A gene delivery system based on the N-terminal domain of human topoisomerase I

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Abstract

The N-terminal 200 amino acid residues of topoisomerase I (TopoN) is highly positive in charge and has DNA binding activity, without DNA sequence and topological specificity. Here, a fusion protein (6×His-PTD-TopoN) containing a hexahistidine (6×His) tag, a membrane penetration domain and TopoN (amino acid 3–200) was designed and developed. The protein can bind to different sizes (3.0–8.0 kb) and forms (circular and linear) of DNA and translocates the bound DNA to the nucleus. The protein also showed low cytotoxicity to GF-1 grouper fish fin cells that were previously very sensitive and difficult to transfect in vitro. Maintaining the hexahistidine tag increased the protein’s transfection efficiency in COS7 African green monkey kidney cells and simplified the purification process. The plasmid pEGFP-N1 was delivered into COS7 cells by the protein in ATP- and temperature-dependent manners. The results indicate that the binding ability of TopoN is very useful for DNA delivery and the carrier protein can be expressed in Escherichia coli without removal of the hexahistidine tag.

Keywords: gene delivery system; protein transduction domain; topoisomerase I
1. Introduction

Efficient delivery of genetic materials through the cell membrane is the key step for success in gene therapy, DNA vaccines and genetically modified organisms. The cell membrane is generally impermeable to exogenous bioactivity molecules such as proteins and peptides.

Techniques used for delivering genetic materials including calcium phosphate precipitation [1], DEAE-dextran-mediated cell fusion [2] and electroporation [3] have been used in vitro and in vivo, virus infection [4], microinjection [5] and liposome mediated transfer[6] have been used in vivo.

Among those methods, the most common is virus infection, which can deliver the exogenous gene into cells and even to insert the gene into a host genome. The gene can maintain and function in the host cell, but is impossible to control and identify the insertion location of the host genome [7–9]. Using the virus as a carrier can have higher transfection efficiency, but also with higher potential risk of inflammatory response and virulence. The application limitations of viral vectors are toxicity, restricted targeting of specific cell types [10], limited DNA carrying capacity, production and packaging problems, recombination and expense [11]. The most wildly used of non-viral vectors for delivering DNA into cells are cationic lipids or polymers [12]. Other methods/vectors such as electroporation, microinjection, cell fusion organic or inorganic nanoparticles [13] are also used for transfection. Cationic liposomes and polymers can form electrostatic complexes with DNA
and protect these complexes from nuclease degradation by condensing DNA [14]. While
non-viral gene delivery systems are safer than viral gene delivery systems [15, 16] they suffer
from complicated operation procedures, low transfection efficiency [9] and may cause
damage to the cell.

Proteins can be taken up into cells through endocytosis. Some proteins, such as bacterial
toxins, growth factors, homeoproteins and viral proteins are able to pass through the cell
membrane when added exogenously [17–19]. Recently, membrane-permeable peptide
delivery systems that include short peptide segments derived from human immunodeficiency
virus type 1 (HIV-1) Tat [20, 21], Drosophila antennapedia (Antp) homeotic transcription
factor [22] and VP22 protein from herpes-simplex-virus-1 [17, 23] – which are termed
cell-penetrating peptide (CPP), protein transduction domain (PTD) or Trojan horse peptides –
have been used to deliver various molecules including proteins, small molecular weight
compounds, oligonucleotides and liposomes into cells [24, 25]. Most of the peptide delivery
vehicles contain fewer than 20 amino acids and are rich in basic or hydrophobic amino acids:
an example is the tryptophan-rich peptide pep-1 (KETWWETWTEWSQPKKRKV) that,
when applied at a certain range of concentrations, can carry proteins and peptides into
mammalian cells without receptor-mediation, input of energy and harm to the cells [26].

In this study, a protein containing DNA-binding and membrane transduction domains
was used to deliver DNA into variety of cell types. The DNA binding and nuclear
localization capabilities of the protein were bestowed by the N-terminal domain of human
topoisomerase I (TopoN), a 765-amino acid protein comprised of an unstructured N-terminal
domain of 200 amino acids, a core domain, a linker domain, and a C-terminal domain [27, 28]. TopoN regulates DNA topology by making single-strand breaks, allowing strand passage,
and then resealing the breaks independent of ATP hydrolysis [29]. It can bind positively and
negatively supercoiled DNA [30], and plays an important role in different aspects of DNA
metabolism such as DNA replication, DNA recombination and transcription [29, 31, 32]. In
addition to its catalytic activity on DNA, TopoN functions as a kinase to phosphorylate RNA
splicing factors [33]. TopoN is poorly conserved, highly positively charged [34], unstructured,
protease sensitive and contains nuclear localization sequences (NLSs) [27, 28, 35–37].
Although the NLSs do not contribute to the catalytic activity, they are essential for the
nuclear translocation of the enzyme [36, 38]. TopoN also binds to DNA [10, 39] without
DNA sequence and topological specificity [10].
The present study was undertaken to develop a new DNA delivery system based on a
short amphipathic peptide carrier, pep-1 [26]. The pep-1 NLS (KKRKV) and spacer domain
(SQP) were removed and the altered peptide was fused to TopoN. A hexahistidine (6×His)
tag was added in the N-terminus of the designed protein to enable purification. A spacer
(SQPGR) between pep-1 and TopoN harbored a proline residue to improve the flexibility and
integrity of the linked peptides.
Materials and methods

