Biotechnology and Bioprocess Engineering 28: 483-490 (2023)
DOI 10.1007/s12257-022-0139-0

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Tumor Homing Peptides as Fusion Partners of Therapeutic Proteins for Efficient Delivery to Cancer Cells

You Eun Lee and Byeong Hee Hwang

Received: 10 May 2022 / Revised: 30 June 2022 / Accepted: 1 July 2022

Abstract For practical protein therapeutics and diagnostics, it is necessary to develop a method that can effectively and specifically deliver proteins into cells. Therefore, the peptide fusion strategy with an enhanced green fluorescent protein (eGFP) was introduced to compare protein delivery efficiency in breast cancer cells among six tumor-homing peptides (THPs). Six THP-fused eGFPs were successfully cloned and expressed recombinantly in *Escherichia coli*. First, CGKRK-fused eGFP showed concentration-dependent and cell-type-specific protein delivery to MDA-MB-231 due to p32 protein. Second, PL3R-fused eGFP of 50 μg/mL showed more efficient protein delivery than eGFP of 50 μg/mL into MDA-MB-231. Finally, under extended comparison among fusion partners, CTTHWGFTLC peptide showed 581% cellular delivery to MDA-MB-231 cells than eGFP. This finding could help increase protein delivery to specific cancer cells for cancer research and diagnostic and therapeutic applications.

Keywords: targeted delivery, tumor homing peptide, recombinant fusion protein, cancer cells, diagnosis

You Eun Lee, Byeong Hee Hwang
Department of Bioengineering and Nano-bioengineering, Incheon National University, Incheon 22012, Korea

Byeong Hee Hwang*
Division of Bioengineering, Incheon National University, Incheon 22012, Korea
Research Center for Bio Material & Process Development, Incheon National University, Incheon 22012, Korea
Institute for New Drug Development, Incheon National University, Incheon 22012, Korea
Tel: +82-32-835-8834; Fax: +82-32-835-0804
E-mail: bhwang@inu.ac.kr

1. Introduction

Therapeutic antibodies are the most important therapeutics for treating various diseases and have a rapidly developing global market size of USD 150 billion in 2019 [1]. Until 2019, US Food and Drug Administration has approved seventy-nine therapeutic monoclonal antibodies [1]. Most antibody pharmaceuticals are most effective in targeting proteins on the surface of cell membranes [2]. However, the therapeutic efficacy of antibodies is limited due to their large size and low availability [2]. To overcome these limitations, it is necessary to develop a method that effectively delivers proteins into cancer cells.

Many methods have been developed to deliver proteins into cells effectively. Typically, there is a method for delivering a protein by wrapping it with a nanocarrier such as a liposome [3] or fusing a cell-permeating peptide to a protein and delivering it [4]. Among them, liposomes are limited in use due to low loading efficiency [3]. Instead, several peptides were reported to deliver biomolecules efficiently due to no need for loading [5-8]. The peptide fusion method has been mainly used for therapeutic protein delivery to cells [4]. Recently, tumor-homing peptide (THP) has been getting attention because of its advantage in specifically delivering biomolecules targeting cancer cells [9-14]. However, the practical application needs to compare the THP sequences and check whether the targeted cell delivery efficiency is excellent when fused with proteins.

Therefore, in this study, six known THPs were fused with enhanced green fluorescent protein (eGFP) to compare which THP had specificity and efficient delivery to cancer cells (Fig. 1). For the first trial, two THP-fused eGFPs (CGKRK and PL3R) would be cloned and tested using a fluorescence assay in MCF-7 and MDA-MB-231 breast cancer cells. For the second extended trial, six THP

