Intracellular Protein Delivery System Using a Target-Specific Repebody and Translocation Domain of Bacterial Exotoxin

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Supporting Information

ABSTRACT: With the high efficacy of protein-based therapeutics and plenty of intracellular drug targets, cytosolic protein delivery in a cell-specific manner has attracted considerable attention in the field of precision medicine. Herein, we present an intracellular protein delivery system based on a target-specific repebody and the translocation domain of Pseudomonas aeruginosa exotoxin A. The delivery platform was constructed by genetically fusing an EGFR-specific repebody as a targeting moiety to the translocation domain, while a protein cargo was fused to the C-terminal end of the delivery platform. The delivery platform was revealed to efficiently translocate a protein cargo to the cytosol in a target-specific manner. We demonstrate the utility and potential of the delivery platform by showing a remarkable tumor regression with negligible toxicity in a xenograft mice model when gelonin was used as the cytotoxic protein cargo. The present platform can find wide applications to the cell-selective cytosolic delivery of diverse proteins in many areas.

Considerable advances in biological and medical sciences have offered the understanding of molecular mechanisms underlying the progression of pathology in various diseases. Consequently, an increasing number of promising druggable targets have been identified, promoting the development of therapeutic agents that can precisely control the unregulated cell signaling processes on the molecular basis of a disease. Compared to small-molecule drugs, protein-based biologics have attracted much attention as the most promising class of targeted therapeutics owing to high efficacy and specificity.1,2 With a growing number of intracellular drug targets, the quest for cytosolic protein delivery in a cell-specific manner has significantly increased in the field of precision medicine.3,4 Diverse delivery systems for proteins have been developed, including liposomes, polymeric or metallic nanoparticles, dendrimers, cell-penetrating peptides, and micelles.1–3 Despite many advances in drug delivery systems, however, the effective intracellular delivery of active proteins remains a challenge for their full therapeutic potential.

Over the past decade, much attention has been paid to the use of bacterial toxins for cytosolic delivery of small-molecule drugs, oligonucleotides, and proteins.9–13 We previously developed a bacterial toxin-based protein delivery system by combining the receptor binding domain of Escherichia coli Shiga-like toxin and translocation domain of Pseudomonas aeruginosa exotoxin A.14 The delivery system was shown to efficiently translocate a protein cargo into the cytosol of mammalian cells in a receptor-specific manner. However, the use of the receptor-binding domain of Shiga-like toxin as a targeting moiety, which is specific for globotriaosylceramide (Gb3), has limitations to versatile applications of the delivery system: its engineering toward other cell surface targets is difficult, and the relevance of Gb3 to diseases is low.

Here, we present a cytosolic protein delivery system based on an EGFR-specific repebody and the translocation domain of Pseudomonas aeruginosa exotoxin A. The repebody scaffold, which is composed of LRR (leucine-rich repeat) modules, was shown to be easy and simple for generating repebodies specific for diverse targets through a phage display selection.15–19 The delivery platform was constructed by genetic fusion of an EGFR-specific repebody as a targeting moiety to the translocation domain of Pseudomonas aeruginosa exotoxin A. Gelonin, which is a ribosome-inactivating plant toxin (MW: 29 kDa), was employed as a cytotoxic protein cargo and fused to the C-terminus of the delivery platform.20 Free gelonin cannot cross the cell membrane owing to the lack of carbohydrate-binding domain, thus showing a strong cytotoxicity when internalized into the cells.21–24 The developed delivery system based on an EGFR-specific repebody exhibited...
a high translocation efficiency of a protein to the cytosol in a target-specific manner. We demonstrate the utility and potential of the delivery platform by investigating cell-selective cytotoxicity and tumor regression in xenograft mice models. Details are reported herein.

RESULTS AND DISCUSSION

Construction of a Cytosolic Protein Delivery Platform.

