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

Weiping Lin¹, Xi Zheng^{1,2}, Huaqian Wang^{1,*}, Lin Yu¹, Xiaofen Zhou¹, Yunxiao Sun³, Zhiyun Du¹, Kun Zhang^{1,4}

1 Allan H Conney Laboratory for Anticancer Research, School of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou, 2 Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, New Jersey, USA.
3 Guangdong Entomological Institute 510160, Guangzhou. 4 Wuyi University, Jiangmen, Guangdong, China.

**For correspondence: huaqianwang@126.com*

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.

Keywords: protein complex; cholera toxin; delivery carrier; EGFP; TAT

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 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.

MATERIALS AND METHODS

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 DH5 α 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-GFP 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).

METHODS

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'-CAGCCAACTCAGCTTCCTT-3') from plasmid pET28a-CTB and cloned into the expression vector of pETduet-1 using BamH I and Hind 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.

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 hours, 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 μ g/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 μ m and 0.45 μ m 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.

(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 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 (0-8M) overnight.

***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 2 M Tris (pH 8.5). Proteins were allowed to renature for at least 1 h. Complexes were analyzed by native PAGE. Protein complex after assembling was observed by blue light transmission instrument (MaestroGen Inc., UltraSlim, USA)

Molecular sieve-type high-performance liquid chromatography assays: Complexes EGFP-CTA2-TAT/(CTB)5, (CTB)5, and EGFP-CTA2-TAT were concentrated and analyzed by chromatographic columns loaded with TSKgel

G2000SWXL (TOSOH, Japan). High-performance liquid chromatography (HPLC) (LC-20A, SHIMADZU, Japan) parameters were: temperature 30°C, flow rate 0.5 mL/min, injection volume 20 µL, and mobile phase PBS. Protein sample was detected at 210 nm.

GM1-enzyme linked immunosorbent assay: Enzyme-linked immunosorbent assays (ELISA) were conducted as previously described [17]. GM1 was dissolved in PBS at 10 µg/mL and added to 96-well plates with 100 µL per well for 12 h at 4°C. GM1 solution was replaced by 100 µL PBS with 10% bovine serum albumin (blocking buffer) at 37°C for 2 h to cover nonbinding-GM1 sites. Plates were incubated with serially diluted protein: native EGFP-CTA2-TAT/(CTB)5 complex, (CTB)5, (CTB)5 (Sigma, New York, USA), or EGFP-CTA2-TAT in 100 µL blocking buffer at room temperature for 1 h and washed three times with 100 µL 0.1% Tween 20 in PBS (PBST). Monoclonal anti-EGFP (1:2000) or anti-CTB (1:500) diluted in PBS was added into the plates for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig)G antibody or HRP-conjugated goat anti-rabbit IgG antibody (1:5000) were added before plates were washed three times with PBST. Chromogenic reactions were analyzed with 3,3',5,5'-tetramethyl benzidine-one reagent (Boster, Wuhan, China) per instructions. Absorbance was evaluated at 450 nm.

Fluorescence microscope analysis:

PC3 cells were seeded 50,000 cells per well in six-well plates (Corning Inc., Corning, NY, USA). Purified EGFP-CTA2-TAT fusion protein, EGFP-CTA2-TAT/(CTB)5

complex protein, or EGFP (0.5 mM) were added to each well at 37°C for 20 min. Then cells were gently washed three times with PBS and observed by fluorescence microscope (Olympus IX71, Tokyo Japan).

RESULTS

Construction, expression, and purification of EGFP-CTA2-TAT and (CTB)5

Design and construction of fusion proteins are shown in Fig. 1A and 1B. EGFP-CTA2-TAT was expressed in soluble form in BL21 (DE3). Expression and purity were studied by SDS-PAGE after purification by nickel affinity chromatography (Fig. 1C). (CTB)5 was expressed in inclusion bodies. Ion-exchange resin was used for monomer CTB purification when the pET28a expression system was used. Native gel electrophoresis was used to detect (CTB)5. The polymer protein had high stability. Purified products were verified by western blot with anti-CTB antibody and anti-EGFP antibody (Fig. 1D).

Assembling EGFP-CTA2-TAT/(CTB)5 *in vitro*

Because of the instability of protein complexes in SDS gels, SDS-PAGE could not directly detect EGFP-CTA2-TAT/(CTB)5 formation. To verify the formation of heterogeneous polymers, native PAGE was used. Purified (CTB)5 and EGFP-CTA2-TAT were used as comparisons (Fig. 2A). After assembly, two main bands were seen in gels. One had a higher molecular weight than (CTB)5 and the EGFP-CTA2-TAT band disappeared. After assembly, proteins retained strong fluorescence (Fig. 2B). One possible reason was that EGFP-CTA2-TAT and CTB5 formed heterogeneous polymers. Green fluorescence was from the protein complexes.

The results suggested that assembly did not destroy the EGFP protein, which maintained high fluorescence.