2.1. Modification of genes and construction of plasmids

6×His-pep1-TopoN (Fig. 1A) and 6×His-pep2-TopoN (Fig. 1B) were modified from pep-1 [26] and pep-2 [40], in which the spacer domain (SQP) and NLS (KKRKV) were replaced by a spacer domain (SQPGR) and 198 amino acids of TopoN (3–200). We have tried different sequences and mainly on the TopoN (N-terminus 198 a.a. and 98 a.a.) which is important on DNA binding and nuclear localization. The result showed that 198 a.a. of human topoisomerase I was better than the shorter one. In addition, use SQPGR as a spacer between pep-1/2 and TopoN was more suitable in this study than SQP. For construction of the plasmid expressing 6×His-pep1-TopoN and 6×His-pep2-TopoN, the oligonucleotides encoding tryptophan-rich peptide (pep1, MGKETWWETWWTEW and pep2, MGKETWFETWFTEW) with spacer domain (SQPGR) were synthesized (Mission Biotech, Taipei, Taiwan), cloned and inserted into the pET15b vector (Novagen, Darmstadt, Germany). The vector contains TopoN (Fig. 1, Suppl. Table 1) and encodes the 6×His tag at the N terminus (Figs. 1A, 1B and 1D; Suppl. Table 1). TopoN was constructed with vector pET15b by use of BamHI and EcoRI. Proteins without the hexahistidine tag (pep1-TopoN; Fig. 1C) and without PTD domain (6×His-TopoN; Fig. 1D) were used as the controls. Gene expression was driven by the T7 promoter and all constructs were sequenced and determined to be error-free.

2.2. Protein expression, protein purification and immunoblotting
6×His-pep1-TopoN and 6×His-pep2-TopoN were expressed in *Escherichia coli* BL21(DE3) by isopropyl-1-thio-D-galactopyranoside (IPTG) induction. The expressed protein was collected by centrifugation and resuspended in 40 ml of 1 × binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9). Cells were broken by sonication. The lysate was centrifuged at 13,000 g for 30 min at 4 °C, and the supernatant was applied to a Ni²⁺-HiTrap® affinity column (Pharmacia Biotech, Upsalla, Sweden) and was concentrated by Centricon® plus-20 (Millipore Asia, Taipei, Taiwan). The fractions were eluted with 250 mM and 300 mM imidazole containing 0.5 M NaCl and 20 mM Tris-Cl, pH 7.9. The sizes of the proteins were determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining (Fig. 2A). Pre-stained protein marker standards (Invitrogen, Carlsbad, CA) were included on each gel for molecular weight estimation. Monoclonal anti-His-tag (diluted 1:2000) and monoclonal rabbit anti-PTD antibodies (diluted 1:3000) were used for the detection of 6×His-PTD-TopoN and PTD-TopoN, respectively. Alkaline phosphatase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA.) diluted 1:2000 was used as the secondary antibody.

2.3. Cells and cell culture conditions

To exam the transfection efficiency of 6×His-pep1-TopoN and 6×His-pep2-TopoN in different cells, COS-7 African green monkey kidney cells (kindly supplied by Dr. Shyh-Yu Shaw, National Cheng Kung University), 3T3 mouse embryo fibroblast cells (Bioresources...
Collection and Research Center (BCRC), Taipei, Taiwan; BCRC 60159) and GF-1 grouper fin cell (BCRC 960094) were used. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Grand Island, NY) and 10% (w/v) fetal bovine serum (FBS). Except for GF-1, the cells were cultured as a monolayer in a humidified atmosphere containing 5% CO$_2$ at 37 °C. GF-1 cells were grown in humidified incubator at 28°C in antibiotic-free L15 medium (Life Technologies, Gaithersburg, MD) supplemented with 5% v/v heat-inactivated FBS [41]. The cells were imaged by fluorescence microscopy (Olympus IX70; Olympus, Tokyo, Japan) using a 488 nm excitation wavelength.

2.4. Lipofectamine transfections

Lipofectamine™ transfection was performed following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Briefly, serum-free DMEM (Gibco) was used to replace all existing medium on cells. For each well of 24-well plate, 1 μg DNA was added to 50 μl DMEM in one tube and lipofectamine™ transfection reagent (Invitrogen) (1 μl was used in Figs. 5C and 5F and 0.5, 1 and 2 μl were used in Fig. 6) with 50 μl DMEM in another tube. The contents of both tubes were mixed and incubated for 20 min then added to cells. After 24 h incubation, the serum-free DMEM was replaced with RPMI-1640. Observation and imaging of green fluorescent protein (GFP) expression in the cells was performed using fluorescence microscopy (Olympus IX70; Olympus).

