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Validation of Heterodimeric TAT-NLS Peptide as a Gene Delivery Enhancer

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Cationic liposomes have been actively used as gene delivery vehicles despite their unsatisfactory efficiencies because of their relatively low toxicity. In this study, we designed novel heterodimeric peptides as nonviral gene delivery systems from TAT and NLS peptides using cysteine-to-cysteine disulfide bonds between the two. Mixing these heterodimeric peptides with DNA before mixing with lipofectamine resulted in higher transfection efficiencies in MCF-7 breast cancer cells than mixing unmodified TAT, NLS, and a simple mixture of TAT and NLS with DNA, but did not show an adverse effect on cell viability. In gel retardation assays, the DNA binding affinities of heterodimeric peptides were stronger than NLS but weaker than TAT. However, this enhancement was only observed when heterodimeric peptides were premixed with DNA before being mixed with lipofectamine. The described novel transfection-enhancing peptide system produced by the heterodimerization of TAT and NLS peptides followed by simple mixing with DNA, increased the gene transfer efficiency of cationic lipids without enhancing cytotoxicity.

Keywords: Cell-penetrating peptide, genetic therapy, liposomes, transfection

Introduction

To overcome the limits of conventional therapeutic modalities, gene therapy has been conducted to treat many diseases, including cancer [1, 14] and heart failure [31, 43]. However, because nucleic acids have the anionic charge and the targets of therapeutics are located around the nucleus, the efficient delivery of therapeutic drugs into cells is more of an issue for nucleic acid drug development than conventional drug development. Nonviral vectors, such as cationic liposomes and polymers, are considered safer tools than viral vectors [48], but the relatively low efficiency of gene delivery is the major drawback of these vectors [47].

Cell-penetrating peptides (CPPs) have the ability to carry "cargo" across the plasma membrane into the cytoplasm and nucleus, and this property has been used for various purposes, including the deliveries of plasmid DNA [15, 16] and antisense oligonucleotides [3] using TAT protein encoded by HIV-1 [11, 12] or the nuclear localization sequence (NLS) of SV-40 [22]. More recently, several peptides, such as antennapedia (Antp) [17], transportan [32], and pVEC [8], were found to be CPPs, and although direct penetration and endocytosis have been proposed as mechanisms [21], the actual mechanisms involved have yet to be elucidated. TAT peptide has been shown to promote the intracellular uptake of cargos [20], and the enhancement of nuclear uptake is considered as the major role of NLS of SV-40 [33]. Here, we designed a heterodimeric peptide to take advantage of these different characteristics of two CPPs. CPPs have been used in several studies to overcome the low efficiencies of liposome-mediated transfections [15, 29, 39], and some have shown cysteine is needed for the efficient transfection of DNA [24, 27]. Addition of terminal cysteines to lysine-rich peptides condensed DNA stably [26], and disulfide bond formation was also found to increase the stability of DNA-peptide interactions [25, 30]. In addition, several studies have shown cysteine residue incorporation into TAT increases liposome-mediated transfection more efficiently than unmodified TAT [24, 27, 47]. These different conflicting results are probably due to the contradictory requirement between the ability of the

gene delivery carrier to protect nucleic acids from the extracellular space and the ease of release of nucleic acids in the cytoplasm [28]. Bioreducible polycation methods such as those based on the use of a disulfide linker have been widely reported to provide this balance between these requirements. Disulfide linkers have been used for drug bioconjugation [28, 44] to take advantage of the biological redox potential difference between the extracellular environment and the intracellular space. Glutathione (GSH) is known to play a critical role in this redox gradient, and although extracellular GSH concentrations are in the micromolar range, its intracellular concentrations are a thousand times greater [10, 18]. Furthermore, amongst intracellular organelles, the nucleus has the strongest reducing environment [2, 4, 45], and the redox active thiols of proteins on cell surfaces can cause the disulfide reduction of bioreducible carriers during cell membrane penetration [9, 36, 37].

In the present study, we designed TAT/NLS heterodimeric peptides by generating disulfide links in the hope of developing an efficient, safe nonviral gene delivery system. The efficacies of the peptides produced were evaluated in the MCF-7 breast cancer cell line by measuring luciferase and GFP gene expression. In addition, physical interactions between these peptides and DNA were investigated using a gel retardation assay.

Materials and Methods

Cell Line and Cell Culture

MCF-7 cells (a human breast cancer cell line) were obtained from the Korean Cell Line Bank. Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and maintained in a humidified incubator in 5% CO_2 at 37°C. The cell culture reagents, including FBS, were purchased from WelGENE (Korea).