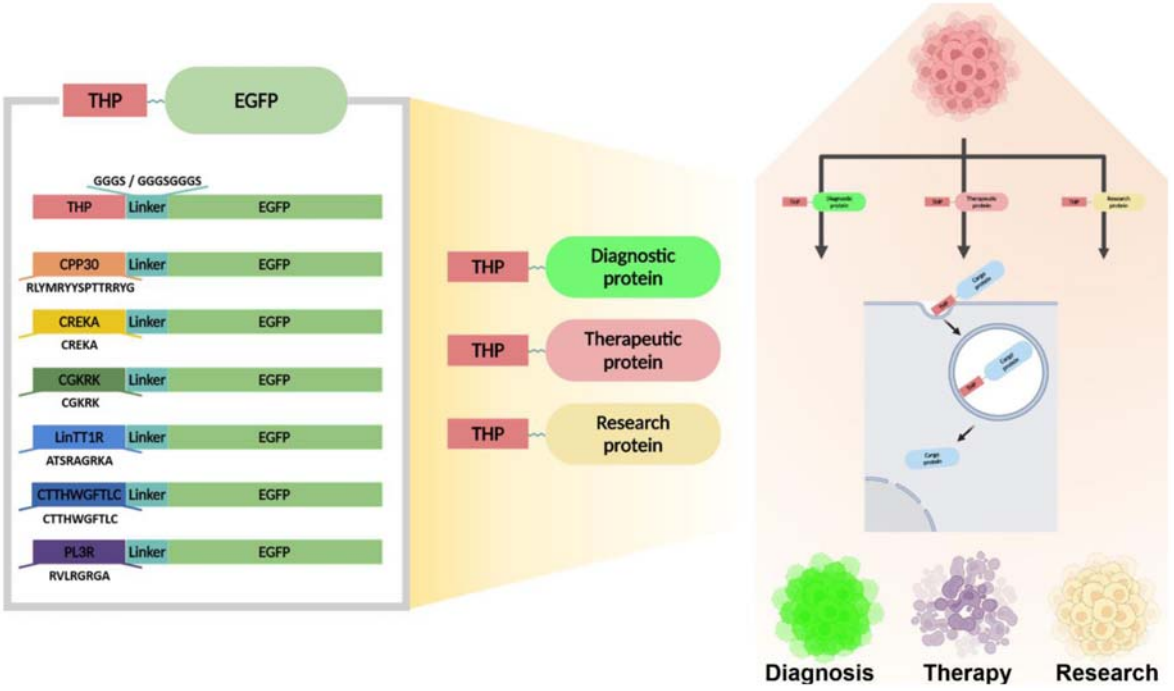

Fig. 1. The tumor homing peptide (THP)-fused enhanced green fluorescent protein (eGFP) and targeted delivery to cancer cells. Six THPs were fused with eGFP: CPP30, CREKA, CGKRK, LinTT1R, CTTHWGFTLC, and PL3R. Furthermore, protein delivery efficiency was analyzed in MDA-MB-231 cells. Screened THP fusion strategy could be applied to diagnostics, therapeutics, and basic research.

Table 1. List of tumor homing peptides

Tumor homing peptide	Sequence	Target	Length (aa)	Final protein product (kDa)	Reference
CGKRK	CGKRK	Heparan sulfate/P32 protein	5	28.305	[9]
CPP30	RLYMRYYSPTTRRYG	Breast cancer	15	28.971	[10]
CREKA	CREKA	Breast cancer	5	28.305	[11]
CTTHWGFTLC	CTTHWGFTLC	MMP2 & MMP9	10	28.86	[12]
LinTT1R	ATSRAGRKA	P32 protein	9	28.749	[13]
PL3R	RVLRGRGA	TNC-C	8	28.638	[14]

aa: amino acid, MMP: matrix metalloproteinase, TNC-C: Tenascin-C.

sequences were collected in Table 1; CGKRK (targeting neovasculature in tumors and premalignant dysplasias) [9], CPP30 (penetrating MCF-7 lineage selectively) [10], CREKA (targeting tumor blood vessels and stroma selectively) [11], CTTHWGFTLC (expressing phage binding to endothelial receptors in angiogenic vasculature to tumors and 10-fold accumulation compared with controls) [12], and LinTT1R (binding to p32 protein and a tumor-penetrating peptide) [13] and PL3R (interacting with the C-isoform of Tenascin-C [TNC-C] upregulated in malignant tissues and neuropilin-1 [NRP-1] as a cell penetration receptor) [14]. Then, six THP-fused eGFPs would be cloned, and the recombinant proteins would be expressed, purified, and evaluated using flow cytometry to MDA-MB-231 cells, a human triple-negative breast cancer cell line. Finally, the optimal fusion THP partner was chosen.

2. Materials and Methods

2.1. Cloning of fusion proteins

THP-fused primers (CPP30, CREKA, CGKRK, Lintt1R, CTTHWGFTLC, and PL3R) were synthesized in Table S1 from Cosmogenetech, Inc. (Seoul, Korea). Each THP-fused eGFP sequence was amplified by an S-Taq PCR Master Mix 2 (Biofact Co., Ltd., Daejeon, Korea) and Thermal Cycler (T100™; Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the pEGFP-N1 template. According to the manufacturer's instructions, 25 μL of 2X S-Taq PCR Master Mix 2, 2 μL of 10 μM primer (forward, reverse, respectively), 10 ng of template DNA (pEGFP-N1), and autoclaved deionized water (DIW) to fill 50 μL were mixed. A thermal cycler was used for a polymerase chain reaction, and the temperature and time settings were performed as follows.

An initial denaturation of 2 min at 95°C was performed, denaturation of 20 sec at 95°C, annealing of 40 sec at Tm-2°C, and extension of 1 min/kb at 72°C were carried out in 40 cycles, and lastly, final elongation of 5 min at 72°C and cooling at 4°C were performed. After TA cloning using the pGEM®-T Easy kit (Promega Co., Madison, WI, USA), each ligated plasmid was transformed into *Escherichia coli* Top10 competent cells cultured in Luria-Bertani (LB) broth. The plasmid was purified using a FavorPrep™ Plasmid Extraction Mini Kit (Favorgen Biotech Co., Ping-Tung, Taiwan). Each purified plasmid was analyzed using a restriction fragment length polymorphism (RFLP) of double digestion of NdeI (Enzynomics, Inc., Daejeon, Korea), XhoI (Promega Co.) with buffer D, bovine serum albumin, autoclaved DIW for 2 h at 37°C. Then, sequences of the chosen plasmids were confirmed using a DNA sequencing service (Cosmogenetech, Inc.) and sequence alignment of BioEdit software. Each confirmed sequence was finally inserted into the pET-23a plasmid using overnight double digestion and a LaboPass™ T4 DNA Ligase kit (Cosmogenetech, Inc.). After transformation and mini-prep of chosen colonies, the purified plasmid was confirmed using RFLP using double digestion. DNA bands were observed using 1% agarose gel electrophoresis at 100 V for 35 min in the Mupid-2plus electrophoresis system (Optima Inc., Tokyo, Japan) with a 1 kbp DNA ladder (Biofact Co., Ltd.). The final cloned plasmid was stored in a deep freezer (Nihon Freezer Co., Ltd., Tokyo, Japan).

