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A protein transduction domain located at the NH_2-terminus of human translationally controlled tumor protein for delivery of active molecules to cells

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ABSTRACT

Protein transduction domains (PTDs) are small peptides, able to penetrate biological membranes and deliver various types of cargo both in vitro and in vivo. Because use of PTDs originating from viral origins resulted in undesired effects, PTDs originating from non-viral origins are needed. Here, we report that a 10-amino acid peptide (MIIYRDLISH) derived from the NH_2-terminus of human translationally controlled tumor protein (TCTP) functions as a PTD. This peptide was internalized through lipid raft-dependent endocytosis and partial macropinocytosis, and did not enter lysosome and nucleus. Beta-galactosidase fused to TCTP-PTD, when injected into mice, was efficiently delivered to liver, kidney, spleen, heart, and lungs of the animals. Preincubation of TCTP-PTD with adenovirus increased adenoviral mediated-gene expression in cells and also improved immune response to intranasally administered adenovirus expressing the triple repeat of G glycoprotein of respiratory syncytial virus (RSV), rAd/3×G. These findings suggest that TCTP-PTD might overcome the limitations of polycation-mediated transduction and serve as an efficient vehicle for drug delivery.

1. Introduction

Many protein transduction domains (PTDs), alternatively named cell penetrating peptides (CPPs) have been used to deliver therapeutic cargo, because of their cell penetration ability. PTDs have been categorized into three groups according to their origin [1]: first group derived from natural proteins such as TAT of the human immunodeficiency virus (HIV), and penetratin of *Drosophila* Antennapedia (Antp), second group are those modified from natural peptides, such as transportan [2] and synB [3] and the third group are those identified by screening random phage display libraries. Regardless of their origin, all PTDs share some features, including an arginine and lysine rich motif, a hydrophobic core sequence, and an amphipathicity or α-helix.

PTDs can transfer a variety of cargos, ranging from small drug molecules to large proteins and nucleic acids, and have therefore been applied as effective vehicles [4,5]. For example, administration of beta-galactosidase fused to TAT to mice resulted in efficient transduction to all tissues, even across the blood-brain-barrier (BBB) [6]. The cross-linking of TAT to siRNA increased cellular uptake of the oligonucleotide without interfering with the perinuclear localization required for RNAi activity [7]. However, several studies of transduction of oligonuceotides with PTD-siRNAs revealed that TAT and penetratin peptide itself affected gene expression [8], underscoring the need for PTDs that do not cause undesirable changes in the organism under study.

We identified a PTD derived from the NH_2 terminus of human TCTP (TCTP-PTD) with the sequence, 'MIIYRDLISH'. TCTP is considered to be a house keeping gene because of its ubiquitous expression, high degree of conservation among species, and occurence in both tumor and normal cells [9]. Human TCTP consists of 172 amino acids and the tertiary structure of fission yeast TCTP shows a possible relationship with Mss4/Dss4 chaperone family [10]. TCTP is known to be involved in human allergic response [11–13], apoptotic regulation [14–16], various cancer-related functions [17–19], and hypertension [20], but its mechanism of the action still remains elusive.

We found that this cell penetrating peptide differs from other well-known PTDs in its amino acid sequence and structure, has the ability to internalize into the cells, and translocate cargo in vitro and in vivo. We characterized the mechanism by which TCTP-PTD internalizes into cells and also examined its usefulness as transduction vehicle using adenoviral gene delivery system.

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2. Materials and methods

2.1. Cell culture

HeLa, NIH-3T3 and MDCK cell lines were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's minimal essential media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 units/ml streptomycin. U-937 cell lines also obtained from ATCC were grown in Roswell Park Memorial Institute (RPMI)-1640 Medium supplemented with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin. Human bronchial epithelial cells, BEAS-2B (ATCC), were maintained in bronchial epithelial growth medium (BEGM, Clonetics, San Diego).

2.2. Peptide synthesis

Highly pure peptides were obtained from Peptron (Daejeon, Korea). Amino-terminals of the peptides were fluorescent labeled with 5-(6)-tetramethylrhodamine (TAMRA), protecting carboxy-terminals by amidation. Freeze-dried peptides were reconstituted in high-purity DMSO (10 mM) and stored at −70 °C until use.

