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A site-specific branching poly-glutamate tag mediates intracellular protein delivery by cationic lipids

Si-Qi Huang [a, b], Bei-Bei Han [a, b], Yan-Mei Li [a, b], Yong-Xiang Chen [b, *]

[a] Beijing Institute for Brain Disorders, Center for Brain Disorders Research, Capital Medical University, Beijing 100069, PR China
[b] Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology (Ministry of Education), Department of Chemistry, Tsinghua University, Beijing 100084, PR China

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ABSTRACT

Intracellular protein delivery is of significance for cellular protein analysis and therapeutic development, but remains challenging technically. Herein, we report a general and highly potent strategy for intracellular protein delivery based on commercially available cationic lipids. In this strategy, a designed double branching poly-glutamate tag is site-specifically attached onto the C-terminal of protein cargos via expressed protein ligation (EPL), which mediates the entrapment of proteins into cationic liposomes driven by electrostatic interaction. The resultant protein-lipid complexes can enter into cytosol with a high efficiency even at the low protein concentration while maintaining protein's biological activity.

1. Introduction

Intracellular protein delivery provides an essential tool for cellular protein analysis and therapeutic development [1]. Since cell membrane separates the interior of cell from external environment, exogenous proteins can hardly pass through this natural barrier to function in the cytosol. As a result, therapeutic proteins for clinical use, including antibodies [2], cytokines and enzymes [3,4], mainly act extracellularly, while their application to access intracellular targets remains elusive. Thus, to fully realize the potential of therapeutic proteins inside cells, various effective methods for cytosolic delivery of proteins have been discovered over the past years, such as cell-penetrating peptides (CPPs) [5–7], lipid and lipid-like nanoparticles [8–10], polymeric vectors [11,12], inorganic nanoparticles and receptor ligands [13,14]. Among versatile delivery platforms, the cationic lipid system attracts intense interest due to its excellent membrane-permeability and efficient endosomal escape of cargos [9,10], leading to its wide use in delivery of DNA and RNA even in clinical trials [15,16]. To date, several groups have successfully applied cationic lipid or lipid-like reagents for intracellular delivery of proteins that are modified with anionic tags to improve the protein-lipids assembling efficiency [8–10]. Nevertheless, the commonly used anionic tags, including negatively super-charged proteins and nucleic acids [9,10,17], still exhibit limitations. For example, the bulky size of fused protein tags like supercharged GFP (~28 kDa) have a risk of affecting the folding and function of delivered protein; global modification by nucleic acid tags might result in a great change of protein surface property. Hence, discovering new anionic tags as well as suitable attachment approaches to overcome the limitations of previous methods is of importance for protein delivery by cationic lipids.

Considering the potency of negatively supercharged proteins as well as its bulky size, we plan to design a branching poly-glutamate (poly-E) peptide tag (Fig. 1) with a much smaller size to mimic the three-dimensional negative charges on supercharged proteins. However, the branching peptide tag cannot be introduced into the protein sequence by recombinant technology. We notice that site-specific protein modification technology has achieved great developments over the past two decades [18–20], offering wide choices of approaches for attaching artificial tags on target proteins. Among versatile modification approaches, the expressed protein ligation (EPL) based on native chemical ligation (NCL) reaction provides a highly efficient way for N- or C- terminal labeling of proteins [21–23], which displays a low risk of affecting proteins' property due to its site-specificity. Herein, we report a general and potent new strategy for intracellular protein delivery based on a commercially available cationic lipid system. This strategy uses EPL method to site-specifically attach a negatively charged branching poly-E tag to the C-terminus of delivered protein, which mediates the entrapment of protein into cationic liposomes driven by

* Corresponding author.
E-mail address: chen-yx@mail.tsinghua.edu.cn (Y.-X. Chen).

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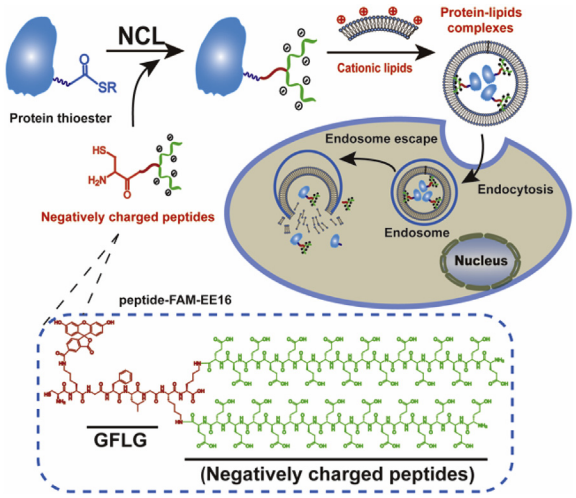

Fig. 1. Schematic illustration of our new strategy for intracellular protein delivery. The steps of this strategy include site-specific protein modification with negatively charged poly-E peptide tag, complexation with cationic lipids and cell membrane permeation.

electrostatic interaction. The resultant protein-lipid complexes have successfully entered into cytosol with a high efficiency while maintaining protein's function.