2.2. Purification and identification of fusion proteins

After the transformation and colony pickup of each cloned plasmid, *E. coli* BL21 (DE3) cells produced the fusion protein through the Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction (1 M solution, Generay Biotechnology, Shanghai, China) of LB media 20 mL at 37°C and 250 rpm for overnight in a shaking incubator (JS Research Inc., Gongju, Korea). After IPTG induction, cell growth curves were drawn by measuring OD600 at intervals of 1 h from 0 h using GENESYS™ 30 Visible Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After that, the cell pellet was harvested using centrifugation at 13,652 × *g* and 4°C for 10 min and resuspended with ice-cooled lysis buffer. Moreover, 100 × 0.2 mg/mL lysozyme was added to the cell lysate. Next, cell lysate was incubated in ice for 20 min and was sonicated using a sonicator (Sonics & Materials, Inc., Newtown, CT, USA) with an amplitude power of 28% for 5 min, turning on and off every 5 sec to lyse cell. When the cell lysate becomes transparent, centrifugation at 13,652 × *g* for 10 min separates soluble and insoluble fractions. Before protein purification, the fluorescence of product protein was measured at a fluorescence excitation/emission of 488-509 nm in 96 well black plates (SPL Life Sciences Co., Ltd., Pocheon, Korea) to select an optimal strain using a microplate reader (SpectraMax M2; Molecular Devices LLC., San Jose, CA, USA). Then, using the Ni-NTA column, each protein was purified through a His-tag purification process (Qiagen, Inc., Hilden, Germany). To purify protein, the lysis buffer, wash buffer, and elution buffer were prepared with 10, 20, and 250 mM imidazole (Sigma-Aldrich, Saint Louis, MO, USA) at pH 8.0 respectively. Eluted proteins were then dialyzed in distilled water. After that, it was stored in 1x phosphate-buffered saline (PBS, Thermo Fisher Scientific) at a 4°C refrigerator in the dark until use. Purified proteins were identified using SDS-PAGE and western blotting.

SDS-PAGE was run at 60 V for 30 min for stacking and 120 V for 90 min for separating in 12% polyacrylamide gel of Mini-PROTEAN system (Bio-Rad Laboratories, Inc.) with prestained protein ladder (Thermo Fisher Scientific). Western blotting was run at 150 mA for 1 h using the same system with Anti-his tag mouse antibody (Sino Biological, Inc., Wayne, PA, USA) and Goat Anti-Mouse IgG Antibody (Merck Inc., Hesse, Germany).

2.3. Fluorescence intensity analysis

The delivery efficiency of THP-fused eGFPs to cancer cells was evaluated using a fluorescence assay according to protein concentration and incubation time. First, 1 mL of 1.0×10^5 cells was dispensed to each well in a 24-well plate and then incubated at 37°C in a 5% CO_2 incubator (Esco Micro Pte. Ltd., Changi, Singapore) for 24 h. For MDA-MB-231 cells, eGFP, CGKRK-fused eGFP, and PL3R-fused eGFP were prepared at 10, 50, 100, 150, and 200 μg/mL in Opti-MEM. For MCF-7 cells, eGFP, CGKRK-fused eGFP, and PL3R-fused eGFP were prepared at 50 and 100 μg/mL in Opti-MEM. After 1 mL sample addition, cells were incubated for 24 h at 37°C in a 5% CO_2 incubator. After three-time washing with Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich) or Hanks' Balanced Salt solution (HBSS), the cells are lysed with 0.1 mL of cell lysis reagent (Promega Co.) for 30 min. After that, 25 μL of lysate was dispensed to wells of a 384-well plate with a transparent bottom (SPL Life Sciences Co., Ltd.), and fluorescence intensity was measured in a SpectraMax M2 microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 509 nm.

For incubation time, MDA-MB-231 cells were prepared following the above procedure. eGFP, CGKRK-fused eGFP, and PL3R-fused eGFP were prepared at 50 and 100 μg/mL in Opti-MEM. After 1 mL sample addition, cells were incubated for 24 and 48 h at 37°C in a 5% CO_2 incubator. The rest procedure is the same as the above.

2.4. Flow cytometry analysis

The fluorescence-positive cells were counted using flow cytometry. First, 2 mL of 5.0×10^5 MDA-MB-231 cells were dispensed to each well in a 6-well plate and incubated at 37°C in a 5% CO_2 incubator for 24 h. Then, after replacing the medium with Opti-MEM, 100 μg/mL of eGFP, CGKRK-fused eGFP, and PL3R-fused eGFP were incubated at 37°C in a 5% CO_2 incubator for 24 h. After washing with DPBS, cells were detached using trypsin-EDTA (Thermo Fisher Scientific), added by 2 mL of medium, then centrifuged at 300 × *g* for 5 min. After supernatant removal and resuspension in DPBS, centrifugation was repeated twice under the same conditions. Finally, the pellet was resuspended in cold PBS, transferred to a flow cytometer tube, and measured using a flow cytometer (CytoFLEX; Beckman, Brea, CA, USA).

2.5. Statistical analysis

The quantitative data were presented as mean ± standard deviation. The statistical significance of the differences was evaluated using a p-value less than 0.05, 0.01, and 0.001 calculated by *t*-test. Each spot represented an independent result.

3. Results and Discussion

The delivery efficiency of THP-fused eGFPs was evaluated by fluorescence assay and flow cytometry for tumor-specific and efficient delivery of proteins. For the first trial, two THP-fused eGFPs were cloned and expressed in *E. coli* recombinantly, and purified THP-fused eGFPs showed different protein delivery efficiency to breast cancer cell types. For the extended trial, six THP-fused eGFPs were successfully cloned, purified, and characterized. Cellular protein delivery efficiency was evaluated using flow cytometry. As a result, CTTHWGFTLC showed the best protein delivery to MDA-MB-231 cells.

