Engineering and cytosolic delivery of a native regulatory protein and its variants for modulation of ERK2 signaling pathway

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Abstract
The modulation of a cell signaling process using a molecular binder followed by an analysis of the cellular response is crucial for understanding its role in the cellular function and developing pharmaceuticals. Herein, we present the modulation of the ERK2-mediated signaling pathway through the cytosolic delivery of a native regulatory protein for ERK2, that is, PEA-15 (phosphoprotein enriched in astrocytes, 15 kDa), and its engineered variants using a bacterial toxin-based delivery system. Based on biochemical and structural analyses, PEA-15 variants with different phosphorylation sites and a high affinity for ERK2 were designed. Semi-rational approach led to about an 830-fold increase in the binding affinity of PEA-15, resulting in more effective modulation of the ERK2-mediated signaling. Our approach enabled an understanding of the cellular function of the ERK2-mediated signaling process and the effect of PEA-15 phosphorylation on its action as an ERK2 blocker. We demonstrated the utility and potential of our approach by showing an efficient cytosolic delivery of these PEA-15 variants and the effective suppression of cell proliferation through the inhibition of the ERK2 function. The present approach can be used broadly for modulating the cell signaling processes and understanding their roles in cellular function, as well as for the development of therapeutics.

KEYWORDS
bacterial toxin, cytosolic delivery, intracellular delivery, protein delivery, protein engineering, signaling modulation

1 | INTRODUCTION

Cellular signaling processes are a key to most biological processes, including the development, regulation, and proliferation of cells (Wells & McClendon, 2007). The modulation of such signaling processes through a molecular binder and a subsequent analysis of the resulting cellular response are essential for understanding their roles in cellular function and drug development. As a cytosolic protein, ERK2 (extracellular signal-regulated kinase 2) is known to play a key role in cell proliferation, acting as a pivotal protein for multiple intracellular signals (Nissan, Rosen, & Solit, 2013; Von Kriegsheim et al., 2009). The signaling axis, RAS-RAF-MEK-ERK, represents a typical feature in the proliferation and invasion of...
cancer cells, and has attracted considerable attention as a target for studying the signaling networks and developing therapeutics for many types of cancers (Blake et al., 2014; Kohno & Pouysségur, 2006; Kolch, 2005; Morris et al., 2013; Plotnikov et al., 2015). Although small molecule inhibitors against RAF and MEK have been approved by the FDA for the treatment of cancers (Menzies & Long, 2014), ERK2 inhibitors have yet to be developed.

PEA-15 (phosphoprotein enriched in astrocytes, 15 kDa) is a naturally occurring regulatory protein for ERK2, and its crystal structure in complex with ERK2 has been recently resolved (Mace et al., 2013). PEA-15 has two phosphorylation sites that are phosphorylated by upstream signaling molecules in the AKT and PKC pathways. Whereas phosphorylated PEA-15 has a negligible inhibitory effect on the function of ERK2, non-phosphorylated PEA-15 is known to directly bind to ERK2 and block its functions, such as substrate binding and nuclear translocation (Callaway, Abramczyk, Martin, & Dalby, 2007; Krueger, Chou, Glading, Schaefer, & Ginsberg, 2005; Trenicia et al., 2003). With such a function, PEA-15 itself has gained significant attention as a potential therapeutic for cancer treatment (Lee et al., 2012; Xie et al., 2013; Xie et al., 2015). The development of PEA-15 as a therapeutic agent has several barriers, however, such as a low binding affinity (~3.4 μM) for ERK2 and the lack of an efficient intracellular delivery system (Mace et al., 2013). Although various delivery methods have been developed, they have certain drawbacks in their practical application in terms of their delivery efficiency, specificity, and safety (Gu, Biswas, Zhao, & Tang, 2011; Gupta, Levchenko, & Torchilin, 2005; Nayerossadat, Maedeh, & Ali, 2012; Torchilin, 2008). Modulation of the cell-signaling processes through the cytosolic delivery of a protein binder is considered more efficient and straightforward, but remains a challenge.