2.5. Electrophoretic mobility shift assay (EMSA)
Reaction mixture totaling 15 μl contained the 6×His-pep1-TopoN and 6×His-pep2-TopoN plasmid and 1× gel retardation assay buffer (20 mM HEPES, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol and 10% glycerol). The plasmids used in this study were pBluescript SK⁺ (pBSK⁺; Stratagene, LaJolla, CA), pCMV-Mx-egfp [37], pEGFP-N1 (Clontech, Mountain View, CA) and pF4X1.4hyg (Jena Bioscience GmbH, Jena, Germany). Within these plasmids, pBluescript SK⁺ and pF4X1.4hyg were cut by BamHI and XhoI, respectively, to obtain linear form DNA. The amount of DNA was fixed to 1 μg and the amounts of protein were very according to the molar ration. The molecular weight (MW) of pep1-TopoN and pep2-TopoN is about 36.5 KDa. The MW of BSA is 66.776 KDa and protease K is 28.9 KDa. 1 base pair MW of dsDNA is 0.66 KDa. The reaction mixture was incubated at 37 ºC for 30 min. Bovine serum albumin (BSA, molar ratio of 40:1 or 100:1) was used as a control protein and loss-of-function was demonstrated by adding protease K (ProK, Sigma-Aldrich, St. Louis, MO) to 6×His-pep1-TopoN. The concentration of protease K followed the manufacture’s recommendation. Each reaction was run on a 0.7% agarose gel during electrophoresis in 1 × TBE buffer, and the DNA was visualized by staining with ethidium bromide.

2.6. Viability and proliferation assay

Cell viability was determined using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as detailed by the manufacturer (Promega, Madison, WI) in a modification of
a previously described protocol [42]. Cells were washed with warm RPMI-1640 without phenol red and a MTT working solution (0.5 mg ml\(^{-1}\) MTT in RPMI-1640 without phenol red) was added (10 \(\mu\)l for each well) into wells of a 96-well plate. Except for GF-1, cells were incubated in a 5% CO\(_2\) incubator at 37 °C for 4 hours. GF-1 cells were incubated at 28 °C. The converted dye was solubilized with 1 ml acidic isopropanol (0.04 M HCl in absolute isopropanol) and the absorbance was measured at 570 nm with background subtraction at 650 nm using a Shimadzu UV-1201 spectrophotometer and disposable plastic cuvettes. Relative cell viability at 4 h was compared to control cells containing cell culture medium without copolymer using the following equation:

\[
\text{Relative cell viability (\%)} = \left( \frac{[\text{OD}]_{\text{test}}}{[\text{OD}]_{\text{total\ cells}}} \right) \times 100\%
\]

\[
[\text{OD}]_{\text{test}} = [\text{OD}]_{\text{sample}} - [\text{OD}]_{\text{medium}}
\]

2.7. Luciferase activity assay

Cells were seeded in wells of 12-well plates and grown to 70% confluence. The control experiment was without BSA in the culture medium. 6×His-PTD-TopoN was mixed with pGL3-Promoter vector (1 \(\mu\)g) (molar ratio of 15:1) and added to each well. After 2 h, the culture medium with BSA was added into each well and cultured for 48 h. Cells were collected and lysed directly in cell lysis buffer (100 mM potassium phosphate pH 7.8, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 7 mM \(\beta\)-mercaptoethanol). Cell lysates were mixed with luciferase substrate (luciferase activity reagent, 25 mM Tricine pH 7.8, 15 mM
potassium phosphate pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM ATP, 0.1 mM dithiothreitol) and measured immediately with a Lumat LB9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). All transfection experiments were performed in triplicate.

2.8 Statistical analysis

The data were obtained from triplicate measurements and summarized as means ± standard deviation (S.D.). Statistical differences ($p < 0.05$) were performed by Student’s $t$-test.
3. Results

3.1. PTD-TopoNs expression and purification

The constructed plasmids were transfected and expressed in *E. coli* BL21(DE3), and production of the protein was induced by IPTG and purified (Fig. 2A). Western blotting confirmed that the 6×His-pep1-TopoN and 6×His-pep2-TopoN proteins were recognized by anti-PTD (data not shown) and anti-hexahistidine-tag monoclonal antibodies (Fig. 2B).

3.2. pep1-TopoN stability

The 6×His-pep1-TopoN and 6×His-pep2-TopoN proteins were transfected with similar efficiency. Hereafter we only focus on 6×His-pep1-TopoN. 6×His-pep1-TopoN was purified and stored at 4 °C, -20 °C and -80 °C (Figs. 2C and 2D). When the proteins were analyzed by 12% SDS-PAGE and Coomassie Brilliant Blue staining, its stability was confirmed, with only a single major band was evident after storage for 3 weeks (Fig. 2C) and 8 months (Fig. 2D). Freshly purified 6×His-pep1-TopoN did not display evident of appreciable degradation when stored at 37°C for 30 min, 1 h and 2 h (Fig. 2E). Experiments in which 6×His-pep1-TopoN was left at room temperature for at least 2 h confirmed the undiminished function of the protein upon translocation.

