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Purification and characterization of a novel cell-penetrating carrier similar to cholera toxin chimeric protein

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ABSTRACT

Developing a recombinant vector for noninvasively delivering biological macromolecules into the brain is important. This study constructed and purified a protein complex based on the cholera toxin (CT) molecular structure. Enhanced green fluorescent protein (EGFP)-modified A2 subunits of CT (CTA2) were used as tracer molecules for introduction of transactivator of transcription (TAT) through the A subunit into cells. The protein complex EGFP-CTA2-TAT/(CTB)5 (CTB: B subunit of CT) was obtained using an *in vitro* recombination method and verified by monosialoganglioside-enzyme-linked immunosorbent assay and high performance liquid chromatography assay. The protein complexes bound more strongly to monosialoganglioside (GM1) than (CTB)5 at low concentrations (0.625–1.25 μg/mL). *In vitro* assays revealed that the transmembrane function of TAT was also maintained. The GM1-binding activity and cell membrane-penetrating ability suggested that a CT structure-based protein complexes could be used to design a delivery carrier for intranasal administration through GM1 binding. The expression vector introduced in this study provides a feasible expression frame for constructing several new macromolecular protein drugs for effective cell penetration.

1. Introduction

Multiple complex barriers of endothelial cells such as the blood brain barrier (BBB) exist widely in all vertebrate organisms. The BBB can impede the entry of unwanted substances and pathogens as well as therapeutic molecules from the blood into the central nervous system (CNS) [1]. Because of low ability to cross the BBB, therapeutic biomacromolecules have limited use in treating CNS diseases [2]. Craniotomy may bypass the BBB and can be used to deliver drugs. But using that method lead to low patient's compliance. Intranasal drug delivery is an effective approach that allows an extensive scope of therapeutic agents including large and small molecules to bypass the BBB [3]. Intranasal drug delivery is assumed to be useful for diagnosing and treating CNS diseases in the future. Therefore, developing new carriers to deliver biomacromolecular drugs into the brain through the intranasal method is important.

Biological macromolecules usually rely on endocytosis to penetrate cell membranes. Endocytosis can lead to lysosome degradation and endosome trapping with little or no macromolecules accessing interstitial targets. Delivery systems based on cell-penetrating peptides (CPPs) can carry macromolecules across cell membranes, and combine low cellular toxicity with high efficiency [4]. Although direct translocation across the cell membrane occurs in some cases, the generally accepted hypothesis is that most CPPs and CPP-cargo complexes enter cells through endocytosis [4–6]. To date, CPPs such as polyarginine sequences Arg8, penetratin and transactivator of transcription-protein transduction domain (TAT-PTD) have been discovered [7]. TAT-PTD is the predominant CPP for delivering small molecules (for example, oligonucleotides and peptides) and large molecules (for example, nanocarriers and full-length proteins), based on its ability to pass through biological

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membranes with different cargoes. New tactics to integrate target proteins with TAT-PTD enable bypassing the BBB by intranasal administration [8]. However, the mechanism of TAT importing is not entirely understood. Researchers found that TAT cannot be used for transcellular delivery across a complete endothelial monolayer *in vitro* [9].

As an endoplasmic reticulum (ER) retention signal, the Lys-Asp-Glu-Leu (KDEL) sequence may prevent proteins from being secreted and trapped in endocytic vesicles through the interaction with KDEL receptors in the Golgi apparatus and intermediate compartment [10]. Fusions containing KDEL and TAT sequences at the C-termini and N-termini efficiently transduce a protein into cells and accurately locate it in the ER [11]. In a recent report, a peptide with the KDEL motif was conjugated to gold nanoparticles. The nanoparticles were internalized through a clathrin-mediated pathway and trafficked to the ER through retrograde transport, bypassing the lysosomal degradation pathway [12].

CTA2, a domain of cholera toxin (CT), has an ER-targeting KDEL motif. CT, expressed by *Vibrio cholerae*, is a large, heteromeric protein of an active A subunit (CTA) and a monosialoganglioside (GM1)-binding pentameric B subunit (CTB). GM1 is a kind of sphingolipid, which is one of the components of the cell membrane of mammals (including humans). *In vivo*, enzymes easily hydrolyze the CTA subunit (28 kDa) to CTA1 and CTA2. CTA1 is the main cause of cholera disease whereas CTA2 serves as a linker to the CTB subunit [13,14]. When CTB binds to GM1 on host intestinal cells, it stimulates the uptake of CT through retrograde endocytosis from the Golgi apparatus to the ER.

In this study, a CT structure-based protein complex was constructed for potential application in intranasal drug delivery. Enhanced green fluorescent protein (EGFP) was the cargo because its intracellular distribution in living cells can be examined immediately using a fluorescence microscope. A new CT-like protein complex, EGFP-CTA2-TAT/(CTB)5, was designed for delivery of EGFP molecules. This study describes the construction and characterization of CT chimeras containing the EGFP, TAT, and KDEL motifs. These chimeras were generated from purified EGFP-CTA2-TAT and CTB proteins. In addition, the ability of the chimeras to transport EGFP into culture cells was characterized using fluorescence microscopy. Successful trafficking *in vitro* is consistent with the goal of effective macromolecular delivery to cells. These experiments suggested the development of CT chimeras as new carriers. With the knowledge gained about the EGFP-CTA2-TAT/(CTB)5 carrier from this study, better carriers can be developed to support intranasal delivery of drugs to the CNS.