Here, we present the modulation of the ERK2-mediated signaling pathway through the cytosolic delivery of PEK-15 and its engineered variants using a bacterial toxin-based system. The PEA-15 variants with different phosphorylation sites and high affinity for ERK2 were designed and used for modulating the ERK2-mediated signaling process. The utility and potential of our approach was demonstrated through the efficient cytosolic delivery of PEK-15 variants and the subsequent suppression of the cell proliferation through the blocking of the ERK2 function in the cells. Details are reported herein.

## 2 METHODS

### 2.1 Cloning, protein expression, and purification

The gene coding for proteins was cloned with his tag into a pET21a plasmid between the NdeI and XhoI sites, and the resulting plasmids were transformed into the Origami B (DE3) strain. The proteins were over-expressed by addition of 0.5 mM of isopropyl-d-1-thiogalactopyranosid (IPTG) when OD600 reached about 0.6 and further incubation at 18°C for 20 hr. Cells were harvested and disrupted through sonication, and the proteins were purified by Ni-NTA agarose resins and size exclusion chromatography as previously described (Ryou et al., 2016).

### 2.2 Imaging

The cells were incubated with 100 nM of proteins fused with a bacterial-toxin based delivery system as described in our previous work (Ryou et al., 2016), followed by washing them twice with DPBS. The cells were fixed using 4% paraformaldehyde for 15 min at room temperature. For the ERK2 blocking experiment, FBS (20%, final concentration) was added to the medium 2 hr prior to fixation. The cells were permeabilized using 0.25% triton X-100 in a PBS (pH7.4), followed by incubation with a blocking buffer (1% BSA in PBST) for 30 min. An FITC-conjugated anti-ERK2 antibody (sc-154 FITC, Santa Cruz, Dallas, TX) in a blocking buffer was added to the cells followed by overnight incubation at 4°C. The nucleus was counter-stained by DAPI in the mounting medium. Zeiss LSM 780 (Carl Zeiss, Germany) was used for the imaging using the same parameters (objective lens, 40x; best signal mode; section thickness, 2 μm; laser power, 2%).

### 2.3 Construction of a phage-displayed peptide library for affinity maturation

A peptide library, which is composed of the C-terminal tail of PEA-15 with five additional randomized residues, was displayed on an M13 phage using a general method (Hamzeh-Mivehroud, Alizadeh, Morris, Church, & Dastmalchi, 2013). Briefly, pTV118N (Takara), a type of phagemid, was engineered to display a peptide library containing the carboxyl-terminal tail of PEA-15 on the minor coat protein (pIII) of the M13 bacteriophages. The modified pTV118N, pBEL4, was used to display the peptide library on the N-terminus of the full form pIII protein without amber codon suppression. A DNA fragment for the peptide library was designed through overlapping PCR using primers containing degenerate codons (GAATTCGCTGAAGAAGAAATCATT AAGCTTGCCCCCGCCACCGAAGAAAGACNNNNKNKNKNKNKNKNC TCGAG). The fragments were inserted into pBEL4, followed by electroporation into XL1-Blue containing F′ episome (Stratagene, San Diego, CA). The phages displaying the peptide library were produced using an SS320 strain and VCS M13 helper phages, and subjected to the panning process.

### 2.4 Affinity maturation of PEA-15AA for ERK2

Bio-panning was conducted repeatedly to increase the affinity of PEA-15 variant (S104A and S116A) toward ERK2, as described in our previous works (Lee et al., 2014). During each round of the panning process, 30 μg/ml of ERK2 was used. To identify the positive clones, a phage ELISA was applied using 5 μg/ml of ERK2, as described in our earlier work (Lee et al., 2014). The affinities of the PEA-15 variants were measured using surface plasmon resonance (Biacore 3000, GE Healthcare, Milwaukee, WI) according to the general procedure (Malmqvist, 1993).

### 2.5 Surface plasmon resonance (SPR) analysis

Binding affinities of PEA-15 variants were measured using a Biacore 3000 system (GE Healthcare). ERK2 was immobilized onto a CM5 chip.
(GE Healthcare) by EDC-NHS coupling method. To evaluate the association and dissociation kinetics, varying concentration of PEA-15 variants were injected, and all data were analyzed by BIA evaluation software.