3.3. DNA binding assay of PTD-TopoNs

6×His-pep1-TopoN and 6×His-pep2-TopoN were mixed with plasmid pBSK+ (3.0 kb) (Fig. 3A), pEGFP-N1 (4.7 kb) (Fig. 3B) or pCMV-Mx-egfp (8.0 kb) (Fig. 3C) in different molar
ratios (5:1, 10:1, 20:1 and 40:1). Both the 6×His-pep1-TopoN and 6×His-pep2-TopoN exhibited binding to plasmids ranging in size from 3.0–8.9 kb (Fig. 3). The control (bovine serum albumin) displayed no binding to DNA observed on the same agarose gels. The gel mobility shift assay revealed decreasing plasmid mobility with increasing molar ratio of 6×His-pep1-TopoN and 6×His-pep2-TopoN (Fig. 4), consistent with the increased binding of DNA to either protein. Similar results were obtained with the circular form of DNA, in which more DNA was bound to 6×His-PTD-TopoN (Fig. 4). When proteinase K was added to digest 6×His-pep1-TopoN, the shift in DNA mobility disappeared. We had done the time course of 0.5, 1, 2 and 4 h. The results showed that after 0.5 h the peptide already bind to DNA and continue to increase the bind amount of DNA until 2 h. No significant difference between 2h and 4h. We then choose the 2h though the whole paper as the reaction time.

3.4. *in vivo* DNA delivery of PTD-TopoNs

COS-7 cells were incubated for 2 h in the presence of 6×His-pep1-topoN and 6×His-pep2-TopoN with 1 μg pGL3-Promoter plasmid encoding the reporter gene luciferase. Use of different molar ratios of 6×His-PTD-TopoN to pGL3-Promoter plasmid (5:1, 10:1, 15:1, 20:1, 25:1 and 30:1) determined that ratios of 15:1 for pep1-TopoN to DNA and 20:1 for pep2-TopoN to DNA produced maximum luciferase activity in COS7 cells (Fig. 5A). The transfection efficiency of 6×His-pep1-TopoN was significant (*p* < 0.005) higher than 6×His-pep2-TopoN in the molar ratio of 15:1. But opposite result was obtain in the molar
ratio of 20:1. 6×His-PTD-TopoN (6×His-pep1-TopoN) was capable of transfecting pEGFP-N1 into COS-7 cells, allowing the expression of GFP. The 7.2% transfection efficiency (Figs. 5D and 5G) was better than the rate achieved using lipofectamine (5.2%) (Figs. 5C and 5F). In the absence of 6×His-pep1-TopoN and lipofectamine, pEGFP-N1 was unable to pass through the cell membrane and no fluorescence was evident in COS7 cells (Figs. 5B and 5E).

3.5. Cytotoxicity test of PTD-TopoNs

COS-7, 3T3 and GF-1 cells were used to test the cytotoxicity of 6×His-pep1-TopoN, 6×His-pep2-TopoN, 6×His-TopoN and lipofectamine. GF-1 cells were very sensitive to commonly-used amount of lipofectamine (0.5, 1 and 2 μl for each well), while cell proliferation was completely unaffected by 6×His-pep1-TopoN and 6×His-pep2-TopoN ($p < 0.005$, Fig. 6A). 3T3 cells were most resistance to exogenous proteins or lipid, with over 80% of the examined populations remaining capable of proliferation (Fig. 6B). COS7 cells were the most sensitive cell type to lipofectamine, 6×His-pep1-TopoN and 6×His-pep2-TopoN (Fig. 6C). The results highlighted the differing responses to different cell types to exogenous proteins or lipid. Even a low amount of lipofectamine (2 μl per well) could similarly affect cells in the presence of higher concentrations of proteins (1,000 μM of 6×His-pep1-TopoN and 6×His-pep2-TopoN). The amount (12, 24 and 48 μl) of lipofectamine indicated in Fig.6 were the total amount been used per 24-well plate.
3.6. Cytotoxicity test of PTD-TopoNs/DNA complex

3T3 and COS-7 cell populations received were used to test different concentrations of 6×His-PTD-TopoNs or 6×His-PTD-TopoNs/DNA complex. Increasing concentration of either preparation produced increased cytotoxicity ($p < 0.005$, Figs. 7A and 7B). Approximately 40% of the 3T3 populations survive after a 3 h exposure to 10 mM 6×His-PTD-TopoNs/DNA at 37 ºC in DMEM supplemented with 10% FCS (Fig. 7B). Treatment with 6×His-PTD-TopoNs under the same conditions resulted in increased cytotoxicity ($p < 0.005$), with only about 27% of the cell populations surviving (Fig. 7A). Lipofectamine produced even higher cytotoxicity at lower concentration (Fig. 6).