2. Materials and methods

2.1. Materials

The pETduet-1 plasmid was donated by Wang Hong, PhD (Rutgers, the State University of New Jersey, New Jersey, USA), *Escherichia coli* strain BL21 (DE3), and DH5α were stored in our laboratory. Plasmid DNA extract, polymerase chain reaction (PCR) reagents, and DNA recovery kits were from Sangon Biological Engineering, Ltd (Shanghai, China). Restriction enzymes and DNA markers were from Thermo Fisher Scientific Inc (Waltham, Massachusetts, USA). Anti-EGFP and anti-His-tag were from Signalway Antibody, Ltd (College Park, Maryland, USA). Antibodies against CTB were from Abcam, Ltd (Cambridge, UK). The pET28a-CTB plasmid was donated by Cao Rongyue, PhD, Nanjing, China Pharmaceutical University. The EGFP-CTA2-TAT gene was synthesized by Sangon Biological Engineering, Ltd (Shanghai, China).

2.2. Methods

2.2.1. Plasmid construction

To construct the pETduet-CTB expression vector, a sequence encoding CTB protein (312 bp) was PCR amplified (primers: 5′-CGCGGATCCAACACCTCAAAATATTACTGATTT-3′ and 5′-CAGC-CAACTCAGCTTCCTT-3′) from plasmid pET28a-CTB and cloned into the expression vector of pETduet-1 using *Bam*H I and *Hin*d III sites. For expression of EGFP-CTA2-TAT (960 bp), the sequence was amplified (primers: CGGAATTCCATATGGTGAGCAAGG and CCGCTCGAGCTGTGGTGGAC) and cloned into the pET22b vector using the Xho I and Nde I sites to obtain the plasmid pET22b-EGFP-CTA2-TAT.

2.2.2. Purification of soluble proteins EGFP-CTA2-TAT from bacterial lysis supernatant

The engineering bacteria *E. coli* BL21 (DE3) with recombinant plasmid pET22b-EGFP-CTA2-TAT were grown in lysogeny broth with 100 mg/mL of ampicillin to optical density at 600 nm = 2.0 and induced with 0.8 mM isopropyl-β-D-thiogalactoside at 25 °C and 120 r/min. After 20 h, cells were collected by centrifugation at 4 °C for 15 min at 10,000 g. Pellets were washed three times with phosphate buffered saline (PBS, pH 7.4, 2 mM potassium dihydrogen phosphate, 10 mM disodium hydrogen phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride) and dispersed in 4 mL lysis buffer of different concentrations in sequence (PBS, pH 7.4 with 200 μg/mL lysozyme) per 100 mL culture. Insoluble cell debris was removed by centrifugation at 10,000 g for 30 min and supernatants sequentially filtered through 15 μm and 0.45 μm filters (Millipore Corp, Billerica, MA, USA). Samples were applied to 5 mL Ni-NTA columns. Unbound proteins were washed from the column with 100 mL Buffer A (PBS, pH 7.4). The column was eluted with 200 mL Buffer B of different concentrations in sequence (PBS, pH 7.4 with 20, 40, 60, 80, 160, 300, or 500 mM imidazole). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze eluted proteins and products containing the EGFP-CTA2-TAT protein were pooled using Amicon ultracentrifugal concentration devices (10,000 Da molecular weight cut-off; Millipore). At each purification stage, target protein was identified and the quality of the final sample was evaluated by SDS-PAGE.

2.2.3. (CTB)5 purification from inclusion bodies and determination by western blot analysis

After lysing by multiple freeze-thaw three times (12 h freeze, 1 h thaw), insoluble cell debris was removed by centrifugation at 10,000 g for 30 min and inclusion bodies were washed three times with Buffer I (pH 8.0, 100 mM NaCl, 2% Triton X-100, 20 mM Tris-HCl, and 5 mM EDTA). Pellets from centrifugation at 10,000 g and 4 °C for 30 min were successively dissolved with 2, 4, 6, and 8 M urea in PBS. Supernatants were collected and SDS-PAGE was used to study dissolved proteins. The western blot analysis was performed as described in detail in our previous publication [15]. CTB monomers in different concentrations of urea were dialyzed against 200 mL Buffer II (PBS, pH 7.4, 1 mM GSH and 0.1 mM GSSH, Ameresco, Solon, OH, USA) at 4 °C, changing Buffer II with sequential dilutions of urea (0–8 M) overnight.

2.2.4. In vitro reassembly of CT-like complexes EGFP-CTA2-TAT/(CTB)5

The assembly protocol for CT-like chimera was similar to previously reported [16]. Purified (CTB)5 and EGFP-CTA2-TAT were loaded into 30-kDa ultrafilters and collected by centrifugation for 15 min at 10,000 g to remove buffer. Protein samples (mass ratio, CTB5: EGFP-CTA2-TAT 5:1) were denatured by 0.5 M citric acid for 20 min at 23 °C to pH 2.3. Solutions were neutralized with 0.12 vol