2.6 | Molecular dynamics simulations

Molecular dynamics simulations were conducted using GROMACS version 4.5.7 (Hess, 2009) with an AMBER03 force field (Duan et al., 2003). The complex of ERK2 with a newly designed PEA-15 variant was placed in a periodic dodecahedron filled with TIP3P water and counterions (0.1 M). Energy minimization was conducted in two steps. The first step included the fixation of ERK2. The peptide up to the first proline and ERK2 were fixed when the remainder of the peptide had relaxed for 5 ns under 250, 300 K, and a variable temperature (from 250 to 1,000 K).

2.7 | Western blot analysis

The cells were harvested and lysed using a RIPA (Radio-Immunoprecipitation Assay) lysis buffer (Sigma–Aldrich, St. Louis, MO). Proteins from the lysed cells were separated through 10% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose blotting membrane (GE, Germany) for 2 hr at 100 V on ice. The membrane was blocked using a PBS-based blocking buffer containing 0.05% Tween-20 and 1% bovine serum albumin for 1 hr at 4°C, incubated with anti-phospho Rsk-1/2 monoclonal antibody (Santa-Cruz), and followed by the addition of an HRP-conjugated anti-rabbit IgG antibody (Biorad, Hercules, CA). The membrane was washed with PBST, and the signals were detected using an enhanced chemiluminescence solution (Millipore, Billerica, MA). For the immunoprecipitation, 1 µM of each protein was treated to HepG2 cells for 3 hr. The cells were homogenized through sonication in the PBST, and the signals were detected using an enhanced chemiluminescence solution (Millipore, Billerica, MA). For the immunoprecipitation, 1 µM of each protein was treated to HepG2 cells for 3 hr.

2.8 | Cell apoptosis viability assays

The delivery system with a cargo protein was added to the cells in a six-well plate. After 24 hr, the cells were washed and trypsinized followed by the addition of an Annexin V-FITC apoptosis detection kit with propidium iodide (Koma Biotech, Seoul, Korea). To assess the cell viability, the cells were seeded into a 96-well plate, and the cells were incubated with a serum-free medium (RPMI) for 12 hr. The proteins were diluted in a serum-free medium at the indicated concentration, and then added to the cells. After incubation for the indicated times, MTT was added at a final concentration of 1 mg/ml and incubated for 2 hr. An unpaired t-test was used to calculate the p-value.

2.9 | In vitro cytotoxicity

The cells were seeded into a 96-well plate and incubated with a serum-free medium (RPMI) for overnight. The proteins were added to each well, followed by incubation for 3 days, and the culture medium was removed. After that, fresh serum-free medium supplemented with a 10% CCK-8 reagent (Dojindo, Japan) was added to the wells and incubated for 1 hr at 37°C. The cell viability was determined by measuring the absorbance at 450 nm.

3 | RESULTS

3.1 | Cytosolic delivery of PEA-15 using a toxin-based system

PEA-15 is a cytosolic protein that inhibits the ERK2-mediated signaling process (Callaway et al., 2007; Krueger et al., 2005; Trencia et al., 2003) (Figure 1a). Prior to modulation of the ERK2-mediated signaling process using PEA-15, we attempted the cytosolic delivery of PEA-15. We previously developed a protein delivery platform comprising a receptor binding domain of Shiga-like toxin (StxB) and a translocation domain of Pseudomonas exotoxin A (TDP) (Figure 1b) (Ryou et al., 2016). The toxin-based delivery system was shown to efficiently transfer diverse proteins into the cytosol of the StxB-targeted Globotriaosylceramide (Gb3) positive cell lines. To check the cytosolic delivery of PEA-15 using the toxin-based platform, we fused EGFP to the N-terminus of PEA-15 and traced the EGFP fluorescence of a construct (StxB-TDP-EGFP-PEA-15) in Gb3-positive HepG2 cells. The EGFP-fused construct (StxB-TDP-EGFP) used as a control. As shown in Figure 1c, EGFP-PEA-15 was spread evenly over the cytoplasm, although a portion of the construct was trapped within the intracellular organelles (Supplementary Figure S1). This result confirms the efficient cytosolic delivery of PEA-15 through the toxin-based platform.