3.7. Energy requirement of PTD-TopoNs

Cells were pre-incubated for 1 h at 4ºC or 37 ºC, or with 10 mM sodium azide and 6 mM 2-deoxy-D-glucose to deplete cellular ATP. Gene delivery via the 6×His-PTD-TopoNs was inhibited at 4ºC ($p < 0.005$) and by depletion of cellular ATP ($p < 0.01$, Fig. 8).

3.8. Effect of His_{6}-tag on pep1-TopoN transfection efficiency

COS7 cells were incubated for 2 h in the presence of 6×His-pep1-TopoN/DNA complexes (15:1) with 1 μg pGL3-Promoter plasmid encoding the luciferase reporter gene. The transfection efficiency of 6×His-pep1-TopoN was higher ($p < 0.01$) than pep1-TopoN (Fig. 9).

3.9. Effect of structural constraints on transfection activity
The 6×His-pep1-TopoN fusion protein was denatured with 6M urea and then tested for DNA binding activity using an agarose gel mobility shift assay. For 1 μg of pCMV-Mx-EGFP-N1 (~8.0kb) plasmid DNA, a mobility shift of the DNA bands was first detected when denatured or native forms of fusion protein were added to plasmid at a 10:1 molar ratio (Fig. 10A). Denatured and native forms of the peptides on transfection to COS-7 cells showed similar results (Fig. 10B).
4. Discussion

The results demonstrate that a fusion protein containing a PTD domain and TopoN can be used to deliver functional exogenous DNA into three different cell types. The fusion protein meets the criteria of a successful delivery system: in order, penetration of the cell membrane, nuclear localization and binding to DNA; as well as cellular/tissue specificity [43] and lack of cell and tissue toxicity.

The most important feature PTD is the ability to transport genes of interest into the cell lines or primary cells; this effectiveness has been confirmed [26]. The designed protein, 6×His-PTD-TopoNs, can bind DNA and transport DNA through the cell membrane by virtue of the TopoN and PTD domains, respectively, and spontaneously locates to cell nuclei due to the NLSs present in TopoN. NLSs function in the active transport of exogenous proteins and probes into the nucleus [44, 45]. This function of the TopoN NLSs was confirmed in the TopoN of 6×His-pep1-TopoN (Figs. 5D and 5G). Unstructured and non-enzymatic functioning TopoN possess DNA binding ability [10], although the details have been unclear. To clarify the binding ability of TopoN, the binding of 6×His-PTD-TopoNs to different sizes and forms of DNA was assessed. 6×His-PTD-TopoNs bound to various sizes of DNA (Fig. 3) and to both circular and linear forms of plasmids (Figs. 3 and 4), which was indicative of a broad application on DNA binding.

Compared with previous transfection methods, the advantages of using PTD to carry
exogenous genetic material into cells is that the PTD domain can significantly increase the transfection efficiency (e.g., in primary lymphocytes) [46, 47]. In *in vivo* experiments, PTD has broad applications, e.g., PTD can even penetrate the blood-brain barrier [48], while viral vectors can only carry foreign genes to the brain artery adventitia. Not only mammalian cell but also the plant cell wall can be penetrated by PTD [49, 50]. Here, we also showed that the COS-7 cell transfection efficiency of the DNA delivery system (7.2 %, Figs. 5D and 5G) is superior than that provided by lipofectamine (5.2 %, Figs. 5C and 5F). The transfection efficiency (7.2 %) of 6×His-PTD-TopoNs was lower than poly-L-lysine-palmitic acid is (~22 %) and Lipofectamine™ 2000 (~11%) [51]. However, compared to other peptide carrier system, 0.165-0.22 μg of 6×His-PTD-TopoNs was required to delivery 1 μg DNA which was much lower than K-Antp (12 μg peptides to deliver 1 μg DNA) [52].

Toxicity is always the major criterion and needs to be considered when designing a gene delivery system. The cytotoxicity of a gene delivery system is cell type-dependent [53] and different cell types were evaluated in this study. A possible concern is that cytotoxicity could be caused by elevated levels of hTopoI. But this topological poison to human cells is from the 3′-terminus of hTopoI [54]. *in vitro* applications of 6×His-PTD-TopoNs showed that different cell types have different responses to the carrier protein (Figs. 6 and 7), and 3T3 and GF-1 cells are resistant (>80% proliferation at different concentrations of exogenous peptides and lipids) to exogenously peptides or lipids. COS7 is more sensitive to 6×His-PTD-TopoNs,
especially 10 µM 6×His-pep1-TopoN (50% proliferation), which had the same cytotoxicity as
12 µl lipofectamine (55% proliferation) at low concentration. The elevated sensitivity of
COS7 cells to 6×His-PTD-TopoNs might be due to the TopoN domain. COS7 and TopoN
both originate from primates and might influence transfection. This might also explain the
superior transfection efficiency in COS7 cells (Fig. 5) as compared to 3T3 and GF-1 cells
(data not shown).

