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Shiga-like toxin-based high-efficiency and receptor-specific intracellular delivery system for a protein

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ABSTRACT

The cell-specific cytosolic delivery of functional macromolecules with high efficiency is of great significance in molecular medicine and biotechnology. Herein, we present a Shiga-like toxin II-based high-efficiency and receptor-specific intracellular delivery system. We designed and constructed the Shiga-like toxin-based carrier (STC) to comprise the targeting and translocation domains, and used it for delivering a protein cargo. The STC was shown to deliver a protein cargo into the cytosol with high efficiency in a receptor-specific manner, exhibiting much higher efficiency than the most widely used cell-penetrating peptide. The general utility of the STC was demonstrated by modulating the targeting domain. The present delivery platform can be widely used for the intracellular delivery of diverse biomolecules in a receptor-specific and genetically encodable manner.

1. Introduction

The cell-specific intracellular delivery of functional macromolecules, including proteins and nucleic acids, is of great significance in molecular medicine and basic research, and many methods for such delivery have been developed. Nanocapsule-based delivery has been attempted for several different proteins [1,2]; however, this approach requires chemical modifications of the protein surfaces, resulting in heterogeneous products. Cell-penetrating peptides (CPPs), composed of 8–25 amino acids, mediate the internalization of charged macromolecules through proteoglycan-dependent mechanisms [3]. Arginine-rich peptides, such as a TAT peptide derived from the HIV virus and poly-arginine (Rn, n > 8), are a major class of cationic CPPs, and are commonly used for intracellular delivery of macromolecules [4,5]. Despite the widespread use of CPPs, they have certain limitations, i.e., internalized macromolecules remain mostly trapped in the endosomes, and are subject to degradation or recycling out of the cells [6]. Moreover, the cell-specific delivery of molecules is nearly impossible because CPPs usually lead to random internalization. Some bacterial toxins are known to have evolved to deliver their cytotoxic domain into the cytosol through specific receptors in the host cells, and a bacterial toxin-mediated delivery of macromolecules has been attempted. Anthrax toxin has been shown to deliver enzymes [7,8], including β-lactamase, and toxins. Although anthrax toxin can be used as a genetically encodable system, it requires co-treatment with translocation molecules, called a protective antigen, limiting the diversity of protein cargos [9]. The translocation domain II of *Pseudomonas aeruginosa* exotoxin A was fused to a CPP to increase the endosome escape of the internalized protein cargo [10]. Shiga-like toxin (SLT) has been attempted for cancer targeting and immunotherapy [11,12]. Especially, the B-subunit of SLT was employed as a delivery carrier targeting Gb3 expressing cells for drugs or prodrugs through chemical conjugation [13,14]. Despite many advances, however, a high-efficiency and cell-specific intracellular delivery system for protein cargos has yet to be developed.

Herein, we present a highly efficient and cell-specific intracellular delivery system based on SLT-II secreted by enterohemorrhagic *Escherichia coli (E. coli)*. This toxin has been revealed to be extremely cytotoxic, and the 50% lethal dose (LD_{50}) in mice is as low as 1 ng [12,15]. Considering the extremely high cytotoxicity and delivery mechanism of SLT-II, we reason that its translocation domain will mediate the intracellular delivery of a protein cargo with high efficiency by hijacking the host cellular machinery. SLT-II is composed of a single A-subunit and five B-subunits, forming an

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AB_5 structure [16]. The B-pentamer is responsible for binding to globotriaosylceramide (Gb3) on the cell surface, and the A-subunit is composed of the translocation and cytotoxic domains, inactivating 28S rRNA and eventually causing cell death. Based on the structure of SLT-II, we designed and constructed the Shiga-like toxin-based carrier (STC) comprising the targeting and translocation domains, and used it for delivering a protein cargo. The intracellular delivery efficiency of the STC was investigated and compared with the most widely used cell-penetrating peptide. To demonstrate the general utility of the STC, we replaced its targeting domain with other binding moieties. Details are reported herein.

2. Material and methods

2.1. Gene cloning

The gene coding for SLT-II was obtained from the genomic DNA of *E. coli* O157:H7 (KCCM, Korea). The gene encoding STC (residues 190 through 297 in the mature form of the A-subunit with a full length of the B-subunit) was cloned into pET21a using NdeI and XhoI sites. An EGFP gene was fused to the 5′ terminus of an STC gene, and cloned into the same vector. Other constructs were also generated through PCR and cloned using the same procedure described above.

2.2. Protein expression and purification

The expression vectors were transformed into Rosetta-gami B competent cells, which were grown in a Luria–Bertani (LB) medium containing antibiotics (100 μg/ml of ampicillin, 50 μg/ml of kanamycin, 34 μg/ml of chloramphenicol, and 10 μg/ml of tetracycline) at 37 °C. When the optical density (OD_{600}) of the culture reached 0.5, isopropyl β-D-1-thiogalactopyranoside was added at a final concentration of 0.5 mM, and further incubated for 24 h at 18 °C and 200 rpm. The culture was harvested by centrifugation, resuspended in the lysis buffer (50 mM tris–HCl, 300 mM NaCl, 10 mM imidazole, and pH 8.0), and lysed through sonication. The lysates were centrifuged, and the supernatants were applied to the Ni-NTA agarose resin (Qiagen, Germany). The hexa-histidine-tagged proteins were eluted from Ni-NTA agarose resin using an elution buffer (250 mM imidazole in the lysis buffer), and further purified through size exclusion chromatography using Superdex75 resin (GE healthcare, USA). The proteins were eluted using PBS at a flow rate of 1 ml/min. To generate TAT-EGFP, a gene of a TAT peptide (YGRKKRRQRRR) was fused to the N-terminus of the EGFP gene and expressed in BL21 (DE3) cells (Novagen, USA) using the same procedure describe above.