3.1. THP-fused eGFPs

Six THP-fused eGFPs were successfully cloned, recombinantly expressed in *E. coli*, and purified using His-tag purification. First, each THP sequence (Table 1) was fused with eGFP through PCR fusion strategy with primer design (Table S1). Next, THP-fused DNA bands were identified with 765-783 bp size using DNA agarose gel electrophoresis (Fig. S1A). After TA cloning, the inserted DNA band were confirmed based on DNA size (Fig. S2). The final plasmids were checked using the size of around 765-783 bp (Fig. S1B). Cloned DNA sequences were confirmed in Fig. S3. Best protein-expressing colonies were picked up from several candidates (Fig. S4). Cell growth curves of chosen colonies were plotted, and optimal harvest time (mostly 4 h) was chosen from the growth curve (Fig. S5). Then, the size and purity of recombinantly expressed THP-fused eGFPs (28.3-29.0 kDa) were checked using SDS-PAGE (Fig. 2A and S6A) and western blotting (Fig. 2B and S6B) after His-tag purification. The concentration was checked as 600 to 900 μg/mL.

3.2. Fluorescence assay of two THP-fused eGFPs

The delivery efficiency of THP-fused eGFPs was evaluated using a fluorescence assay depending on protein concentration. Relative fluorescence intensity normalized by the cell only was compared to unmodified eGFP in MDA-MB-231 triple-negative breast cancer cells (Fig. 3) and MCF-7 luminal breast cancer cells (Fig. 4). For MDA-MB-231 cells in Fig. 3A, cellular delivery represented 105% for CGKRK-fused eGFP and 113% for PL3R-fused eGFP compared to 100% for eGFP. PL3R-fused eGFP showed statistically significant difference (p-value based on cell only: CGKRK; 0.014 / PL3R; 0.013 | p-value based on

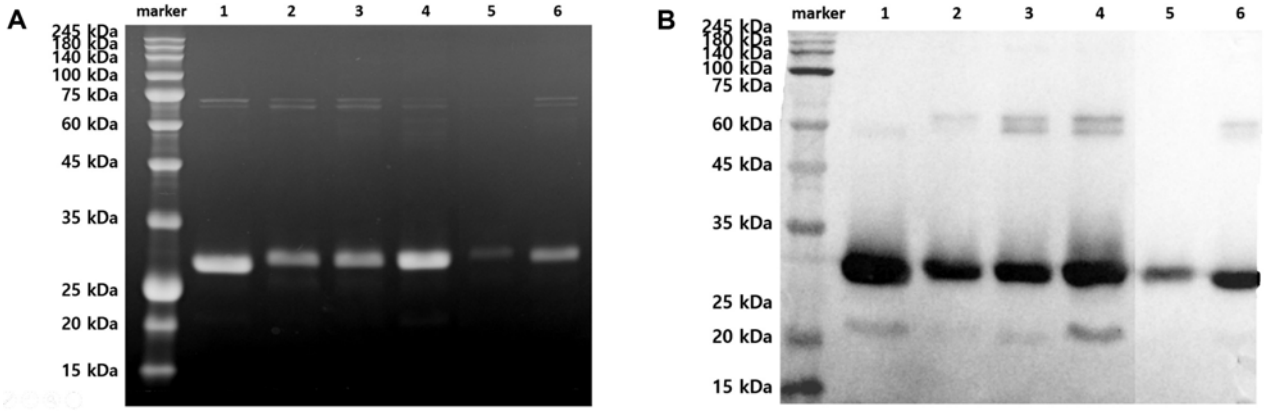

Fig. 2. The purified proteins were checked using size analysis of (A) SDS-PAGE and (B) western blotting. Marker: protein marker, Lane 1-6: eGFP, CPP30, CREKA, CGKRK, LinTT1R, and CTTHWGFTLC. eGFP: enhanced green fluorescent protein.