2. Materials and methods

2.1. Solid-phase peptide synthesis of peptides

Peptides were prepared by the standard method of Fmoc solid-phase peptide synthesis (SPPS) as described previously [24]. Briefly, Fmoc-Lys (ivDde)-Wang resins (Loading: 0.231 mmol/g, GL Biochem) were swelling in DCM for 30 min. Fmoc group was removed by 20% piperidine in DMF twice for 5 min and 10 min. For coupling reactions, the resins were treated with Fmoc-amino acid (4 eq.) in the presence of 1-Hydroxy-7-azabenzotriazole (HOAt, 4 eq.), 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 3.6 eq.) and N, N-diisopropylethylamine (DIEA, 8 eq.) for 1 h at room temperature. The coupling of glutamic acids in side chains and the introduction of 5(6)-carboxyfluorescein (FAM) were carried out after the protection groups ivDde and mtt were respectively removed by 2% hydrazine hydrate ($N_2H_4 \cdot H_2O$) and 1% trifluoroacetic acid (TFA) in DCM 4 times for 10 min. After drying in vacuum for 3 h, the resins were treated with a solution of TFA/phenol/TIS/H_2O (88:5:5:2, v/v) to release the peptides, which were precipitated from the solution by cold diethyl ether. The crude products were further purified by preparative HPLC to afford pure peptides that were further identified by analytic HPLC (C18 column) and ESI-MS.

2.2. Preparation of protein thioester

The plasmid of KRas $(G12V)^{1-174}$ (pTwin1-KRas$(G12V)^{1-174}$-intein-CBD) was transformed into *E.coli* BL21 (DE3) cells and the expression of KRas proteins was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG, 1 mM) when the optical density of bacteria solution (OD_{600}) reached 0.6 (16 °C, 200 rpm, 12 h). Saporin (pET28a-Smt_3-Saporin-intein CBD) was expressed in *E.coli* BL21(DE3) pLysS cells for 3 h at 30 °C (1 mM IPTG, $OD_{600} = 0.8$, 200 rpm). After harvested and resuspended in buffer (50 mM Hepes, 500 mM NaCl, pH 7.4), bacteria were split by ultrasonication in lysis buffer with the addition of phenylmethanesulfonyl fluoride (PMSF, 100 μg/mL) and Triton X-100 (1%, v/v). Then the supernatant of recombinant protein was purified by chitin affinity column and protein-MESNa thioester was released from the column by treatment with sulfhydryl reagent, sodium 2-mercaptoethanesulfonate (MESNa, 500 mM), followed by concentration via ultrafiltration (Amicon Ultra-15, Millipore).

2.3. Ligation of protein thioester with negatively charged peptide tags

The conjugation of protein thioester with negatively charged peptide tags was performed via native chemical ligation (NCL) reaction [25]. Protein-MESNa thioester (KRas-SR or Smt_3-Saporin-SR, 1 eq.) and excessive peptide tags (Peptide-FAM, Peptide-FAM-E8, Peptide-FAM-EE8, Peptide-FAM-E16 or Peptide-FAM-EE16, 5 eq.) were dissolved in the ligation buffer (50 mM Hepes, 500 mM NaCl, pH 7.4) supplemented with 4-mercaptophenylacetic acid (MPAA, 120 mM) and tris (2-carboxyethyl) phosphine (TCEP, 200 mM). The reaction mixture was adjusted to pH 7.2 and incubated at 16 °C for 5 h. Then, the crude ligation products were purified by fast protein liquid chromatography (FPLC, AKTA purifier-10, GE) using desalting column (HiPrep™ Desalting, 5 mL) and ionic exchange column (HiPrep™ Q HP, 1 mL). Low-salt buffer (50 mM Hepes, 2 mM DTT) and high-salt buffer (50 mM Hepes, 1 M NaCl, 2 mM DTT) were used for ionic exchange chromatography. The purified proteins were concentrated by size exclusion filtration (Millipore) and characterized by SDS-PAGE analysis and ESI-MS. Protein concentrations were determined by Nanodrop (IMPLEN) and Peirce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).

2.4. Cell culture

All cell lines (HeLa, N2a, A549) were cultured in a T flash with 5% CO_2 in a 95% humidified atmosphere (37 °C). HeLa cells were maintained in DMEM medium, which was supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. N2a cells were maintained in 50% DMEM and 50% α-MEM and A549 cells were maintained in McCoy's 5A Medium. When reaching 90% confluency, cells were harvested by trypsinization and collected for subsequent experiments.

2.5. Intracellular protein delivery

Before intracellular protein delivery, 1×10^5 cells were seeded into 24-well plates with 1 mL cell culture medium overnight. For preparation of protein:lipid complexes, different concentration of proteins were diluted with 50 μL serum-free Opti-MEM medium in a tube followed by adding 3 μL P3000 regent at room temperature for 10 min. 3 μL lipofectamine 3000 reagent were diluted with 50 μL serum-free Opti-MEM medium in another tube at room temperature for 10 min. Then, diluted proteins were added into the diluted lipofectamine 3000 reagent to form protein:lipid complexes at room temperature for 15 min. For intracellular protein delivery, different ratios of protein:lipid complexes were added to 24-well plates with a final volume of 300 μL in each well. After incubation for 4 h at 37 °C, protein delivery efficiencies were quantitatively determined by flow cytometry (BD FACS Calibur). The intracellular distribution of delivered proteins was observed by a confocal laser scanning microscopy (Zeiss LSM780).

2.6. Flow cytometry and confocal microscopy analysis

After protein transfection for 4 h, cell culture medium was removed. The proteins:lipid complexes bound on the outer face of cell membrane were washed away with PBS buffer for 3 times. Through trypsinization (trypsin-EDTA) and centrifugation

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