3.2 | Mutational effect of PEA-15 phosphorylation site on ERK2-mediated signaling

It is known that PEA-15 has two phosphorylation sites, S104 and S116. Whereas phosphorylated PEA-15 has a negligible effect on the ERK2-mediated signaling pathway, non-phosphorylated PEA-15 binds directly to ERK2 and inhibits the ERK2-mediated signaling (Callaway et al., 2007; Krueger et al., 2005; Trencia et al., 2003). To examine the effect of PEA-15 phosphorylation on the ERK2-mediated signaling, we constructed PEA-15 variants with no phosphorylation sites (S104A and S116A) and pseudo-phosphorylation (S104E and S116E), namely, PEA-15AA and PEA-15EE, respectively. We genetically fused the PEA-15 variants to the ER. The resulting constructs (StxB-TDP-PEA-15AA and StxB-TDP-PEA-15EE) were expressed in Escherichia coli (E. coli) and purified. StxB is a pentamer, and each delivery module has five cargo molecules (Figures 1b and S2). In the cytosol, a portion of the TDP remained at the N-terminus of the cargo proteins. To check whether the remaining part of the TDP has an effect on the interaction with ERK2, we determined the affinities of
the PEA-15AA and TDPcleaved-PEA-15AA for ERK2 using surface plasmon resonance, and observed a negligible effect on the binding affinity for ERK2 (Supplementary Figure S3).

To test whether the PEA-15 variants expressed in E. coli can also bind to endogenous ERK2, two his tag-fused PEA-15 variants (PEA-15AA and PEA-15EE) were delivered to HepG2 cells and subjected to an analysis of the immuno-precipitates from the cell lysates after elution through a Ni-NTA column (Figure 2a). The delivered PEA-15AA and PEA-15EE were shown to bind to endogenous ERK2 in a dose-dependent manner, whereas ERK2 was not detected in the control experiment using EGFP. This result indicates that the delivered PEA-15 variants maintain their binding ability toward ERK2 in the cells, supporting the binding of non-phosphorylated and pseudo-phosphorylated forms of PEA-15 to ERK2 (Callaway et al., 2007). Although PEA-15 retained its binding affinity for ERK2 regardless of the phosphorylation states, phosphorylated PEA-15 is known to lose its inhibitory effect on the function of ERK2. It is known that ERK2 is translocated into the nucleus when the cells are stimulated by serum, whereas non-phosphorylated PEA-15 blocks the translocation of ERK2 into the nucleus (Mace et al., 2013). To investigate the effects of PEA-15 phosphorylation on its function, PEA-15AA and PEA-15EE were delivered to serum-starved HepG2 cells followed by stimulation with fetal bovine serum (FBS) for 2 hr, and their inhibitory activity toward ERK2 was analyzed (Figure 2b). As a result, the cytosolic delivery of PEA-15AA was shown to reduce the nuclear translocation of ERK2. In contrast, PEA-15EE, a pseudo-phosphorylated form of PEA-15, had little effect as a PBS control. The fluorescent signal from ERK2 was observed mainly around the nuclear region.

Activated ERK2 is known to phosphorylate downstream signaling molecules, such as Rsk-1/2 and Elk-1. Thus, blocking the ERK2 action will lower the level of phosphorylated Rsk-1/2 and Elk-1. To obtain insight into how PEA-15AA and PEA-15EE affect the phosphorylation of downstream signaling molecules, HepG2 cells were incubated in a serum-free medium overnight followed by incubation with 1 µM of StxB-TDP-PEA-15AA or StxB-TDP-PEA-15EE for 8 hr and subsequent stimulation with Phorbol-12-Myristate-13-Acetate (PMA) for 2 hr. The delivery of PEA-15AA was shown to significantly lower the level of phosphorylated Rsk-1/2, whereas PEA-15EE and EGFP had no effect as a PBS control (Figure 2c). We checked the minimum delivery time of PEA-15 giving rise to an efficient inhibition effect on the ERK2 function. The cells were treated with 1 µM of StxB-TDP-PEA-15AA for different incubation times, and the levels of phosphorylated Rsk-1/2 and Elk-1 were analyzed (Figure 2d). The levels of phosphorylated Rsk-1/2 and Elk-1 decreased after 4 hr. These results indicate that non-phosphorylated PEA-15 efficiently blocks the ERK2 function in 4 hr.