The mechanism of cellular uptake of PTD is controversial. One hypothesis posits that
the cell-penetrating peptide that delivers molecules into cells is independent of temperature
and does not require energy or receptors. In this scenario, PTD-mediated gene delivery is
probably through the non-endosomal pathway [21, 55–58]. More recent hypotheses include
the artifactual uptake of peptides upon even mild cell fixation [59], improved endosomal
escape as the result of photochemical reactions initiated by photosensitization of compounds
localized in endocytic vesicles, which induces rupture of these vesicles upon light exposure
[60], ATP- and temperature-dependent involvement of endocytosis [59, 61], which was
subsequently identified as macropinocytosis [24, 25, 62], clathrin-dependent endocytosis [61]
and endosomal acidification [63]. Here, experiments conducted with cells that were
pre-incubated for 1 h at 4 °C [43] or with 10 mM sodium azide and 6 mM 2-deoxy-D-glucose
to deplete cellular ATP [59] revealed that the cellular uptake of 6×His-PTD-TopoNs/DNA
complexes was energy- and temperature-dependent (Fig. 8), implicating
6×His-PTD-TopoNs-mediated gene delivery through the endosomal pathway.

The 6×His-PTD-TopoNs developed in this study not only provide a DNA delivery system but also allow the easy and inexpensive preparation of protein. Re-folding is not necessary for purification of 6×His-PTD-TopoNs because there was no difference in DNA binding and transfection efficiency between soluble and 8M urea-denatured forms (Fig. 10).

6×His-PTD-TopoNs were expressed in *E. coli*, which lacks posttranslational modification machinery, and were functionally active, negating the necessity of posttranslational modifications for biological functions of the proteins. Moreover, the absence or presence of a hexahistidine tag did not influence the DNA binding ability of PTD-TopoNs (Fig. 9), which simplifies PTD-TopoNs production, since removal of the hexahistidine tag is unnecessary.

Presence of a hexahistidine tag at the N-terminus might not affect the functional C-terminal peptide sequences, and even can increase the transmembrane ability of PTD-TopoNs (Fig. 9). Aa arginine-rich basic PTD domain is the common use for DNA delivery. However, a histidine-rich PTD also has the same cell membrane penetrating ability [66]. This may answer the question why PTD-TopoNs with N-terminal poly-histidine can have a higher transfection efficiency than PTD-TopoNs.
5. Conclusion

This study demonstrates a DNA delivery system by a fusion protein containing a PTD and TopoN. PTD can cross biological membranes independent of transporters or specific receptors. TopoN can bind to DNA regardless of DNA size and topology, and contains five NLSs that lead the protein to the nucleus. In addition, this protein can deliver biological active DNA into different cells (3T3, GF-1 and COS7).
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References


[40] Morris MC, Chaloin L, Choob M, Archdeacon J, Heitz F, Divita G. Combination of a new generation of PNAs with a peptide-based carrier enables efficient targeting of cell


Gentry, AC, Juul, S, Veigaard, C, Knudsen, BR, Osheroff, N. The geometry of DNA supercoils modulates the DNA cleavage activity of human topoisomerase I. Nucl Acid Res 2010; Doi:10.1093/nar/gkq822.


Gupta B, Levchenko TS, Torchilin VP. Intracellular delivery of large molecules and


[63] Richard JP, Melikov K, Brooks H, Prevot P, Lebleu B, Chernomordik LV. Cellular uptake of unconjugated TAT peptide involves clathrin-independent endocytosis and


**Figure captions**

**Fig. 1.** Schematic structure of plasmids used in protein transduction. The constructions were inserted into the pET15b system and the expression was driven by T7 promoter. 

- **A.** 6×His-pep1-TopoN with hexahistidine tag at N-terminus; **B.** 6×His-pep2-TopoN with hexahistidine tag at N-terminus; **C.** pep1-TopoN without hexahistidine tag; **D.** 6×His-TopoN with hexahistidine tag at N-terminus. T7, T7 promoter; 6xHis, hexahistidine tag; pep1, PTD domain pep1 (KETWWETWWTEW); pep2, PTD domain pep2 (KETWFETWFTEW); S, a spacer domain (SQPGR); TopoN, N terminal human topoisomerase I (3–200 amino acids.). The NLSs are located in positions 59–65, 117–146, 150–156, 174–180 and 192–198 [32, 55].

**Fig. 2.** Protein expression by pET system and storage. 

- **A.** IPTG induction of 6×His-PTD-TopoN for 3 hours in *E. coli* BL21(DE3) and separation by 12% SDS-PAGE with Coomassie Brilliant Blue staining. **B.** Western blotting assay. Rabbit anti-PTD antibody was used to identify 6×His-PTD-TopoN (6×His-pep1-TopoN and 6×His-pep2-TopoN). M, protein marker; P, purified protein. Stored 6×His-pep1-TopoN was separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lanes 1–3, stored at 4 ºC; lanes 4–6, stored at -20 ºC; lanes 7–9, stored at -80 ºC for 3 weeks (C) and 8 months (D). 