HPLC analysis of EGFP-CTA2-TAT/(CTB)5 formation

To further verify the formation of six heterogeneous polymers, HPLC was used (Fig. 3). The retention time of purified EGFP-CTA2-TAT (37.0 kDa) is 16.154 min (Fig.3A). The retention time of purified (CTB)5 (66.3 kDa) is 20.151 min (Fig.3B). In the chromatographic conditions, large molecular weight peak time is greater than the small molecular weight peak time. When we loaded the assemble complex to the column, the results showed that the peak of EGFP-CTA2-TAT disappeared. A new peak appeared whose retention time is 22.919 min (Fig. 3C). The result was consistent with the native PAGE results. These results provided further evidence of assembly of CT class-six polymers EGFP-CTA2-TAT/(CTB)5.

Binding activity of EGFP-CTA2-TAT/(CTB)5 complex to GM1

As a carrier molecule, the efficacy of CTB for intranasal drug delivery is associated with ability to bind as a pentamer to GM1[15]. GM1-ELISA was used to analyze the ability of purified protein complexes to bind to GM1. Binding curves showed that the protein complexes EGFP-CTA2-TAT/(CTB)5 bound GM1 at the same degree as (CTB)5. At low concentrations, the binding activity of the protein complexes was higher than (CTB)5 (Fig. 4).

***In vitro* transduction**

Prostate cancer (PC-3) cells were used to investigate the transmembrane activity of the protein complexes EGFP-CTA2-TAT/(CTB)5. Purified EGFP-TAT from our

laboratory was used as the negative control. Our experiments showed fluorescence after 20 min with EGPF-CTA2-TAT (20 $\mu\text{g/mL}$) (Fig. 5). Purified EGFP-TAT (20 $\mu\text{g/mL}$) was used as negative control. The literature reported that EGFP-TAT (295 $\mu\text{g/mL}$) can enter the cells in 1h [18]. However, our results suggest that when the concentration is 20 $\mu\text{g/mL}$, no fluorescence will be observed after incubation for 20 min (Fig 6A(2), B(2)). The protein complex EGFP-CTA2-TAT/(CTB)5 also crossed the membrane efficiently (Fig 6D (2)).

Discussion

This study aimed to develop and express a new carrier for delivering biomacromolecules and determine whether the carrier had the ability to penetrate the cell membrane. Two plasmids that expressed either CTB or EGFP-CTA2-TAT were transformed into *E. coli* and purified protein from the cells were recombined as protein complexes *in vitro*. Several TAT fusion proteins infiltrate into different types of cells and tissues with high effectiveness [4]. CTA2 has the ER sequence KDEL, which supports protein localization to the ER instead of degradation in the lysosome; this is believed to increase the transmembrane efficiency[12]. Recombinant proteins with KDEL fused at the C-terminus effectively transduce into cells and locate accurately to the ER [19, 20]. In our study, when the C-terminus of a fusion protein was fused with TAT, the new fusion protein, EGFP-CTA2-TAT, demonstrated high penetrance in PC-3 cells from the TAT penetrating activity and the CTA2 localization function. Fluorescence was observed after 15 min with EGPF-CTA2-TAT in at the

low dose of 5 $\mu\text{g/mL}$ (data not shown).

This study further developed a carrier that could be used for intranasal administration of CNS drugs. CPPs can transport nucleic acids to poorly accessible tissues such as the skin and brain. In mice, TAT-functionalized micelles deliver a dual formulation of siRNA and camptothecin to a glioma through intranasal administration [21]. Similarly, a polyarginine CPP and a transferrin-targeting ligand improve delivery to the brain of plasmid DNA encapsulated in a liposome [22]. In addition to delivering nucleic acid, CPPs also improve the delivery of protein. Lou et al. [23] found that when TAT-haFGF (FGF: fibroblast growth factor) (14–154) was used to study the impact of haFGF on senescence-accelerated mouse prone-8 (a mouse model of Alzheimer's disease), TAT-haFGF greatly enhanced the concentration of haFGF in the brain. In their study, the protein drug needed to be liposome-encapsulated to promote absorption by nasal mucosa; otherwise, nasal absorption of protein drugs was not optimal.

GM1 receptors exist in the majority of mammalian cells. CTB has high-affinity binding to GM1 and can be effectively used for intranasal administration [24, 25]. Currently, recombinant CTB is safe for drug use and is used for a globally licensed oral cholera vaccine. Therefore, chimera proteins containing CTB as a chaperone were used in our study. We hypothesized that when the CTB subunits bound to GM1 on nasal epithelial cells, noncovalent binding of CTA subunits would cross into cells through the hole formed by CTB [26]. This would avoid degradation by proteases in the nasal cavity and might replace the steps of liposome packaging. Binding activity

data on EGFP-CTA2-TAT/(CTB)5 to GM1 supported this hypothesis. PC-3 cells were used to investigate transmembrane activity of the protein complex. The data showed that the chimera protein EGFP-CTA2-TAT/(CTB)5 crossed the membrane efficiently and quickly. Although *in vitro* studies demonstrated the ability of EGFP-CTA2-TAT/(CTB)5 to bind GM1 and penetrate into culture cells, and animal trials, which are currently in development, must be completed to determine the delivery capabilities of this carrier candidate.