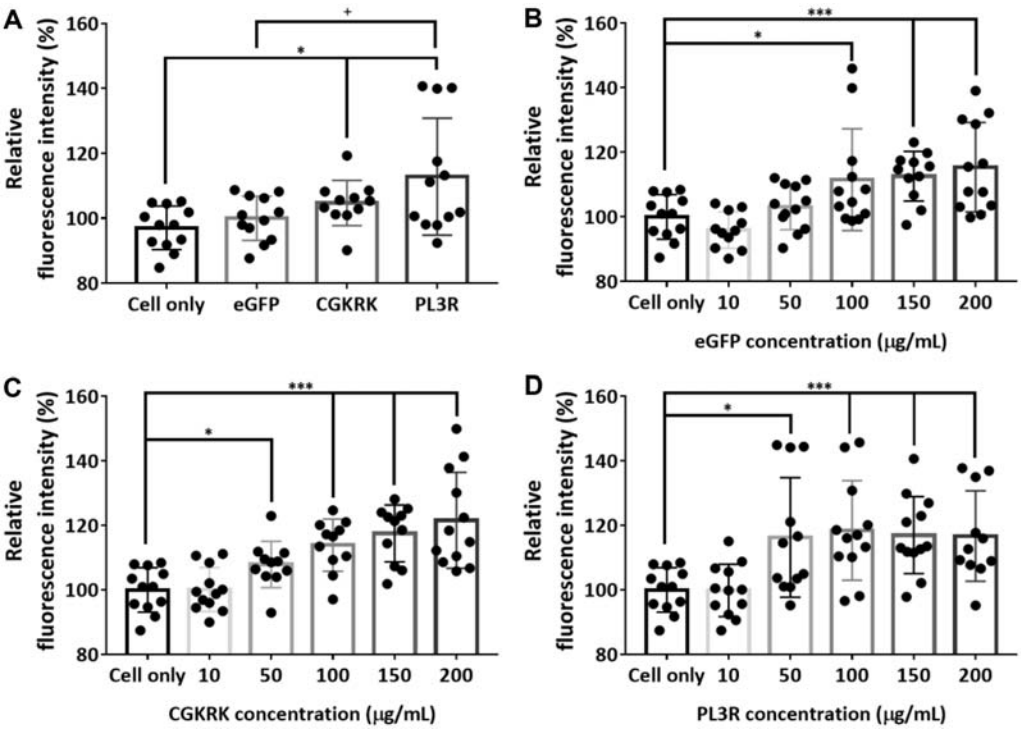

Fig. 3. The delivery efficiency of THP-fused eGFPs to MDA-MB-231 cells was evaluated using fluorescence assay according to protein concentration. (A) Relative fluorescence intensity was plotted for eGFP, CGKRK-fused eGFP, and PL3R-fused eGFP of 50 μg/mL normalized by the cell only. Relative fluorescence intensity was plotted for (B) eGFP, (C) CGKRK-fused eGFP, and (D) PL3R-fused eGFP of 10, 50, 100, 150, and 200 μg/mL normalized by the cell only. *, +, and *** mean $P < 0.05$, $P < 0.05$, and $P < 0.001$ by T-test (n = spot number). THP: tumor homing peptide, eGFP: enhanced green fluorescent protein.

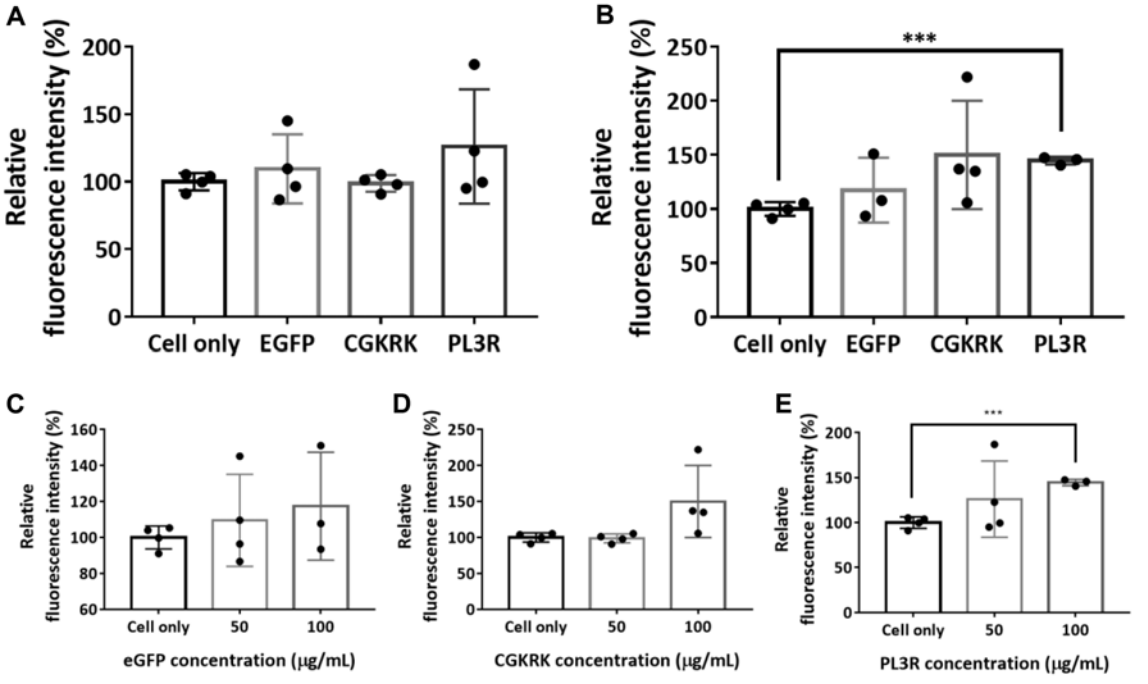

Fig. 4. The delivery efficiency of THP-fused eGFPs to MCF-7 cells was evaluated using fluorescence assay according to protein concentration. Relative fluorescence intensity was plotted for eGFP, CGKRK-fused eGFP, and PL3R-fused eGFP of (A) 50 μg/mL and (B) 100 μg/mL normalized by that of the cell only. Relative fluorescence intensity was plotted for (C) eGFP, (D) CGKRK-fused eGFP, and (E) PL3R-fused eGFP of 50 and 100 μg/mL normalized by that of the cell only. *** means $P < 0.001$ by T-test (n = spot number). THP: tumor homing peptide, eGFP: enhanced green fluorescent protein.

eGFP: PL3R; 0.037). eGFP fluorescence intensity rapidly increased to 100 μg/mL and gradually increased to 200 μg/mL (Fig. 3B). CGKRK-fused eGFP showed a gradual fluorescence increase to 200 μg/mL (Fig. 3C). PL3R-fused eGFP presented a dramatic fluorescence increase to 50 μg/mL and maintained fluorescence to 200 μg/mL (Fig. 3D). Interestingly, all fluorescence values do not exceed 120%, probably a limitation of protein delivery to MDA-MB-231 cells. Efficient delivery of PL3R-fused eGFP in low concentration might be explained by the PL3 peptide having a high affinity on the surface proteins of MDA-MB-231 cells.