Peptide Synthesis and Modification

The peptides used in the present study were derived from the TAT peptide of HIV-1 and SV-40 NLS peptide. The TAT peptide corresponds to the 12-amino-acid (RKKRRQRRRPPQ) nuclear localization signal sequence of HIV-1, and the 13-amino-acid peptide NLS (CGYGPKKKRKVGG) was derived from SV-40. These two peptides were modified at either their N- or C-termini by the addition of cysteine (Table 1). All peptides described in this study were synthesized by Peptron, Inc. (Korea) using the Fmoc-SPPS solid phase method and purified by reverse phase HPLC using a Vydac Everest C18 column (250 mm × 22 mm, 10 μm). Elution was carried out with a water-acetonitrile linear gradient (3~40% (v/v) of acetonitrile) containing 0.1% (v/v) trifluoroacetic acid. To form homodimeric peptides, each monomeric peptide was dissolved in 0.1 M ammonium bicarbonate in acetonitrile/water (1:1 (v/v)) (1 mg/ml). Reaction mixtures were stirred under ambient conditions until the reactions were complete. Reactions were monitored using the Ellman test [6] and LC/MS. Subsequently, the reaction mixtures were lyophilized and purified by reverse-phase HPLC. Average homodimeric conjugation yields were over 80% as determined by HPLC (data not shown). The molecular weight of the purified peptide was confirmed by LC/MS (Agilent HP1100 series; USA). Purified peptides were resuspended at 10 μg/μl in distilled water and stored at −70°C for future use.

Formation of Complex and Transfection

pcDNA-Luc containing the firefly luciferase reporter gene and the plasmid coding for green fluorescent protein (pCMVTnT-GFP) were purchased from Welgene (Korea). DNA plasmids were amplified in *Escherichia coli* XL1-Blue strain and purified using a Maxi-kit (Qiagen Inc., USA). DNA purities were determined by agarose gel electrophoresis and by measuring optical densities (OD). DNAs having $OD_{260}/OD_{280} \geq 1.8$ were used in this study. pcDNA-Luc and pCMVTNT-GFP were used at a concentration of 0.2 μg/well, unless specified otherwise. Plasmid DNAs, peptides, and liposome solution were prepared in Transfection Optimizing Medium (TOM; WelGene, Korea) in a volume of 50 μl; plasmid DNAs were first mixed with peptides. After incubating DNA:peptide complexes for 10 min, we mixed the complexes with liposome and incubated them for 15 min at room temperature. Twenty-four hours prior to transfection, the cells were transferred to 48-well culture plates at a density of 40,000 cells/well. Thirty minutes before transfection, the medium was removed and cells from each well were briefly washed with 100 μl of sterile phosphate-buffered saline (PBS). TOM (150 μl) was then added to each well, followed by 150 μl of DNA:peptide:lipid complex, and the plates were incubated for 4 h. An additional 300 μl of medium (20% FBS) was then added to each well to achieve a final serum concentration of 10%, and plates were incubated for a further 24 h. Commercial liposomes were also tested within the range allowed by the manufacturer's protocol in order to identify optimal conditions.

Table 1. List of peptides.

Name	Sequence	Number	Modification	Molecular weight
TAT	RKKRRQRRRPPQ	12		1,661
NLS	CGYGPKKKRKVGG	13		1,377
TN-CTHtD	RKKRRQRRRPPQC, GYGPKKKRKVGGC	26	C-Terminal disulfide heterodimer	3,122
TN-NTHtD	CRKKRRQRRRPPQ, CGYGPKKKRKVGG	26	N-Terminal disulfide heterodimer	3,122

Luciferase Assay and GFP Expression

Twenty-four hours after transfection, the media were aspirated and the wells were washed twice with 200 μl of ice-cold PBS. To each well, 100 μl of 1× reporter lysis buffer (Promega Corp., USA) was added and cells were then lysed for 1 h in an ice tray. Lysates were placed in Eppendorf tubes and centrifuged (15,000 ×*g*, 4°C) for 5 min. The supernatants were then transferred to Eppendorf tubes in ice and subjected to luciferase and protein assays. Then 20 μl of a cell lysate was transferred to a white opaque 96-well plate and the luciferase activity was assessed directly using an LMax II 384 luminometer (Molecular Devices Corp., USA) and a luciferase assay kit (Promega Corp., USA). Protein contents were quantified using a bicinchoninic acid (BCA) assay (Pierce, USA) according to the manufacturer's instructions. Briefly, an aliquot of cell lysate (40 μl) was mixed with 1 ml of BCA reagent in an acrylic cuvette and incubated for 1 h at 37°C. Light absorption was then read at 562 nm using a DU-600 spectrophotometer (Beckman Coulter, USA), and the protein content was determined versus bovine serum albumin standards. Luciferase activities were normalized with respect to protein contents and expressed in relative luminescence units per microgram of protein (RLU/μg protein). To observe GFP expression, 24 h after transfection, the medium was removed and cells were rinsed twice with PBS. Fluorescence protein was observed under a Nikon ECLIPSE TE300 fluorescence microscope (Japan).