Researchers shown that the CTB can self-assemble *in vitro* and form stable B pentamers (CTB)5 [27]. In CT structure, CTA1 is the enzymatic subunit that causes the profuse secretory diarrhea of V. cholera infection. Simulation of the CT and polymerization structure to replace the CTA2 subunit will not affect the combination of CTA2 and (CTB)5 and can carry proteins of at least 42 kDa [28]. In previous studies the toxic CTA1 domain could be replaced by several other domains of antigenic proteins to generate holotoxins such as chimeras that could be used as latent mucosal vaccines [17,28, 29]. Two methods can obtain protein complexes and one is coexpression *in vivo*. In the periplasm, the protein concentration is important for effective assembly [30]. Unique systems have been used to transport subunits to the periplasm for recombination [31]. Leader peptides were adopted to transport modified CTA2 and CTB to the periplasm for assembly of protein complexes in the same manner as native CT [29, 31, 32]. Another method for obtaining protein complexes is by recombining purified subunits *in vitro*. The first segregation and re-association of

CTB and CTA subunits *in vitro* [33] was reported by Finkelstein et al. in 1974. The assembly of B subunit pentamers of LTI and CT was studied by Ruddock et al. CTB assembly-competent monomers can efficiently form through exposure of CTB pentamers to pH no more than 2.0 for a short period, followed by quick neutralization to pH 7.0 [34]. Our study used the *in vitro* renaturation method. The results showed that EGFP-CTA2, modified with TAT in the C-terminal, keep the ability to dock with the (CTB)₅. EGFP-CTA2-TAT/(CTB)₅ chimeras possess a number of advantages for use, including noncovalent association of the cargo to a functional CTB subunit, the absence of the toxic domain and maintenance of the endoplasmic reticulum (ER)-targeting KDEL motif contained within the CTA2 domain [28, 35]. Our study confirmed that using TAT as a C-terminal modification peptide did not affect CTA2 docking with (CTB)₅. TAT is useful for increasing the activity of membrane proteins. It can be located in the C-terminus or N-terminus of the fusion protein [36]. In this study, when TAT was placed at the C-terminus and labeled with six His residues, its membrane activity was not affected. Guo et al. prepared four TAT-EGFP conjugates and found that TAT-fused conjugates could be generated in flexible and convenient formats for biopharmaceutical applications. His-tags have no influence on the cell-penetrating activity of fused proteins [37].

The construction of EGFP-containing chimeras could be a widely used method for CNS drug carrier development. This study used CT and TAT combinations for developing intranasal delivery carriers. Also, the expression vectors constructed in this study are a useful protein expression frame for developing novel macromolecular

proteins to deliver drugs to the CNS through intranasal administration.

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Figure Legends

Figure 1. Construction, purification, and identification of EGFP-CTA2-TAT and CTB.

(A) Strategy for plasmid mapping of pet22b-EGFP-CTA2-TAT. CTA2 and HIV-derived TAT (47-57) were linked to the C-terminus of EGFP. (B) Strategy for plasmid mapping pETduet-CTB. (C) Electrophoresis of purified (CTB)₅ and EGFP-CTA2-TAT. Lane M: molecular weight markers (116, 66.2, 45, 35, 25, 18.4, and 14.4 kDa); lane 1: purified CTB (13.3 kDa); lane 2: purified EGFP-CTA2-TAT (36.30 kDa); lane 3: purified CTB pentamer (66.3 kDa). (D) Western blots identified purified proteins using (1) antibodies against EGFP and (2) antibodies against CTB.

Figure 2. Identification of protein complex. (A) Native PAGE to verify protein assembly by 12% PAGE of purified proteins. Lane M: molecular weight markers (116.0, 66.2, 45.0, 35.0, 25.0, 18.4, and 14.4 kDa); lane 1: EGFP-CTA2-TAT/(CTB)₅ assembling complex (102.6 kDa); lane 2: CTB pentamer (66.3 kDa); lane 3: EGFP-CTA2-TAT (36.3 kDa). (B) Protein complex observed under after assembling blue light transmission instrument.

Figure 3. HPLC analysis of EGFP-CTA2-TAT/(CTB)5 protein complex. (A) Purified fusion protein EGFP-CTA2-TAT. (B) Purified protein (CTB)5. (C) Assembled EGFP-CTA2-TAT/(CTB)5 protein complex.

Figure 4. GM1 binding analysis of EGFP-CTA2-TAT/(CTB)5 protein complex. Proteins were diluted to different concentrations (0.625, 1.25, 2.5, 5.0, 10 $\mu\text{g/mL}$). EGFP-CTA2-TAT and pentameric CTB were used as negative and positive controls, respectively. Data are average of triplicates.

Figure 5. Bright-light and fluorescent images of the same view field of PC-3 cells after treatment with EGFP-CTA2-TAT, EGFP, or EGFP-CTA₂-TAT/ (CTB)5 protein complex (20 $\mu\text{g/mL}$) at 37°C for 20 min.