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Human-derived fusogenic peptides for the intracellular delivery of proteins

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ABSTRACT

The cytosolic delivery of therapeutic proteins (*e.g.*, antibodies or enzymes) by cell-penetrating peptides (CPPs), such as a human immunodeficiency virus-derived TAT peptide, is facilitated by fusogenic peptides (FPs). For instance, we recently demonstrated that an FP, B18, which is derived from a sea urchin gamete fusion protein, promotes endosomal escape of an enhanced green fluorescent protein (eGFP)-TAT fusion protein directly conjugated to it. However, the potential clinical use of FPs raises concerns because all conventional FPs are non-human-derived. To solve this problem, we have attempted to identify novel human-derived FPs from two human proteins, including a human sperm protein, IZUMO1, which is involved in gamete recognition and fusion, and a human endogenous retroviral envelope protein, Syncytin1, which is involved in placental morphogenesis. Partial peptides from the core domains of the abovementioned proteins were chosen as candidates to generate human-derived FPs. We prepared fusion proteins of these peptides with eGFP and TAT in *Escherichia coli* and observed the localization of these fusion proteins in HeLa cells using confocal microscopy. Our results suggested that a 19-residues peptide of Syncytin1 (positions 322–340), named S19, possessed strong intracellular uptake activities with no detectable cytotoxicity. In addition, we estimated the number of molecules that escaped from endosomes using a nuclear localization signal, suggesting that the S19 peptide stimulated the intracellular delivery of TAT-fused eGFP by ~90-fold. Furthermore, we confirmed that S19 promoted the intracellular delivery of eGFP to various human cell lines, including HeLa, A431, HepG2, and SK-N-SH. In addition, we demonstrated that not only eGFP but also SNAP-tag and β-galactosidase were delivered efficiently and retained their activities.

1. Introduction

The intracellular delivery of therapeutic proteins, such as antibodies or enzymes, is important for the treatment of many diseases. Although cell-penetrating peptides (CPPs) [1], such as HIV-derived TAT [2], *Drosophila* Antennapedia-derived penetratin [3], and artificial polyarginine [4] and polyhistidine [5], have so far been applied to intracellular protein delivery, the CPP-mediated delivery of cargo (*e.g.*, protein) suffers from a problem (Fig. 1A). After the uptake of the cargo-CPPs *via* endocytosis, many remain trapped in the endosomes, which subsequently causes lysosomal degradation. Therefore, the amount of cargo localized to the cytoplasm and exhibiting the expected biological activity is very low [6–8].

To overcome the problem, non-cationic fusogenic peptides (FPs) have been designed and used to improve the efficiency of protein endosomal escape [9–12] (Fig. 1B). Wadia et al. demonstrated that a hemagglutinin HA2 peptide derived from the influenza viral envelope protein enhances endosomal escape efficiency [9]. Recently, we demonstrated that the B18 peptide, which is derived from a sea urchin gamete fusion protein called Bindin, promotes endosomal escape [10]. Several studies have demonstrated that FPs are useful for the intracellular delivery of not only proteins but also various other cargos [13–17]. However, all conventional FPs are non-human-derived. Therefore, for clinical applications, the immunogenicity of conventional FPs is of major concern.

In this study, we sought to identify novel human-derived FPs from human proteins involved in membrane fusion. Because the sea urchin gamete fusion protein-derived B18 peptide has no homolog in humans, we selected another human gamete fusion protein as the source of human FPs, namely the sperm protein IZUMO1 [18]. We also selected the human placental morphogenesis protein Syncytin1 [19], also known as human endogenous retroviral envelope protein, which is involved in trophoblast cells fusions, because some FPs were identified from viral envelope proteins [9,11]. To isolate FPs from these human proteins, we prepared eGFP-fusion proteins with a variety of FP-candidate peptides. Each candidate peptide comprised a region potentially involved in membrane fusion. We first observed the localization of the candidate fusion proteins in HeLa cells with confocal microscopy, looking for peptides that could enhance the endosomal escape of the eGFP-TAT fusion proteins. Consequently, a 19-residues Syncy-

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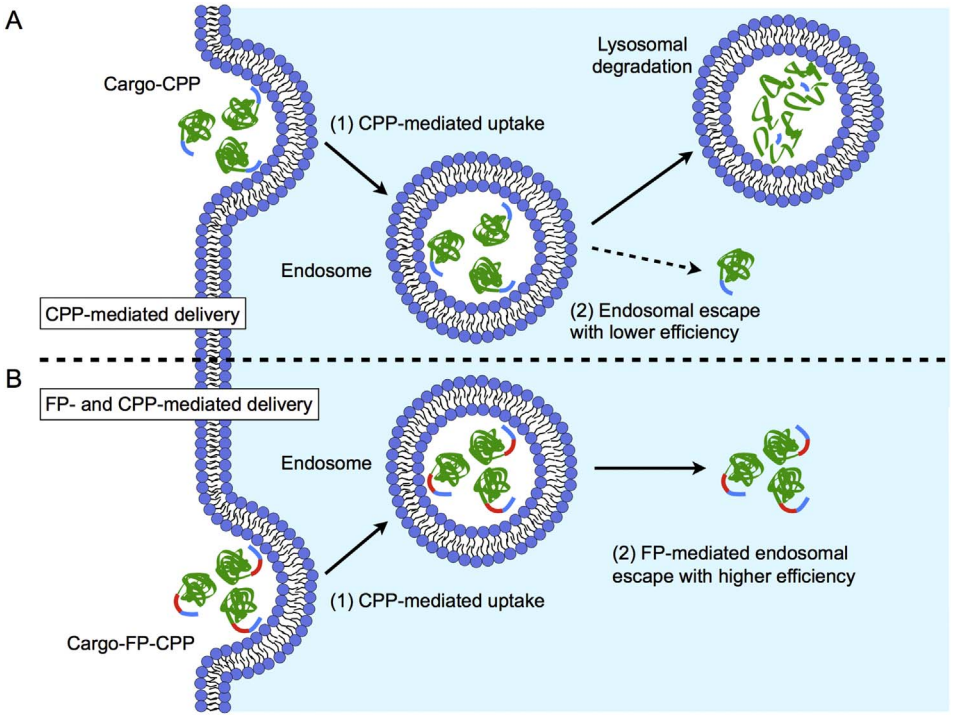