We previously developed a protein delivery system by combining the receptor-binding domain of the Escherichia coli Shiga-like toxin and translocation domain of Pseudomonas exotoxin A.14 The delivery system was shown to efficiently translocate diverse protein cargos into the cytosol of GB3-expressing mammalian cells. However, the receptor binding domain of Shiga-like toxin is specific for Globotriaosylceramide (Gb3) and was hard to diversify toward other cellular targets, limiting versatility of the previous delivery system. We thus wanted to develop a generally applicable protein delivery platform by replacing the Gb3-binding domain with a target-specific repebody. The repebody scaffold, which is composed of LRR (leucine-rich repeat) modules, was previously developed as a nonantibody protein scaffold. The repebody scaffold was shown to be stable and able to easily generate repebodies specific for diverse targets through a phage display selection.15−19

A repebody (rEgH9) specific for EGFR ectodomain with a binding affinity of 301 pM was employed as a targeting moiety and genetically fused to the N-terminus of the translocation domain of Pseudomonas aeruginosa exotoxin A.14 EGFR is known to be overexpressed in a variety of cancer cells,25,26 and has been used as a cell surface target for the delivery of various cargos to the tumor sites.27 The translocation domain (residues 252 through 364) of Pseudomonas aeruginosa exotoxin A (TDP) was used owing to its favorable property of soluble expression in E. coli. To check the performance of the delivery platform, we chose enhanced green fluorescent protein (EGFP) as a protein cargo and investigated the delivery efficiency (Figure 1A). EGFP was genetically fused to the C-terminus of the EGFR with a binding affinity of 301 pM to facilitate the retrograde transport.30 A schematic representation and complete amino acid sequence of the resulting construct, Rb-TDP-EGFP, are shown in Supporting Information Figure S1. The Rb-TDP-EGFP was eluted as a monomeric peak on a size-exclusion chromatogram and a single band on SDS-PAGE gel (Supporting Information Figure S2).

Figure 1. Construction of a protein delivery platform by combining a target-specific repebody and translocation domain of Pseudomonas aeruginosa exotoxin A. (A) Schematic representation of the delivery platform composed of an EGFR-specific repebody and translocation domain (TDP). EGFP was used as a protein cargo and fused to the C-terminal end of the translocation domain. (B) Western blot analysis of intracellular delivery of EGFP in a dose dependent manner. (C) Target-specific EGFP delivery of different cancer cell lines through receptor-mediated endocytosis. (D) Intracellular delivery of Rb-TDP-EGFP in A431 cells. A431 cells were incubated with Cell tracker or ER tracker for 30 min and washed with DPBS. (E) The Z-stack images of A431 cells treated with 2 μM of Rb-TDP-EGFP for 6 h. Scale bar = 20 μm.
EGFR expression, exhibited a negligible increase in the green fluorescence. Based on the result, it is likely that EGFP was translocated to the cytosol by the delivery platform in a receptor-specific manner.

To check the entrapment of EGFP in the ER compartment, we traced the localization of EGFP using the cell and ER trackers in A431 cells (Figure 1D). The fluorescence signals were shown to mainly come from the cytosol, supporting efficient translocation of a cargo to the cytosol by the delivery platform. It is worth noting that the internalized cargo did not colocalize with the ER trackers and distributed evenly over the entire cytosol of the cells. To further confirm the cytosolic delivery of EGFP, the Z-stack images of A431 cells were obtained after treatment with 2 μM of Rb-TDP-EGFP for 6 h. As a result, strong fluorescence signals were observed from the cytosol (Figure 1E). To test the effect of the concentration of the delivery platform, we performed an additional experiment with 50 nM of Rb-TDP-EGFP (Supporting Information Figure S3). Distinct fluorescent signals were also detected from EGFP even though the signal intensity was weak compared to 2 μM of Rb-TDP-EGFP. To estimate the delivery efficiency, subcellular fractionation was carried out after treatment with 2 μM of Rb-TDP-EGFP, and the amount of EGFP in the cytosol was determined by Western blot using an anti-EGFP antibody (Supporting Information Figure S4). About 80% of total internalized EGFP was detected in the cytosolic fraction.