For MCF-7 cells, cellular delivery of 50 μg/mL was 90% for CGKRK-fused eGFP and 115% for PL3R-fused eGFP without a statistically significant difference (Fig. 4A). Moreover, cellular delivery of 100 μg/mL represented 128% for CGKRK-fused eGFP and 123% for PL3R-fused eGFP compared to 100% for eGFP without a statistically significant difference (Fig. 4B). eGFP fluorescence intensity gradually increased to 100 μg/mL (Fig. 4C). CGKRK-fused eGFP showed a dramatic fluorescence increase from 50 to 100 μg/mL (Fig. 4D). PL3R-fused eGFP presented a rapid fluorescence increase to 100 μg/mL (Fig. 4E). Similar to MDA-MB-231 cells, PL3R-fused eGFP showed the most efficient delivery to MCF-7 cells. This result might be the same reason above.

The delivery efficiency of THP-fused eGFPs to MDA-MB-231 cells was evaluated using a fluorescence assay depending on concentration and incubation time (Fig. 5). For 50 μg/mL and 48-h incubation, PL3R-fused eGFP showed the highest fluorescence intensity (Fig. 5A). CGKRK and PL3R-fused eGFPs represented the highest fluorescence intensity for 100 μg/mL and 48-h incubation (Fig. 5B). As time increased, the fluorescence intensity of 50 μg/mL proteins decreased in all cases (Fig. 5C). For 100 μg/mL, the fluorescence intensity of eGFP decreased, and CGKRK and PL3R-fused eGFPs were maintained as time increased (Fig. 5D). Thermal denaturation or protease degradation might affect reduced fluorescence intensity in low protein concentration over time. On the other hand, a sufficient concentration of proteins could maintain fluorescence intensity over time.

CGKRK peptide is homing to neovasculature in tumors and transplanted tumors from PDSC5, MDA-MB-435, and C8161 cell lines using phage display. Moreover, it was suggested that CGKRK peptide could be internalized by heparan sulfate binding and KRK acts as nuclear localization sequence [15]. Furthermore, PL3 peptide was identified as TNC-C binding by *in vitro* biopanning on recombinant TNC-C. PL3 peptide could be internalized by NRP-1 overexpressing tumor cell lines, including U87-MG glioma cells and PPC1 prostate carcinoma cells [14]. Therefore,

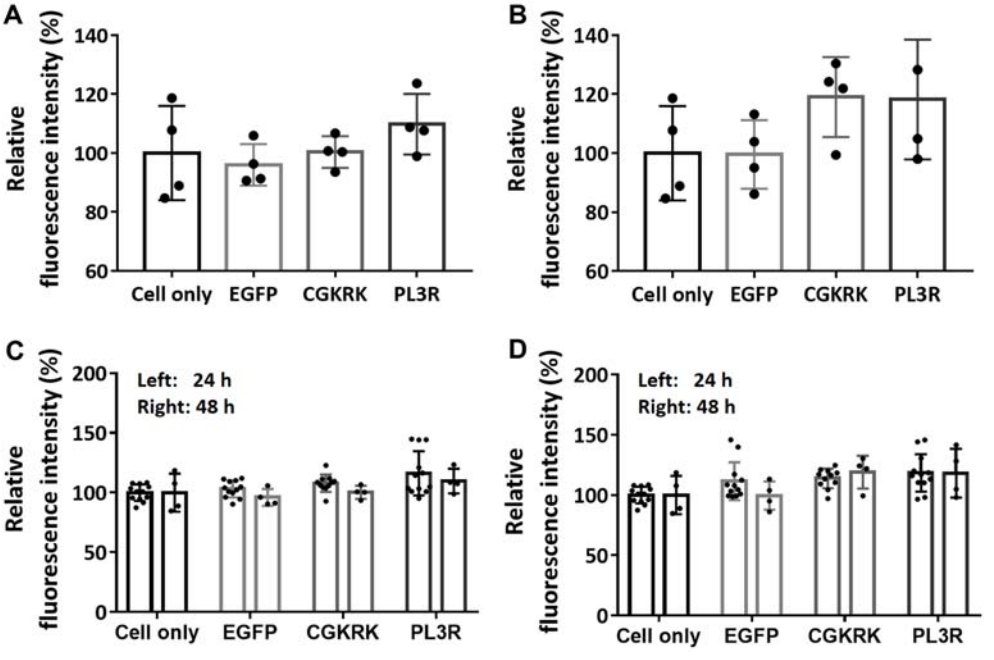

Fig. 5. The delivery efficiency of THP-fused eGFPs to MDA-MB-231 cells was evaluated using fluorescence assay according to incubation time. Relative fluorescence intensity was plotted for eGFP, CGKRK-fused eGFP, and PL3R-fused eGFP of (A) 50 μg/mL and (B) 100 μg/mL for 48 h normalized by that of the cell only. Relative fluorescence intensity at 24 and 48 h were plotted for (C) 50 and (D) 100 μg/mL of eGFP, CGKRK-fused eGFP, and PL3R-fused eGFP normalized by that of the cell only. THP: tumor homing peptide, eGFP: enhanced green fluorescent protein.

the higher delivery efficiency of PL3R-fused eGFP might be explained by TNC-C or NRP-1-mediated delivery to MDA-MB-231 and MCF-7 cells.