2.3. Protein expression

TAT regions of pTAT-HA-LacZ vector (kindly provided by S. F. Dowdy, UCSD, CA, USA) were removed or substituted by TCTP-1-10. These plasmids were expressed in E.coli BL21(DE3) pLysS (Novagen) and purified on a His binding column of nickel-nitrilotriacetic acid (Ni^{+}-NTA) agarose resin (Qiagen). After replacing the buffer with PBS using PD-10 column, proteins were quantitated with Bradford Protein Assay Dye (Bio-Rad). Purity of proteins was examined with Coomassie staining by SDS-PAGE and the activity of beta-galactosidase was assessed with 15 mM O-nitrophenyl-β-D-galtopyranoside (ONPG) dissolved in 0.1 M Na.Pi (pH 7.4) buffer. The absorbance was measured at 405 nm with ELISA microplate reader. For preparation of intact and truncated TCTPs, *Escherichia coli* BL21(DE3)pLysS cells transformed with the pRSET A/TCTP or pRSET A/deleted TCTPs, were overexpressed and purified as previously described [13].

2.4. Peptide and protein uptake assay

1.0×10^6 cells/well were seeded onto 6-well plates 24 h before incubation with peptides or proteins. The cells were then washed with serum-free media and incubated with peptides or proteins in serum-free media. To study the effect of low temperature on the cellular internalization of peptides, HeLa cells were washed with serum-free DMEM and preincubated in serum-free DMEM for 1 h at 4 °C. The cells were incubated with 10 μM peptide in serum-free DMEM for 2 h at 4 °C. For energy depletion experiments, HeLa cells were washed with serum free DMEM and pre-incubated in serum- and glucose-free DMEM containing 6 mM 2-deoxy-D-glucose (Sigma) and 10 mM sodium azide (Sigma) for 1 h at 37 °C. The cells were incubated with 10 μM peptide in the same DMEM for 2 h at 37 °C. To study the effect of specific endocytosis inhibitors (all were obtained from Sigma), HeLa cells were washed with serum-free DMEM and pretreated for 30 m at 37 °C with 0–15 μM chlorpromazine (CPZ), 0–5 mM methyl-β-cyclodextrin (MβCD), 0–10 mM EIPA, 200 μM chloroquine or 25 μg/ml heparin. Then the cells were incubated with the 10 μM peptide in serum-free DMEM containing each inhibitor for 2 h at 37 °C. Following the above procedures, the cells were washed two times with PBS and immediately trypsinized (1 mg/ml) for 10 min at 37 °C to remove extracellular peptides or proteins and then washed two times with PBS. Analysis of peptide internalization was performed by flow cytometry (Becton Dickinson). Flow cytometric analyses were accomplished using WinMDI version 2.8 software (free ware).

2.5. Western blotting

When BEAS-2B cells became 80% confluent, they were passaged in 48-well culture plates, incubated for 20–24 h in bronchial epithelial basal medium (BEBM), washed twice with 1% penicillin-streptomycin/BEGM, and then incubated for 24 h with 5 μg/ml or 1 M of proteins in 1% penicillin-streptomycin/BEBM. The supernatants were saved for western blotting, and the pellets were washed twice with PBS and treated with 20 mM of trypsin at 37 °C for 10 min before denaturing in a sample buffer containing AEBSF (50 mM Tris, pH 6.8, 2.5% SDS, 22% glycerol, 5% β-mercaptoethanol, 1 mM AEBSF). The resulting supernatants and cell extracts were subjected to western blotting.

2.6. Confocal microscopy

Cells (1.0×10^5/well) were seeded on to glass cover slips 24 h before incubation with peptides or proteins. After washing with serum-free DMEM, the cells were treated with 10 μM TAMRA-labeled peptide for 2 h at 37 °C. For subcellular localization of TCTP-PTD peptide in live cells, the cells were coincubated with fluorescence markers (Mito-Tracker, Lyso-Tracker, Alexa flour 488 labeled trasferrin, Oregon green-labeled neural dxtran-70 kD, Alexa flour 488 labeled cholera toxin B) in serum-free DMEM for 1 h at 37 °C. All fluorescence markers were obtained from Molecular Probe. The cells were washed and mounted in phenol red-free DMEM (JBI) and immediately visualized by confocal microscopy (Carl Zeiss).