2.3. FITC labeling of proteins

Purified proteins were incubated with FITC-NHS (10 fold higher concentration the proteins; Thermo Scientific, USA) for 1 h at room temperature in PBS (pH 7.5). Free FITC-NHS was eliminated using a PD-10 column (GE healthcare, UK).

2.4. Intracellular delivery and imaging

Vero (monkey kidney epithelium, ATCC No. CCL-81) and U86MG (human glioblastoma, ATCC No. HTB-14) cells were cultivated in DMEM media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, USA). K-562 (human chronic myeloid leukemia, ATCC No. CCL-243), HT-29 (human colon adenocarcinoma, ATCC No. HTB-38), HeLa (human cervical carcinoma, ATCC No. CCL-2), and SkBr3 cells (human breast adenocarcinoma, ATCC No. HTB-30) were cultivated in RPMI1640 (10% FBS). All cell cultures were incubated in 5% CO_2 at 37 °C. For the delivery tests, the cells were attached to a slide glass overnight. The media were changed to the serum free media containing proteins at the indicated concentration for the indicated time. The cells were washed twice with DPBS and fixed with 4% paraformaldehyde for 20 min at room temperature. The nuclei were counterstained with DAPI in a mounting medium. Lysosome tracker (Invitrogen L-7528, USA), ER-tracker (Invitrogen E34250), and cell tracker (Invitrogen C34551) was added 30 min before fixation at a final concentration of 75 nM, 1 μM, and 1 μM, respectively. Endosome tracker (Invitrogen D-22913) was treated for the same time with the proteins at the concentration of 1 mg/ml. Golgi tracker (Invitrogen, B-34400; the final concentration, 5 μM) was treated for 30 min at 4 °C followed by 30 min incubation at 37 °C in the fresh media.

Images were obtained using Zeiss LSM 710 (except images of live cell, trackers, and U87MG: LSM 780) using the same parameters (objective, 40×; scanning properties, best signal mode (individually obtaining image of each channel); section thickness, 2 μm; laser power, 2%). Live cell image was obtained in 5% CO_2 at 37 °C (objective, 40×; section thickness, 2 μm; laser power, 2%), and section interval of z-stack image was 1 μm. The mean fluorescence intensity of the cells and overlap coefficient were calculated using the ZEN 2011 program, which evaluated more than ten cells from three images for each condition. Each individual cell was cropped as a region of interest (ROI), and the fluorescence intensity of each ROI was quantified in arithmetic mean intensity. Co-localization quantification of ROI was calculated as Manders overlap coefficient in the Zen 2011 program.

For analysis using flow cytometry, the cells were attached to a 6-well plate, and STC-EGFP was treated using the same procedure described above. After 3 h, the cells were washed and analyzed using FACS Calibur (BD Biosciences, USA). The same cells were used in western blot analysis. A total of 20 μg of cell lysate in a lysis solution (Triton X-100 1%, 50 mM tris–HCl, 150 mM NaCl, and pH 7.5) was loaded into 12% SDS-PAGE gel for separation, followed by transfer to a nitrocellulose membrane. The antibodies used were β-actin, sc-47778, Santa-cruz; and GFP, sc-9996, Santa-cruz.

3. Results

3.1. Construction of a Shiga-like toxin-based intracellular delivery system

Based on the structure of SLT-II, we constructed an intracellular delivery system as depicted in Fig. 1a. Both the A- and B-subunits of SLT-II exist in one transcriptional unit in the genomic DNA of *E. coli*, and the genes coding for the STC were cloned between the T7 promoter and terminator in the pET21a expression vector. The resulting construct possesses two ribosome binding sites (RBSs) for each subunit. For purification, a hexa-histidine tag was attached to the C-terminus of the B-subunit. As a model cargo, EGFP was fused to the N-terminus of the A-subunit$^{190-297}$ replacing the cytotoxic domain (Fig. 1a, STC-EGFP). The remaining A-subunit$^{190-297}$ includes the translocation domain and the B-subunit association domain (Fig. 1b). The constructed STC also has a furin protease-sensitive loop (residues 241 to 260) and a disulfide bond (Cys241 – Cys260), which are key components for releasing the protein cargo from the receptor binding domain for retro-translocation from the ER to the cytosol [17]. Because only the B-subunit has a hexa-histidine tag at the C-terminus, both the holo-protein and the isolated B-subunit pentamer were easily purified through affinity purification using a Ni-NTA agarose resin followed by size-exclusion chromatography (SEC). The purified proteins were analyzed using SEC, SDS-PAGE, and MTT for the complex association, purity, and cytotoxicity (Supplementary Fig. S1). The A- and B-