3.3. Flow cytometry of THP-fused eGFPs

The fluorescence-positive MDA-MB-231 cells by THP-fused eGFPs were counted using flow cytometry (Fig. 6A). The green count was plotted for each THP-fused eGFPs using the FITC-A filter compared to the red count of the cell only. The percentage represented the fluorescence-positive cell population; 0.47% (the cell only), 10.1 (eGFP), 19.7% (LinTT1R), 25.0% (CREKA), 35.5% (CGKRK), 38.3% (CPP30), 39.0% (PL3R), and 41.3% (CTTHWGFTLC). Mean fluorescence intensity (MFI) was plotted for each THP-fused eGFP of 100 μg/mL for 24 h normalized by the cell only (Fig. 6B). MFI values represented 135% (CREKA), 167% (CPP30), 205% (LinTT1R), 216% (PL3R), 217% (CGKRK), and 581% (CTTHWGFTLC) than eGFP. Especially, LinTT1R, PL3R, CGKRK, and CTTHWGFTLC showed statistically significant differences compared to eGFP. Therefore, depending on the THP, the THP fusion strategy increased the fluorescence-positive cell population and MFI in human triple-negative breast cancer cells.

LinTT1 is a linear THP that binds to p32 protein. p32 is a mitochondrial chaperon expressed at the cell surface of malignant cells and activated tumor macrophages. After cleavage by uPA, LinTT1 contains the CendR motif, which binds to the NRP-1 protein. NRP-1 binding triggers an increase in the tumor tissue permeability, and the cargo is internalized into the tumor [16]. CREKA peptide was identified in breast tumors of MMTV-PyMT transgenic mice by phage display. CREKA peptide recognizes clotted plasma proteins and selectively homes to tumor blood vessels and stroma by binding to fibrin-like structures and does not penetrate the cellular membrane [11]. CPP30 peptide represents a cell-penetrating peptide designed by amino-acid substitution of CPP7 (KLWMRWWSPTTRRYG) and selectively penetrated MCF-7 breast cancer cells. Moreover, a dynamin inhibitor blocked the internalization

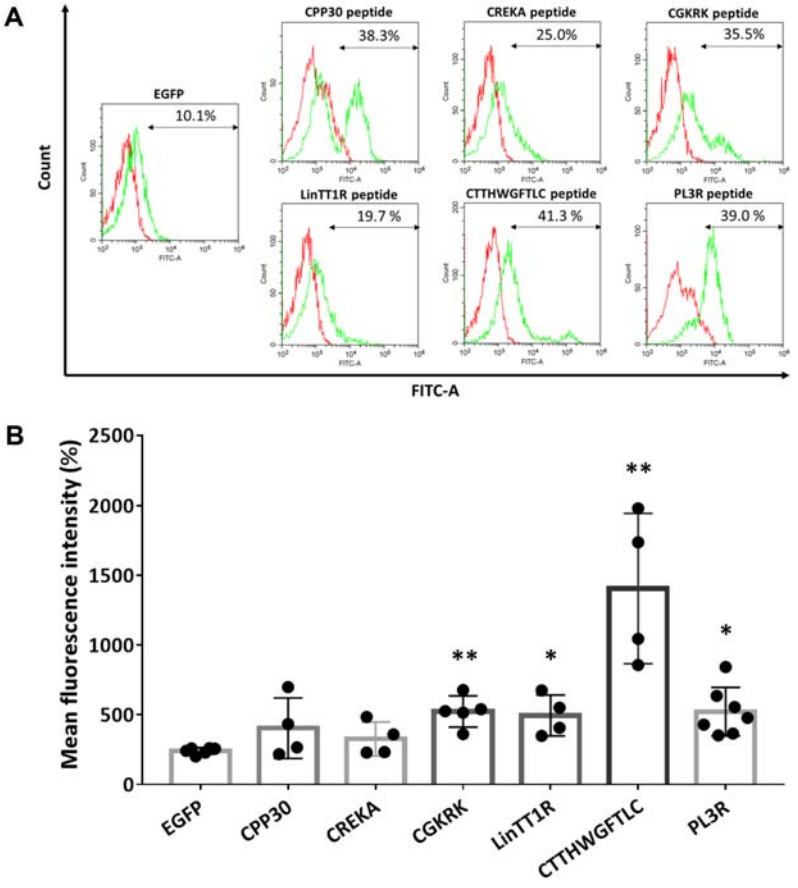

Fig. 6. The fluorescence-positive cells of THP-fused eGFPs were counted using flow cytometry. (A) The green count was plotted for each THP-fused eGFPs using the FITC-A filter compared to the red count of the cell only. The percentage represented the fluorescence-positive cell population. (B) Mean fluorescence intensity was plotted for each THP-fused eGFP of 100 μg/mL for 24 h normalized by the cell only. * and ** mean $P < 0.05$ and $P < 0.01$ by T-test (n = spot number). THP: tumor homing peptide, eGFP: enhanced green fluorescent protein.

of CPP2 and CPP44, similar to CPP30 [10]. Cyclic CTTHWGFTLC peptide was identified as a selective gelatinase inhibitor and suppressed migration of tumor cells and endothelial cells *in vitro* and was specifically homed to tumor vasculature *in vivo* by phage display [12]. However, the cellular internalization mechanism of the CTTHWGFTLC peptide was not revealed.