Fig. 1. Schematic representation of FP and CPP-mediated protein delivery. (A) A conventional CPP-mediated delivery. (1) A cationic CPP (blue) interacts electrostatically with the anionic cell-surface, and a CPP-fused cargo (green) is internalized into the endosome by endocytosis. (2) Because the endosomal escape efficiency of CPP is low, the cargo-CPP is subjected to lysosomal degradation. (B) FP- and CPP-mediated delivery. (1) An FP (red)- and CPP-fused cargo is internalized into the endosome by endocytosis. (2) Because the efficiency of FP-mediated endosomal escape is relatively high, the cargo-FP-CPP is efficiently transferred from the endosome to the cytoplasm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tin1$_{322–340}$ peptide, named S19, exhibited strong endosomal escape activity and was used for the delivery of several proteins into the cytoplasm of various human cell lines. This human-derived fusogenic peptide could be useful for clinical applications.

2. Materials and methods

2.1. Plasmid construction

All primers were purchased from Eurofins Genomics (Tokyo, Japan), and their sequences are indicated in Supplementary Table S1. The construction of the plasmids peGFP, peGFP-B55, peGFP-HA2, peGFP-TAT, and peGFP-NLS has been previously described [10,20].

The IZUMO1$_{78–134}$ and Syncytin1$_{320–440}$ genes (Supplementary Table S2), optimized for the *E. coli* expression system, were synthesized by Eurofins Genomics. Each sequence possessed *Bam*HI and *Xho*I cleavage sites at the 5′ and 3′ terminus, respectively. The GS-Syncytin1$_{320–340}$, Syncytin1$_{352–392}$, and Syncytin1$_{407–440}$ genes were amplified from the Syncytin1$_{320–440}$ gene using the Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA) and the specific primer pairs GS-Syncytin1-F and Syncytin1-R1, Syncytin1-F2 and Syncytin1-R2, or Syncytin1-F3 and Syncytin1-R3, respectively. Each FP gene was cloned into the pEGFP-B55 backbone at the *Bam*HI and *Xho*I sites, resulting in peGFP-FP plasmids.

A series of peGFP-FP-TAT plasmids and peGFP-FP-TAT-NLS plasmids were constructed based on the peGFP-FP plasmids as described in Supplementary Methods.

The O^6-alkylguanine-DNA alkyltransferase (SNAP-tag) gene was amplified from synthesized pUC-SNAP (GenScript, Piscataway, NJ) using the SNAP-F and SNAP-R primer pair and cloned into the *Nde*I and *Bam*HI sites of peGFP-TAT or peGFP-S19-TAT (See Supplementary Methods), resulting in pSNAP-TAT or pSNAP-S19-TAT, respectively. Similarly, the β-galactosidase (β-Gal) gene was amplified from *E. coli* K12 genomic DNA using the β-Gal-F and β-Gal-R primer pair and cloned into the above plasmids, resulting in pβ-Gal-TAT or pβ-Gal-S19-TAT, respectively.

2.2. Protein expression and purification

All proteins were expressed in *E. coli* BL21(DE3)-CodonPlus-RIPL cells (Agilent Technologies, Santa Clara, CA). Each FP-fused eGFP protein was expressed as previously described [10]. Briefly, the expression of FP-fused eGFP was performed by cultivation at 20 °C for 72 h in the absence of IPTG. For FP-fused SNAP or β-Gal proteins, *E. coli* transformed with each plasmid encoding the corresponding protein were grown in LB medium at 37 °C until the OD_{600} reached 0.5. Then, 0.4 or 1.0 mM of IPTG was added, and the cells were cultivated at 37 °C or 25 °C for 4 h, respectively. The eGFP-IZUMO1$_{78–134}$, eGFP-IZUMO1$_{78–134}$-TAT, eGFP-GS-Syncytin1$_{320–340}$-TAT, and eGFP-GS-Syncytin1$_{320–340}$-TAT-NLS proteins were purified from the bacterial insoluble fraction and refolded by dialysis with Amicon Ultra-4 (30k; Merck Millipore, Billerica, MA, USA). Other proteins were purified from the soluble fraction, as previously described [10], except that PBS (Nacalai Tesque, Kyoto, Japan) supplemented with 400 mM of NaCl and 0.1% of Tween 20, PBS supplemented with 400 mM of NaCl and 300 mM of imidazole, and PBS supplemented with 400 mM of NaCl were used as the wash, elution, or exchange buffer, respectively. The expression and purification of each protein was confirmed using 12.5% SDS-PAGE with Coomassie brilliant blue staining, and the protein concentration was estimated using bovine serum albumin as the reference.

2.3. Human cell lines

The human cervical cancer cell line HeLa, the human epidermoid carcinoma cell line A431, the human hepatocellular carcinoma cell line HepG2, and the human neuroblastoma cell line SK-N-SH were purchased from the RIKEN Cell Bank (Ibaraki, Japan). The cells were maintained in DMEM (Nacalai Tesque) supplemented with 10% FBS