It is well-known that the translocation domain of bacterial exotoxin A is released from the receptor binding domain to the cytosol through the cleavage of a furin-sensitive loop (RHRQPRG) and reduction of a disulfide bond in endosomes (Figure 2A). To get insight into the translocation mechanism of a cargo by the delivery platform, we conducted the time-course analysis of the localization of EGFP (Figure 2B). Following the binding to EGFR at the cell membrane, the delivery platform was shown to be internalized through endocytosis, exhibiting strong fluorescence signals in endosomes after 1 h of incubation. EGFP appeared to be colocalized with ER trackers at 3 h around the nucleus, and fluorescence signals were distributed throughout the entire region of the cells at 5 h. This result indicates the retrograde transport of EGFP by the delivery platform as bacterial exotoxin. To examine the role of each domain, we constructed the delivery platform with an off-target repebody (Rboff) or without the translocation domain and tested the resulting constructs, Rboff-TDP-EGFP and Rb-EFGP, in terms of translocation of EGFP (Supporting Information Figure S5). The Rboff-TDP-EGFP was shown to result in a negligible delivery of EGFP to the cytosol, and most of the Rb-EFGP was located on the plasma membrane or remained trapped in the endosomes. Based on the results, it is likely that the EGFR-specific repebody led to the receptor-mediated endocytosis of the cargo, and TDP facilitated the retrograde translocation of the cargo to the cytosol from the endosomes. It seems that protein cargo can escape from the endosomes to the golgi and translocate to the cytosol through the endoplasmic reticulum (ER). The translocation domain of Pseudomonas aeruginosa exotoxin A is likely to undergo proteolysis and reduction of the disulfide bond, releasing a protein cargo from the repebody.

**In Vitro Performance of the Delivery Platform.** We tested in vitro performance of the delivery platform using gelonin as a cytotoxic protein cargo. Gelonin, which is a 29 kDa single-chain ribosome-inactivation protein (RIP), is known to act as an N-glycosidase in cells and inactivate the 60S ribosomal subunit. Gelonin (Glo) was genetically fused to the C-terminal end of the delivery platform (Figure 3A and Supporting Information Figure S6). The resulting constructed (Rb-TDP-Glo) was observed to be expressed as a soluble form in E. coli. Various cancer cell lines expressing different EGFR levels were treated with Rb-TDP-Glo, and their viabilities were assayed. A431 and MDA-MB-468 were known to express a high level of EGFR, while H1650 cells were revealed to have a moderate EGFR level. MCF7 cells were used as an EGFR-negative control. As a result, the Rb-TDP-Glo was shown to induce dose-dependent mortality in A431 and MDA-MB-468 cells, whereas no significant effect on the viability of MCF7 cells was observed (Figure 3B).

Both Rboff-TDP-Glo and free repebody were observed to have a negligible cytotoxicity in all the tested cell lines. This result indicates that the delivery platform efficiently translocated gelonin to the cytosol of the EGFR-expressing cells in a target-specific manner and consequently induced the cell death. We also conducted a competitive cytotoxicity assay to examine a receptor-mediated cytotoxicity (Supporting Information Figure S7). For this, 1 μM of free repebody (rEgH9) was preincubated as a competitor in EGFR-positive cells. In contrast to the above result, the cell viability was significantly suppressed in the presence of free repebody (rEgH9), indicating the EGFR-mediated endocytosis and subsequent cell death. Diverse target-specific repebodies can be easily obtained through a phage display selection, and the present delivery platform seems to have the potential to be broadly applicable.