- **E.** 6×His-pep-1-TopoN was stored at 37 ºC for 0.5 h (lanes 1–3), 1 h (lanes 4–6) and 2 h (lanes 7–9).

**Fig. 3.** PTD-TopoN binding to the circular form of DNA. Gel retardation electrophoresis
assay on the binding ability of 6×His-PTD-TopoNs (6×His-pep1-TopoN and 6×His-pep2-TopoN) to different sized plasmids. A. pBSK⁺ (~3.0kb); in molar ratio of 5:1, 10:1, 20:1 and 40:1 (protein: DNA), the amount of protein is 9.125×10⁻²μg, 1.825×10⁻¹μg, 3.65×10⁻¹μg and 7.3×10⁻¹μg, respectively. B. pCMV-Mx-egfp-N1 (~8.0kb); in molar ratio of 5:1, 10:1, 20:1 and 40:1 (protein: DNA), the amount of protein is 3.5×10⁻²μg, 7.0×10⁻²μg, 1.4×10⁻¹μg and 2.8×10⁻¹μg, respectively. C. pEGFP-N1 (4.7kb); in molar ratio of 5:1, 10:1, 20:1 and 40:1 (protein: DNA), the amount of protein is 5.8×10⁻²μg, 1.16×10⁻¹μg, 2.32×10⁻¹μg and 4.64×10⁻¹μg, respectively. Different molar ratio (5:1, 10:1, 20:1 and 40:1) of 6×His-PTD-TopoN and plasmid DNA (6×His-PTD-TopoN:DNA). 1.3 μg of bovine serum albumin (BSA, molar ratio = 40:1) was used as a control protein. 5.8×10⁻¹μg protease K (ProK, molar ratio = 40:1) was added with 6×His-PTD-TopoN and plasmid DNA as control.

M, DNA marker. C, plasmid only as control.

**Fig. 4.** 6×His-pep1-TopoN binding to the linear form of DNA. Gel retardation electrophoresis assay of 6×His-pep1-TopoN to two different sizes of the linear form of DNA. A. pBSK⁺ (~3.0kb); in molar ratio of 10:1, 20:1 and 40:1 (protein: DNA), the amount of protein is 1.825×10⁻¹μg, 3.65×10⁻¹μg and 7.3×10⁻¹μg, respectively. B. pF4.1Xhyg (~8.9kb); in molar ratio of 10:1, 20:1 and 40:1 (protein: DNA), the amount of protein is 6.205×10⁻²μg, 1.241×10⁻¹μg and 2.482×10⁻¹μg. Different molar ratio (10:1, 20:1 and 40:1) of 6×His-pep1-TopoN and plasmid DNA (6×His-pep1-TopoN:DNA). 1.3 μg of bovine serum
albumin (BSA, molar ratio = 40:1) was used as a control protein. M, DNA marker. C, linear form DNA.

**Fig. 5.** PTD-TopoN-mediated DNA delivery into mammalian cells. A. 6×His-PTD-TopoNs (6×His-pep1-TopoN and 6×His-pep2-TopoN) concentration-dependent DNA delivery. Both DNA (1 μg of pGL3-promoter [5kb]) were incubated with different concentrations of 6×His-PTD-TopoNs from 0.05 mM (ratio 5:1) to 5 mM (ratio 40:1), in serum-free cell culture medium for 2 h. In molar ratio of 5:1, 10:1, 15:1, 20:1, 25:1 and 30:1 (protein: DNA), the amount of protein is 5.5×10⁻²μg, 1.1×10⁻¹μg, 1.65×10⁻¹μg, 2.2×10⁻¹μg, 2.75×10⁻¹μg and 3.3×10⁻¹μg, respectively. Following this transfection step, fresh DMEM supplemented with serum was added for another 48 h. Cells were then extensively washed and examined by luciferase activity. B–G. Comparison of lipofectamine and 6×His-pep-1-TopoN on delivery of pEGFP-N1 into COS7 cells. B and E. COS7; C and F. Lipofectamine with pEGFP-N1; D and G. 6×His-pep1-TopoN with pEGFP-N1 (10:1). B, C and D were excited with 490 nm light and merged with bright field images. E, F and G were excited with 490 nm light. Bar = 20μm. *p < 0.005 when compared the transfection efficiency of 6×His-pep1-TopoN to 6×His-pep2-TopoN.

**Fig. 6.** Cell proliferation assays of exogenous proteins and lipids. A. GF-1. B. 3T3. C. COS7. The amount of lipofectamine (total volume of 0, 12, 24 and 48 μl per experiment) reflected the manufacture’s recommendation. For the other added peptides or protein (group a), the
concentrations were 0, 10, 100 and 1,000 μM. *p < 0.005 when compared the cytotoxicity of lipofectamin to grouper a protein.