**FIGURE 1** Cytosolic delivery of PEA-15 through a toxin-based system. (a) Schematic of the PEA-15 role in the ERK2-mediated signaling process. PEA-15 inhibits both the activation of RSK and MNK as well as nuclear translocation of ERK2. RTK indicates receptor tyrosine kinase. (b) Schematic of a toxin-based delivery platform comprising a receptor-binding domain of Shiga-like toxin and a translocation domain of Pseudomonas exotoxin A. The protein cargo is genetically fused to the C-terminal of the translocation domain followed by fusion of the KDEL sequence. The receptor-binding domain of Shiga-like toxin is a pentamer, and five molecules of the cargo are loaded into one delivery module. The translocation domain is cleaved by furin protease before the cargo is retro-translocated into the cytosol (dotted line). (c) Fluorescence image of HepG2 cells after intracellular delivery of EGFP-fused PEA-15. EGFP-PEA15 was genetically fused to the delivery platform followed by incubation with HepG2 cells for 2 hr. The nucleus was counter-stained using DAPI. Scale bar: 20 µm
3.3 In vitro inhibitory effect by PEA-15AA on the cell proliferation

PEA-15 plays a crucial role in ERK2-mediated cell proliferation, and phosphorylated PEA-15 may act as a tumor-promoter (Sulzmaier, Opoku-Ansah, & Ramos, 2012). We assessed the effect of the PEA-15 variants on the proliferation or apoptosis of the cells. To this end, we constructed another variant, PEA-15SA and PEA-15AS, with a single phosphorylation site. After serum-starvation overnight, HepG2 cells were incubated with 100 nM of each construct (StxB-TDP-PEA-15AA, StxB-TDP-PEA-15EE, StxB-TDP-PEA-15SA, and StxB-TDP-PEA-15AS), and the cell proliferation was measured (Figure 3a). Interestingly, PEA-15 variants with pseudo-phosphorylation and a single phosphorylation site, namely PEA-15EE, and PEA-15SA, PEA-15AS had little effect on the cell proliferation. Only PEA-15AA with no phosphorylation site exhibited an inhibitory effect on the cell proliferation. This result implies that PEA-15SA and PEA-15AS with a single phosphorylation remained inactive in the cells. A similar effect by the PEA-15 variants with single phosphorylation site and single pseudo-phosphorylation suggests that PEA-15 underwent immediate phosphorylation in the cytosol of the cancer cells even without serum stimulation. We checked whether serum stimulation would modulate the inhibitory effect by the PEA-15 variants on the cell proliferation. The PEA-15 variants were delivered to HepG2 cells followed by serum stimulation (Figure 3b). As a result, the cytosolic delivery of PEA-15AA was shown to have an inhibitory effect on the cell proliferation, whereas the other PEA-15 variants resulted in a slight degree of inhibition. The inhibitory effect of PEA-15AA was distinct when the cells were stimulated with a serum, which acts as a signal of the proliferation. This result indicates that non-phosphorylated PEA-15 significantly inhibits the cell proliferation. No significant difference was observed between PEA-15EE and PEA-15SS. We next tested whether PEA-15AA induces apoptosis of the cells. Serum-starved HepG2 cells were treated with 100 nM of StxB-TDP-PEA-15AA or StxB-TDP-PEA-15EE for 24 hr, and subjected to an apoptosis assay. For PEA-15AA, apoptotic cells accounted for around 10% of the total cells, whereas
PEA-15AA is thought to have a low binding affinity for ERK2 as PEA-15 (~3.4 μM) (Supplementary Figure S3). PEA-15AA with a higher affinity for ERK2 is therefore expected to modulate the ERK2-mediated signaling pathway more effectively. We intended to rationally design the PEA-15AA with a high affinity for ERK2 based on the crystal structure of PEA-15 in complex with ERK2. A structural analysis reveals that PEA-15 binds to ERK2 in a bipartite manner. The C-terminal segment of PEA-15 (residues 122–127) interacts with the D recruitment site (DRS) of ERK2, which consists of a hydrophobic pocket and negatively charged domain (termed the CD domain) (Xu, Stippec, Robinson, & Cobb, 2001). Interestingly, the C-terminal segment of PEA-15 only utilizes the hydrophobic pocket, and is unlikely to extend to two aspartates (D316 and D319) in the CD domain owing to its short length (Mace et al., 2013). Accordingly, the interaction interface is small, resulting in a low binding affinity for ERK2. This "minimal" interacting mode can be demonstrated by comparing the structure of PEA-15 with that of MNK1 in complex with ERK2 (Garai et al., 2012) (Figure 4a). Based on this analysis, we reasoned that five additional residues at the C-terminus of PEA-15 would lead to an additional interaction between PEA-15AA and the CD domain of ERK2, consequently increasing the binding affinity for ERK2.