We investigated the mode of action by Rb-TDP-Glo in MDA-MB-468 cells using the TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling, Figure 3C). MDA-MB-468 and MCF-7 cells were...
treated with 1 μM of Rb-TDP-Glo, followed by fluorescent labeling of apoptotic cells by the TUNEL assay. MDA-MB-468 cells exhibited a strong fluorescence signal, whereas negligible green fluorescence was detected in MCF-7 cells, which indicates that apoptosis occurred through internalization of Rb-TDP-Glo. MDA-MB-468 cells treated with only gelonin showed no green fluorescence due to the lack of EGFR-specific repebody. Based on the results, it is likely that the cytotoxic effect of Rb-TDP-Glo in the cells was linked to an apoptotic mechanism.

In Vivo Performance of the Delivery Platform. We investigated the in vivo antitumor activity of Rb-TDP-Glo in xenograft mice with MDA-MB-468 cells. When the tumor volume reached about 100 mm³, the mice were subjected to intravenous injection of the Rb-TDP-Glo (10 mg/kg) five times within 15 days (Figure 4A). Free EGFR-specific repebody (4.2 mg/kg) was also injected into mice intravenously every 3 days as a control. Treatment of xenograft mice with Rb-TDP-Glo resulted in a remarkable suppression of tumor growth, whereas the tumor continued to grow when treated with PBS and free repebody. The tumor volume of mice treated with Rb-TDP-Glo reduced to 137 mm³ on day 30, whereas the tumor volume increased to 465 mm³ when treated with PBS (Figure 4B and C). The overall health status and body weight of mice were monitored twice a day during treatment with Rb-TDP-Glo, and no significant change in body weight was observed on day 30 (Figure 4D). We also observed a negligible effect of Rb-TDP-Glo on the levels of aspartate and alanine aminotransferase (AST and ALT) as well as creatine and blood urea nitrogen (BUN) even after 30 days (Figure 4E and F), implying a negligible toxic effect of Rb-TDP-Glo on the liver and kidneys. We also examined the toxicity of TDP-Glo without a targeting moiety (Supporting Information Figure S8). No significant effects on body weight, hepatotoxicity, and nephrotoxicity were observed, implying a negligible toxicity of free gelonin. Taken together, our results demonstrate that the delivery platform efficiently translocated the protein cargo to the tumor site in a target-specific manner and effectively suppressed the tumor growth with negligible cytotoxicity in xenograft mice models.

Conclusion. We have demonstrated that the delivery platform enabled efficient translocation of a protein cargo into the cytosol in a target-specific manner through the endocytic pathway. Our delivery platform enabled an efficient translocation of a protein cargo to the cytosol in a target-specific manner. Furthermore, our platform was simple and cost-effective to produce in homogeneity by a bacterial expression system through a genetic fusion. A protein cargo-fused delivery platform was well expressed as a soluble form in E. coli, and this seems to be due to the translocation domain of Pseudomonas aeruginosa exotoxin A. Treatment of xenograft mice with Rb-TDP-Glo led to a remarkable suppression of...
tumor growth in a target-specific manner with negligible toxicity. An EGFR-specific repebody was employed as a model targeting moiety and genetically fused to the N-terminus of the translocation domain to construct the delivery platform. The repebody scaffold, which is composed of LRR (leucine-rich repeat) modules, was shown to be stable and to easily generate repebodies specific for diverse targets through a phage display. The binding affinity of a repebody could be matured to a subnanomolar range by modular engineering. Taken together, target-specificity of the delivery platform can be easily modulated by replacing a repebody with another one, which allows broad utility of the delivery platform.