4. Conclusion

Six THP-fused eGFPs were successfully cloned and recombinantly expressed in *E. coli*. First, CGKRK-fused eGFP showed concentration-dependent and cell-type-specific protein delivery to MDA-MB-231 due to p32 protein. Second, PL3R-fused eGFP of 50 μg/mL showed more efficient protein delivery than eGFP of 50 μg/mL into MDA-MB-231 due to TNC-C and NRP-1 proteins. Finally, under extended comparison among six fusion partners, CTTHWGFTLC peptide showed the most efficient delivery of 581% MFI to MDA-MB-231 cells than eGFP. This finding could help increase the delivery efficiency of proteins to specific cancer cells for cancer research, diagnostic and therapeutic applications.

Acknowledgements

This research was supported by the Incheon National University Research Grant in 2019.

Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-022-0139-0) contains supplementary material, which is available to authorized users.

References

1. Lu, R.-M., Y.-C. Hwang, I.-J. Liu, C.-C. Lee, H.-Z. Tsai, H.-J. Li, and H.-C. Wu (2020) Development of therapeutic antibodies for the treatment of diseases. *J. Biomed. Sci*. 27: 1.
2. Slastnikova, T. A., A. V. Ulasov, A. A. Rosenkranz, and A. S. Sobolev (2018) Targeted intracellular delivery of antibodies: the state of the art. *Front. Pharmacol*. 9: 1208.
3. Ray, M., Y.-W. Lee, F. Scaletti, R. Yu, and V. M. Rotello (2017) Intracellular delivery of proteins by nanocarriers. *Nanomedicine (Lond.)* 12: 941-952.
4. Yu, S., H. Yang, T. Li, H. Pan, S. Ren, G. Luo, J. Jiang, L. Yu, B. Chen, Y. Zhang, S. Wang, R. Tian, T. Zhang, S. Zhang, Y. Chen, Q. Yuan, S. Ge, J. Zhang, and N. Xia (2021) Efficient intracellular delivery of proteins by a multifunctional chimaeric peptide *in vitro* and *in vivo*. *Nat. Commun*. 12: 5131.
5. Ryu, Y. C., K. A. Kim, B. C. Kim, H. D. Wang, and B. H. Hwang (2021) Novel fusion peptide-mediated siRNA delivery using self-assembled nanocomplex. *J. Nanobiotechnology* 19: 44.
6. Jeong, S. G., Y. C. Ryu, and B. H. Hwang (2021) Synergistic gene delivery by self-assembled nanocomplexes using fusion peptide and calcium phosphate. *J. Control. Release* 338: 284-294.
7. Ryu, Y. C., Y. E. Lee, and B. H. Hwang (2022) Efficient and safe small RNA delivery to macrophage using peptide-based nanocomplex. *Biotechnol. Bioeng*. 119: 482-492.
8. Wu, Y., J. Li, and H. J. Shin (2021) Self-assembled viral nanoparticles as targeted anticancer vehicles. *Biotechnol. Bioprocess Eng*. 26: 25-38.
9. Hoffman, J. A., E. Giraudo, M. Singh, L. Zhang, M. Inoue, K. Porkka, D. Hanahan, and E. Ruoslahti (2003) Progressive vascular changes in a transgenic mouse model of squamous cell carcinoma. *Cancer Cell* 4: 383-391.
10. Kondo, E., K. Saito, Y. Tashiro, K. Kamide, S. Uno, T. Furuya, M. Mashita, K. Nakajima, T. Tsumuraya, N. Kobayashi, M. Nishibori, M. Tanimoto, and M. Matsushita (2012) Tumour lineage-homing cell-penetrating peptides as anticancer molecular delivery systems. *Nat. Commun*. 3: 951.
11. Simberg, D., T. Duza, J. H. Park, M. Essler, J. Pilch, L. Zhang, A. M. Derfus, M. Yang, R. M. Hoffman, S. Bhatia, M. J. Sailor, and E. Ruoslahti (2007) Biomimetic amplification of nanoparticle homing to tumors. *Proc. Natl. Acad. Sci. U. S. A*. 104: 932-936.
12. Koivunen, E., W. Arap, H. Valtanen, A. Rainisalo, O. P. Medina, P. Heikkilä, C. Kantor, C. G. Gahmberg, T. Salo, Y. T. Konttinen, T. Sorsa, E. Ruoslahti, and R. Pasqualini (1999) Tumor targeting with a selective gelatinase inhibitor. *Nat. Biotechnol*. 17: 768-774.
13. Simón-Gracia, L., P. Scodeller, S. Salazar Fuentes, V. Gómez Vallejo, X. Ríos, E. San Sebastián, V. Sidorenko, D. Di Silvio, M. Suck, F. De Lorenzi, L. Yokota Rizzo, S. von Stillfried, K. Kilk, T. Lammers, S. E. Moya, and T. Teesalu (2018) Application of polymersomes engineered to target p32 protein for detection of small breast tumors in mice. *Oncotarget* 9: 18682-18697.
14. Lingasamy, P., A. Tobi, K. Kurm, S. Kopanchuk, A. Sudakov, M. Salumäe, T. Rätsep, T. Asser, R. Bjerkvig, and T. Teesalu (2020) Tumor-penetrating peptide for systemic targeting of Tenascin-C. *Sci. Rep*. 10: 5809.
15. Craggs, G. and S. Kellie (2001) A functional nuclear localization sequence in the C-terminal domain of SHP-1. *J. Biol. Chem*. 276: 23719-23725.
16. Teesalu, T., K. N. Sugahara, V. R. Kotamraju, and E. Ruoslahti (2009) C-end rule peptides mediate neuropilin-1-dependent cell, vascular, and tissue penetration. *Proc. Natl. Acad. Sci. U. S. A*. 106: 16157-16162.