We first conducted a directed evolution approach to generate an additional interaction between PEA-15AA and ERK2. Because the C-terminal segment of PEA-15AA can bind independently to ERK2 without an N-terminal part, we used 15 amino acids at the C-terminus as a template for affinity maturation (Figure 4b). A library was constructed by introducing five random amino acids at the C-terminus, and subjected to a phage display selection. Through a panning process against ERK2, we identified five clones showing high signals in the phage-ELISA. Interestingly, five clones were shown to have the same sequence (WWRVK) on the mutation sites. We determined the binding affinity of the PEA-15AA containing five additional residues (termed PEA-16 owing to a size of 16 kDa). The KD value of PEA-16 was estimated to be 4.1 nM, which corresponds to approximately an 830-fold increase compared to PEA-15 (Figures 4c and S5). To confirm the contact of the five additional residues with the CD domain of ERK2, we changed two aspartates in the CD domain to asparagine (D316N/D319N). As expected, the D316N/D319N mutations had a critical effect on the binding of PEA-16 to ERK2, whereas the mutations caused a negligible change in the affinity of PEA-15AA to ERK2 (Supplementary Table S1). We conducted an alanine-scanning mutagenesis in the five additional residues (WWRVK) of PEA-16. The mutation of the basic residues into alanine resulted in a significant decrease in the affinity for ERK2 (Supplementary Figure S6). Based on the result, it is likely that the additional region of PEA-16 interacts with the CD domain of ERK2, as expected. To further confirm the additional contact by PEA-16, we conducted the molecular dynamics simulations at various temperatures (Figure 4d). The key basic residues, R133 and K135 (where the residue number was assigned after the final residue of PEA-15), were shown to face the acidic residues of the CD domain, indicating a significant charge interaction between them. W131 and R133 exhibited the strongest bonds with ERK2, and this interaction was maintained even at 720 K (Supplementary Figure S7). While W131 was deeply buried in the interface, R133 is thought to have formed a salt bridge with ERK2 (Figure 4e), contributing significantly to an increase in the binding affinity for ERK2.

3.5 Inhibitory effect of high affinity variant, PEA-16

We attempted to check whether PEA-16, which is a high-affinity variant of PEA-15AA with five additional residues, has a stronger inhibitory effect on the ERK2-mediated signaling pathway. PEA-16 was delivered to the HepG2 cells, and its inhibitory effect was
investigated (Figure 5a). PEA-16 was shown to more effectively lower the level of phosphorylated Rsk than PEA-15AA. To assess the potential of PEA-16 as a therapeutic agent, we measured the cell viability three days after the cytosolic delivery of PEA-16 to the serum-starved HepG2 cells. As shown in Figure 5b, PEA-16 showed a much higher cytotoxic effect than PEA-15AA and PEA-15SS (native form). This result indicates that PEA-16 with a higher affinity for ERK2 leads to a more effective modulation of the ERK2-mediated signaling pathway in the cells.