**MATERIALS AND METHODS**

**Protein Expression and Purification.** All genes coding for various fusion proteins were cloned into the Ndel and Xhol restriction enzyme sites of a PET21a vector (Novagen). DNA encoding the Gelonin was PCR-amplified from a pMal-E4rGel (Addgene) and digested with XhoI and HindIII. The fragment was then ligated into the corresponding sites in DNA encoding the C-terminal region of a repebody (rEgH9)-TDP, which generated the expression construct rEgH9-TDP-Gelonin. The construct rEgH9-TDP-EGFP was generated by replacing DNA encoding gelonin with EGFP. Each gene has the C-terminal hexa-histidine tag for affinity purification. The vectors were transformed into E. coli BL21 (DE3), and IPTG (0.5 mM) was added for induction when the optical density reached OD₆₀₀ between 0.5 and 0.7. Induced cells were further grown at 18 °C for 20 h and harvested by centrifugation at 7000 rpm. The cells were resuspended in a lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole, at pH 8.0) and disrupted through sonication. Following centrifugation at 13,000 rpm for 1 h, supernatant was loaded onto a Ni-NTA Superflow (Qiagen). The solution was washed with three column volumes of a buffer (50 mM NaH₂PO₄, pH 8.0) containing 300 mM NaCl and 50 mM imidazole. Hexahistidine tagged fusion proteins were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole, pH 8.0). The proteins were further purified by gel permeation chromatography (Superdex200, GE Healthcare) with a phosphate-buffered saline (PBS; pH 7.4).

**Mammalian Cell Culture.** A431 (human epidermoid carcinoma, ATCC No. CRL-1555) cells were cultivated in a DMEM medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone). MDA-MB-468 (human adenocarcinoma, ATCC No. HTB-132) cells were cultivated in Leibovitz’s L-15 Medium (Gibco) supplemented with 10% (v/v) FBS. H1650 (human adenocarcinoma, ATCC No. CRL-5883) and MCF7 (human adenocarcinoma, ATCC No. HTB-22) cells were cultivated in RPMI1640 Medium (Gibco) supplemented with 10% (v/v) FBS. All cell cultures were incubated in a 5% CO₂ chamber at 37 °C.

**Cytosolic Delivery of EGFP by the Delivery Platform.** For the delivery of proteins, the respective cells were attached into a culture slide. Cells were seeded in an eight-well chamber slide (SPL) at a density of 1 × 10⁴ cells/well. After overnight incubation, serum-containing media with a predetermined concentration of repebody-TDP-EGFP were treated for 6 h. The Cell tracker (Invitrogen...
C345S1) and ER tracker (Invitrogen E34250) were treated for 30 and 20 min, respectively. The cells were washed twice with DPBS and fixed with 4% (w/v) paraformaldehyde for 20 min. Cell nuclei were counterstained with DAPI in the mounting medium (Vector). Cell imaging was performed using a Zeiss LSM 780 confocal microscope with a x63 Apochromat 1.0 numerical aperture (NA) objective (Carl Zeiss). The section interval of the z-stack image was 0.7 μm.

**In Vitro Cytotoxicity.** Respective cells were seeded in a 96-well plate (SPL) at a density of 5 × 10^4 cells/well. The cells were seeded in an eight-well chamber slide (SPL) at a density of 1 (Takara) according to the manufacturer's instructions. Brieﬂy, cells were seeded in an eight-well chamber slide (SPL) at a density of 1 × 10^3 cells/well, and the medium was changed to a serum-free medium containing each protein. Different concentrations of the repepbody-TDP-Gelonin were added to each well, followed by incubation for 3 days, and the serum-free medium supplemented with 10 μL of CCK-8 reagent (Dojindo) was added to the wells and incubated for 2 h at 37 °C. The cell viability was determined by measurement of OD_{450} using an Inﬁnite M200 plate reader (Tecan).

**Western Blot Assay.** The cells were washed with DPBS and homogenized in a lysis buffer (Tris-HCl 50 mM, pH 8, NaCl 150 mM, and 1% (v/v) Triton X-100). The supernatant fractions were separated using 12% SDS polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Bio-Rad) at 100 V for 2 h in ice. The membrane was incubated with a blocking buffer and phosphate-buffered saline with Tween 20 (PBST)-bovine serum albumin (BSA; PBS containing 0.1% (v/v) Tween-20 and 1% (v/v) BSA) and subjected to a Western blot analysis using the goat anti-GFP polyclonal antibody (Abcam) and rabbit antigoat IgG-HRP conjugated antibody (Bio-Rad). The blots were visualized by an enhanced chemiluminescence solution (Millipore) using a LAS-3000 imaging system (Fuji-Film).