**Fig. 7.** Cytotoxicity of PTD-TopoNs to cells. 3T3 and COS-7 cells were incubated with 1 μM to 1 mM of 6×His-PTD-TopoNs at 37 ºC in DMEM supplemented with 10% FCS. A. 6×His-PTD-TopoNs only. B. 6×His-PTD-TopoNs with pEGFP-N1 (6×His-pep1-TopoN: DNA = 15:1; 6×His-pep2-TopoN: DNA = 20:1). Cytotoxicity was assessed using MTT. *p < 0.005 when compared the cell viability of control group to other experiment groups.

**Fig. 8.** PTD-TopoNs-mediated gene delivery is energy- and temperature-dependent. COS7 cells were incubated for 2 h in the presence of 6×His-pep1-TopoN/DNA and 6×His-pep2-TopoN/DNA complexes formed at a molar ratio of 15:1 and 20:1, respectively, and 1 μg pGL3-Promoter plasmid encoding the reporter gene luciferase was added. Cells were pre-incubated for 1 h at 4ºC, or with 10 mM sodium azide and 6 mM 2-deoxy-D-glucose to deplete cellular ATP. *p < 0.005 when compared the ATP depletion group to control group (37ºC). #p < 0.01 when compared the 4ºC group to control group (37ºC).

**Fig. 9.** Effect of hexahistidine tag on transfection activity. COS7 cells were incubated for 2 h in the presence of 6×His-pep1-TopoN/DNA complexes (molar ratio = 15:1) and with 1 μg pGL3-Promoter plasmid encoding the reporter gene luciferase. #p < 0.01 when compared the transfection efficiency of 6×His-pep1-TopoN to pep1-TopoN.

**Fig. 10.** Effect of soluble and denatured pep1-TopoN on DNA binding. A. Gel retardation
electrophoresis assay of soluble and denature forms of 6×His-pep1-TopoN mixed with pCMV-Mx-EGFP-N1 (~8.0 kb). Different molar ratios (10:1, 20:1, 40:1, 80:1 and 100:1) of 6×His-pep1-TopoN and plasmid DNA (6×His-pep1-TopoN:DNA) were used. In molar ratio of 10:1, 20:1, 40:1, 80:1 and 100:1 (protein: DNA), the amount of protein is 7.0×10⁻² μg, 1.4×10⁻¹ μg, 2.8×10⁻¹ μg, 5.6×10⁻¹ μg and 7.0×10⁻¹ μg, respectively. B. A 15:1 molar ratio of 1 μg of 6×His-pep1-TopoN and pGL3-promoter plasmid were mixed and transfected to COS-7 cells. After 48 h, the cell lysate was collected to analysis the activity of luciferase. 3.25 μg of bovine serum albumin (BSA, molar ratio = 100:1) was used as a control protein. M, DNA marker. *p < 0.005 when compared the soluble or denature form of 6×His-pep1-TopoN to native form.
Fig. 1
Fig. 2

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Fig. 4

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[Images of gel electrophoresis results for A and B]
Fig. 5

A

B

C

D

E

F

G
Fig. 6

The diagram shows the relationship between the amount of lipofectamine (μl) and proliferation (%) of cells at varying concentrations of group a protein (μM). The x-axis represents the amount of lipofectamine (μl) with values at 0, 12, 24, and 48 μl. The y-axis represents proliferation (%) with values ranging from 0 to 120. The concentration of group a protein (μM) is shown on the x-axis with values at 0, 10^1, 10^2, 10^3, and 10^4 μM. The different symbols and markers represent different treatments, including 6xHis-pep1-TopoN, 6xHis-pep2-TopoN, 6xHis-TopoN, DSA, and Lipofectamine.
Fig. 8

![Bar chart showing luciferase activity (RLU/mg) at different temperatures.

- 37°C: 6×His-pep1-TopoN (white bar) with error bars, 6×His-pep2-TopoN (black bar) with error bars.
- ATP depletion: 6×His-pep1-TopoN (white bar) with error bars, 6×His-pep2-TopoN (black bar) with error bars.
- 4°C: 6×His-pep1-TopoN (white bar) with error bars, 6×His-pep2-TopoN (black bar) with error bars.

Key:
- # indicates statistical significance with p < 0.05.
- * indicates statistical significance with p < 0.01.
Fig. 9

![Graph showing relative fold change for different samples. The x-axis represents different samples: pep1-TopoN, 6xHis-pep1-TopoN, 6xHis-TopoN. The y-axis represents relative fold change from 0 to 3. The graph indicates a significant increase in relative fold change for 6xHis-pep1-TopoN, as indicated by a hash symbol (#).](image-url)
Fig. 10

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B  

Relative fold change

6xHis-TopoN  | Soluble | Denature |
6xHis-pep1-TopoN
Supplementary Data

Supplemental Table 1. The protein sequences used in this study.

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*Letters in red indicates the hexahistidine (6×His) tag at the N terminus of 6×His-pep1-TopoN; letters in purple indicates the pep1 sequences; letters in blue indicates the spacer domain; letters in orange indicates the N-terminal (3–200 amino acids.) domain of human topoisomerase I; letters in dark blue indicates the NLSs.