and nystatin (25 μ g/ml) for the disruption of caveolar structure and function. FITC-linked Pep-c19 peptide was supplemented to the medium and after 6 h of incubation, HeLa cells were vigorously washed three times with PBS. The intracellular FITC-Pep-c19 was measured by green fluorescence using spectrofluorometer (ex. 485 nm/em. 535 nm). * $p < 0.05$, compared with the control group ($n = 3$). Error bars represent standard deviation.

Table 1
Comparison of Pep-c19 with other cell-penetrating peptides.

Name	Origin	Sequence (No. of amino acid)	Features
Tat	Human immunodeficiency virus-1 trans-activating transcriptional activator (HIV-1 TAT); residues 48-60	GRKKRRQRRRPPQ (13)	Able to transduce 15 – 120 kDa proteins into cells with high efficiency (~100%). <i>In vivo</i> delivery after 4 h into various tissues and across BBB.
VP22	Herpes simplex virus-1 protein 22 (HSV-1); residues 266-301	DAATATGRSAASRPTERR APARSASRRRPVD (34)	38 kDa size with last 34 residues responsible for penetration. Efficacy varies with cell type but quite inefficient.
Antp (Penetratin)	Antennapedia homeodomain; residues 43-58	RQIKIWQNRRMKWKK (16)	Consists of 3 alpha-helices. Third helix responsible for penetration; <i>in vivo</i> delivery into brain within 30 min.
Nona-arginine (R9)	Polyarginine	RRRRRRRRR (9)	Polyarginine peptides of 4-16 residues tested with 8 and 9 being optimal. Able to transduce molecules into a variety of cells in culture.
Pep-c19	<i>Bombyx mori</i> 30Kc19 protein; residues 45-57	VVNKLIRNNKMNC (13)	28 kDa size with 13 residues in third helix responsible for penetration; <i>in vivo</i> delivery into various tissues except brain.

Red color: positive amino acid; blue color: hydrophobic amino acid [86].

exist among the CPPs, the major advantage of Pep-c19 is that it is not a virus-derived cell-penetrating peptide meaning it is applicable as a therapeutic tool, short length, and is not toxic. Currently, we are now comparing the Pep-c19 with other known CPPs for assessment of uptake efficiency, delivery kinetics and toxicity test for potential use of this Pep-c19.

4. Conclusions

We have identified a new CPP; VVNKLIRNNKMNC, from 30Kc19 protein and demonstrated that 30Kc19 exhibited a cell-penetrating property due to the presence of a cell-penetrating peptide at 45–57. Our results strongly suggest that Pep-c19 has great potential for the efficient delivery of micro- and macromolecules including drugs and proteins to target tissues for therapeutic purposes. Since Pep-c19 is a cell-penetrating peptide derived from the first cell-penetrating protein that has been found in insect hemolymph, we

anticipate that other cell-penetrating peptides that have similar properties to Pep-c19 will be identified from other proteins sourced from insects.

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