**TUNEL Assay.** A TUNEL assay was used to evaluate the apoptotic cells in mammalian cells using the In Situ Apoptosis Detection Kit (Takara) according to the manufacturer's instructions. Brieﬂy, cells were seeded in an eight-well chamber slide (SPL) at a density of 1 × 10^3 cells/well. The cells were ﬁxed in 4% (w/v) formaldehyde solution for 15 min and washed with DPBS, followed by permeabilization with permeabilization buffer for 20 min. The cells were quenched with 3% (v/v) hydrogen peroxide, washed with DPBS, and labeled with TdT (Terminal deoxynucleotidyl Transferase) enzyme. The slide with cells was incubated at 37 °C humidified chamber for 1 h and then treated with DPBS as a stop buffer.

**Animals and Tumor Cells for Xenograft.** The colorectal carcinoma cell line MDA-MB-468 was obtained from ATCC. MDA-MB-468 cells were cultured in RPMI (Life Technologies, Inc.), supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. Cell cultures were maintained at 37 °C in 5% CO2 incubator. Six-week-old male BALB/c nu/nu mice were purchased from Orient Bio Inc., Seoul, Korea. All animals were acclimatized to controlled conditions of temperature (23 ± 2 °C), humidity (55 ± 5%), and light (12 h light/dark cycle) at the Central Animal Research laboratory, Advanced Radiation Technology Institute (ARTI), Korea Atomic Energy Institute (KAERI). The animals were provided a sterile pellet diet and water ad libitum. The experiment was approved by the Institutional Animal Ethical Committee and was performed in strict compliance with the guidelines prescribed by the committee.

**Determination of Toxicity in Mice.** The animals were divided into three groups of six male BALB/c nu/nu mice. Each group of animals was given injections in the tail vein. Animal mortality was observed over 4 weeks.

**Antitumor Activity.** MDA-MB-468 (5 × 10^6 cells with 100 μL of Matrigel) was inoculated subcutaneously (s.c.) into the right lateral flank of the mice. After about 7 days, the mice were used in the experiments when the tumor size was established at approximately 100 mm^3. The study was divided into three groups with six mice in each group. To evaluate the antitumor activity of Rb-TDP-Gel fusion proteins in the MDA-MB-468 xenograft mouse model, the mice were intravenously (i.v.) injected with PBS, Rb (4.2 mg/kg), and Rb-TDP-Gel (10 mg/kg) on days 0, 3, 6, 9, and 12. The mice were injected with equivolumar amounts of each protein (27 μM/Dose). The body weight and tumor size was measured individually twice daily for 30 days. The tumor volume was measured macroscopically with a caliper, and tumor volume was calculated according to the formula (mm^3) \[ \text{length} \times \text{width}^2 \times 0.5 \]. Mice were euthanized when tumors reached a volume of 650 mm^3 or when tumors showed skin ulcerations.

**Determination of Hepatotoxicity and Nephrotoxicity.** The blood was allowed to clot, and plasma was separated by centrifugation (3000 for 20 min) and stored at 4 °C. Hepatotoxicity was evaluated based on aspartate, and alanine transaminases (AST and ALT) in serum were assayed using enzymatic kits (Asan Pharmaceuticals, Co., Ltd.). Nephrotoxicity was assessed by measuring the levels of blood urea nitrogen (BUN) and creatine using a Fuji dri-Chem NX 7000i (Fuji Photo Film Co., Ltd.).

**Statistical Analysis.** The values are expressed as means ± SD of six mice per group. Statistical analyses were performed using a one-way analysis of variance (ANOVA), and intergroup comparisons were made using Tukey's multiple comparison test. A value of \( P < 0.05 \) was considered statistically significant.

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These authors contributed equally to the work.

**Notes**

The authors declare no competing financial interest.

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