2.7. Beta-galactosidase staining

4–6 weeks-old Balb/c mice were injected intraperitoreally with 400 μg of β-gal control protein and TCTP-PTD/β-gal protein in 0.5 ml of PBS. After 2 or 4 h, mice were sacrificed. Tissues were harvested and washed with PBS, fixed in 0.25% glutaraldehyde for 1 h, and developed overnight in 0.2% X-gal staining solution. All animal procedures were performed in accordance with institutional guidelines.

2.8. Infection of the cells

For the preparation of adenovirus complex with a TCTP-PTD, adenovirus was incubated with TCTP-PTD peptide in 100 μl of serum-free DMEM for 30 min at room temperature. 6.0×10^4 cells/well were seeded on to 24-well plates 24 h before infection and washed with serum-free DMEM. The complex was added to the cells, left to incubate for 4 h and changed with 10% serum containing DMEM for 24 h.

2.9. Immunization

All animal experiments were performed according to the National Institute of Health Publication No. 8523: Guide for Care and Use of Laboratory Animals (Accession No. ELAGC-08-1021). 6- to 8-week-old female BALB/c mice were kept under specific-pathogen free conditions. For immunization, mice were lightly anesthetized by ether/chloroform inhalation and intranasally administered 5×10^6 PFU of replication-defective adenovirus complex with TCTP-PTD in a volume of 70 μl. For measuring total IgG titer, after 2 weeks of infection, blood was obtained from the retro-orbital plexus with a heparinized capillary tube and centrifuged, and serum was stored at −20 °C. For measuring secretory IgA titer, bronchoalveolar lavage (BAL) fluid was obtained from a lung airway. RSV G protein-specific antibody titers in immunized mice were measured by a direct enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase-conjugated affinity-purified rabbit anti-mouse total IgG or IgA secondary antibody (Zymed Laboratoris).

2.10. Toxicity of TCTP-PTD in mice

TCTP-1-10 peptide was intravenously injected into five mice (1.6 mg/kg) every day for 2 weeks. To assess liver and kidney toxicity, levels of aspartate aminotransferase (AST), alanine aminotrasferase (ALT), blood urea nitrogen (BUN) and creatinine in the blood were determined and body weight was monitored.

2.11. Statistical analysis

Data are presented as mean ± SD. Comparison of differences was conducted by using an unpaired, two-tailed Student *t*-test. The difference was considered statistically significant when the *P* value was ≤ 0.05.

3. Results

3.1. Identification of a protein transduction domain

We fortuitously observed the translocation of TCTP into the RBL-2H3 cell line while performing confocal microscopic experiments using purified full length TCTP. Based on this fortuitous finding, we postulated that TCTP contains a PTD in its sequence. While attempting to locate the PTD present in TCTP protein, we constructed protein expression vectors of full length TCTP as well the several amino terminal truncated proteins, containing only the indicated amino acids residues: TCTP-11-172 and TCTP-35-172. We also purified other truncated derivatives of TCTP including TCTP-1-38, TCTP-39-110 and TCTP-111-172. In BEAS-2B cells, after 24 h incubation with full length TCTP and the truncated derivatives, TCTP-11-172 and TCTP-35-172, we harvested the supernatants and then subjected them to western blotting using anti-TCTP antibody. We noted that the full length TCTP disappeared from the supernatant, but the amino terminal truncated proteins, TCTP-11-172 and TCTP-35-172, remained in BEAS-2B cell supernatants, suggesting that amino-terminus of TCTP is necessary for internalizing into cells (Fig. 1A). In the next set of experiments, different types of truncated derivatives, TCTP-1-38, TCTP-39-110 and TCTP-111-172 were treated for 5 min and 30 min in BEAS-2B cells. As shown in Fig. S1, only TCTP-1-38 disappeared rapidly from the cell