We next tested whether PEA-16 has a cytotoxic effect on the other cell lines, including the Gb3-negative cell lines (NIH-3T3, CHO-K1, and K-562). Serum-starved cells were treated with 200 nM of

**FIGURE 4** Affinity maturation of PEA-15 based on structural analysis. (a) Binding modes of MNK1/ERK2 (PDB ID: 2Y9Q) and PEA-15/ERK2 (PDB ID: 4IZ5). Crystal structure of MNK1 in complex with ERK2 was compared with that of PEA-15 in complex with ERK2. MNK1 (blue stick) interacts with both the CD domain (red sphere) and the hydrophobic pocket (black sphere) of ERK2 (gray cartoon). Yellow sticks are the key basic residues contacting the CD domain. PEA-15 (green stick) interacts with the hydrophobic pocket, but has no interaction with the CD domain (dot circle). The leucine residue, a key residue in the interaction with the hydrophobic pocket, is shown in magenta. (b) Schematic of an additional segment at the C-terminus of PEA-15 for increasing the interaction with ERK2. Five residues (dotted lines) added to the C-terminus of PEA-15 were randomized for a directed evolution. (c) Amino acid sequence of selected PEA-16 from residues 121 to 135. Five additional residues from the C-terminus of PEA-15 are shown. The sequence of PEA-16 is aligned with those of MNK1 and PEA-15. The key basic residues are shown in bold. Numbers indicate the position of the amino acid residue in the respective proteins. (d) Predicted binding mode of PEA-16 with ERK2 through a molecular dynamics simulation. Basic residues (yellow) show the interaction with the CD domain. The leucine residue is shown in magenta as in (a). (e) A close view of the binding interface between PEA-16 and ERK2. Acidic residues of ERK2 participate in Coulombic interactions with PEA-16. Residues of PEA-16 are underlined.
StxB-TDP-PEA-16 for 3 days, and the cell viability was measured (Figure 5c). The cytotoxic effect differed depending on each cell line, which seems to be due to the fact that the expression level of Gb3 and the dependency of the cell proliferation on the ERK2-signaling vary with the cell lines. Gb3 is a cell surface target for the receptor-binding domain of the delivery system. Interestingly, two pancreatic cancer cell lines were shown to respond differently according to the dependency of the cell proliferation on the ERK signaling. The proliferation of Panc-1 cells is known to be independent of the ERK-signaling (Buck et al., 2006), and accordingly, the viability was maintained at over 90%. On the other hand, Capan-1 cells are dependent on the ERK signaling (Buck et al., 2006), and only 10% of the cells survived. These results indicate that PEA-16 effectively blocks the ERK2-mediated signaling pathway in various cell lines, implying a therapeutic potential in cancers showing the dependence of proliferation on the ERK-mediated signaling pathway.

We further investigated the general utility of our delivery system by changing a receptor binding domain into a repebody (rEgH9), specifically binding to EGFR ectodomain III with sub-nanomolar affinity, because the epidermal growth factor receptor (EGFR) has been known to be the cell surface target of many tumors (Lee et al., 2015, 2017). The repebody was genetically fused to the N-terminus of TDP-PEA-16 or TDP-PEA-15AA as a tumor-targeting moiety instead of StxB (Supplementary Figure S8a). The resulting two constructs were added to the EGFR-overexpressing cancer cells (H358), and Rsk phosphorylation and cell viability were investigated (Supplementary Figure S8b). As a negative control, low EGFR-expressing HepG2 cells were used. As a result, the delivery of PEA-16 was shown to more
effectively inhibit the Rsk phosphorylation than PEA-15AA in the high EGFR-expressing cells, whereas no significant change in the Rsk phosphorylation was observed for the low-EGFR expressing cells. These results support that the present delivery system can be broadly used for cytosolic delivery of a protein cargo by changing the targeting moiety.

4 | DISCUSSION

We have demonstrated the modulation of the ERK2-mediated signaling process in cells through the cytosolic delivery of the PEA-15 variants using a bacterial toxin-based system. The modulation of the cellular signaling process using a target-specific molecular binder, followed by an analysis of the cellular response, is a key to understanding its role in the cellular function and drug development. PEA-15 is a naturally occurring regulatory protein for ERK2, and its function is known to suppress the cell proliferation through a specific interaction with ERK2. Non-phosphorylated PEA-15 is known to act as a tumor suppressor, whereas phosphorylated PEA-15 is considered a tumor promoter (Sulzmaier et al., 2012). Based on this, we constructed the PEA-15 variants with or without phosphorylation sites and a high affinity for ERK2. The intracellular delivery of the PEK-15 variants led to a modulation of the ERK-mediated signaling process in a different manner, resulting in distinct cellular responses. Phosphorylated PEA-15 is unlikely to act as a tumor promoter in the case of hepatocellular carcinoma, and in terms of cell proliferation. On the other hand, a PEA-15 variant with no phosphorylation site was shown to effectively block the endogenous ERK2-mediated signaling pathway, suppressing the cell proliferation. This result also provided a direct clue regarding the effect of phosphorylation and the role of PEA-15 on the cell proliferation. Moreover, a high-affinity variant, PEA-16, showed a higher cytotoxic effect on the cell proliferation, suggesting the potential of a native regulator as a therapeutic agent. The use of the EGFR-specific repebody as a targeting moiety instead of original receptor binding domain (StxB) demonstrated general applicability of our approach to effective cytosolic delivery of a protein cargo and consequent modulation of cellular processes in a target-specific manner. A method to effectively modulate the cellular signaling processes using a protein binder has great utility in understanding the function and network of cellular signaling processes as well as the development of pharmaceuticals. Therefore, the present approach can find wide applications in modulating the cell-signaling process, leading to a low cell-to-cell variation and a more druggable format compared to other methods.

The design of PEA-16 with a higher affinity for ERK2 enabled a much stronger modulation of the ERK-mediated signaling process, demonstrating the utility of a naturally occurring regulatory protein in the development of potential therapeutics. ERK2 is known to interact with many intracellular proteins, and has two major binding sites controlling and regulating many cellular functions (Lee et al., 2004; Tanoue, Adachi, Moriguchi, & Nishida, 2000). One is the DEF-docking site (Docking site for ERK, FxF), and the other is the DRS (Futran, Kyin, Shvartsman, & Link, 2015). The interface between ERK2 and PEA-15 occupies both sites. The N-terminal domain of PEA-15 (residues 1–86) binds to the DEF-docking site, and the C-terminal segment of PEA-15 (residues 122–127) occupies the DRS. However, the C-terminal segment of PEA-15 does not completely occupy the DRS, which is composed of a hydrophobic pocket and the CD domain. The crystal structure (PDB ID: 4IZ5) revealed no interaction between the CD domain of ERK2 and PEA-15, mainly owing to a short C-terminal segment of PEA-15. Based on this analysis, we successfully designed PEA-16 showing an 830-fold higher affinity toward ERK2 than PEA-15AA through a rational structure-based design and a phage display of the additional segment at the C-terminus of PEA-15. A molecular dynamics simulation confirmed that the elongated segment of PEA-16 makes contact with the CD domain of ERK2, suggesting that Trp131 may play an important role in the interaction with ERK2 by filling in the pocket formed by the aromatic residues of ERK2. A high-affinity variant, PEA-16, showed a more potent inhibitory effect on the ERK2-mediated signaling process, suppressing more effectively the cell proliferation than PEA-15AA. This approach would be useful for developing potential therapeutics based on native regulatory proteins.

In conclusion, we have shown that the ERK2-mediated signaling process can be effectively modulated by the cytosolic delivery of PEA-15 and its variants using a toxin-based platform. The use of an EGFR-specific repebody as a targeting moiety demonstrated the general applicability of the present delivery system. Our approach has enabled an understanding of the cellular function of the ERK2-mediated signaling process and the effect of EPA-15 phosphorylation on its action as an ERK2 blocker. Furthermore, the design of a high-affinity variant showing a much stronger inhibitory effect on the cell proliferation demonstrated the utility of a native regulatory protein as a potential therapeutic. Based on the results, the present approach can be effectively used for modulating the cellular signaling process, and consequently understanding its role in cellular function, assisting in the development of various therapeutics.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

AUTHORS’ CONTRIBUTIONS

JHR, YKS, and HSK designed the research; JHR, YKS, and DGK conducted the research; HHK applied the computational methods;
JHR, YKS, and HSK analyzed the data; and JHR, YKS, and HSK wrote the paper.

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REFERENCES


Callaway, K., Abramczyk, O., Martin, L., & Dalby, K. N. (2007). The anti-apoptotic protein PEA-15 is a tight binding inhibitor of ERK1 and ERK2, which blocks docking interactions at the D-recruitment site. Biochemistry, 46(32), 9187–9198.


SUPPORTING INFORMATION

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