Gel Retardation Assay

For agarose gel retardation assays, 0.25 μg of pcDNA-Luc was mixed with peptide and/or cationic lipid at various concentrations. DNA:peptide or DNA:peptide:lipid complex solution (10 μl) was mixed with 2 μl of 6× loading buffer and loaded onto a 1% agarose gel containing ethidium bromide. Electrophoresis was carried out for 45 min in 1× TBE running buffer. Images were taken using an UV light illuminator.

Cell Viability Assay

According to the transfection protocol described above, 24 h after transfection, 20 μg of MTT in 20 μl of PBS was added to each well and incubated for 3 h. The media were carefully removed and 200 μl of dimethyl sulfoxide (DMSO) was added to each well. The formazan crystals produced were then completely dissolved in DMSO and absorbance was determined at 550 nm. Cells treated with only pcDNA-Luc solution without liposome were used as OD controls. Cell viability was calculated using the following equation:

Cell viability (%) = OD sample/OD control × 100

Results and Discussion

TAT-NLS Heterodimeric Peptides Enhanced the Transfection Efficiency of Cationic Lipid Without Inducing Significant Cytotoxicity

The modified heterodimer derived from HIV-1 TAT and

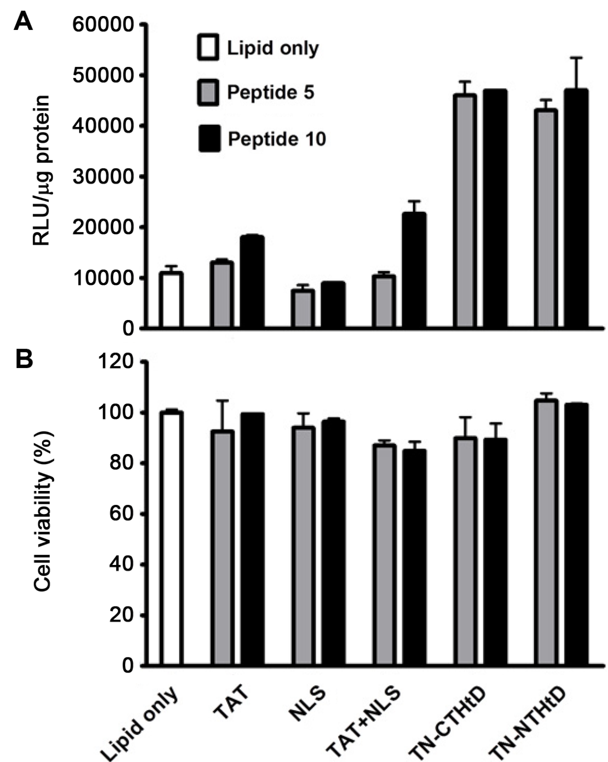

Fig. 1. Effects of peptides on transfection efficiency and cell viability.

Luciferase activities (**A**) and cell viabilities (**B**) were measured in MCF-7 breast cancer cells 24 h after transfection. DNA was used at a concentration of 0.2 μg per well in 48-well culture plates, and the cationic lipid (Lipofectamine) was mixed at a weight ratio of 1:5 (DNA:lipid). Peptides were mixed at ratios of 1:5 or 1:10 (DNA:peptide). Cell viabilities were determined using an MTT assay. The results shown represent the mean ± SD of triplicate experiments. (TAT+NLS, simple mix of TAT and NLS peptide; TN-CTHtD, TAT-NLS C-terminal heterodimeric peptide; TN-NTHtD, TAT-NLS N-terminal heterodimeric peptide)

SV40 NLS peptide by disulfide bond formation was designed to develop a peptide with an enhanced ability to transfect DNA. The addition of heterodimers (TN-CTHtD, C-terminal disulfide heterodimer; TN-NTHtD, N-terminal disulfide heterodimer) to plasmid DNA before mixing with cationic lipid increased the transfection efficiency by over 4 fold as compared with lipid only (Fig. 1A). In addition, this enhancement of transfection efficiency was obviously greater than those of TAT peptide, NLS peptide, and simple mixture of TAT and NLS. CPPs have been known to enhance cellular uptake and the nuclear entry of nucleic acids and proteins. SV40 NLS peptide is known as a more classical type of nuclear localization signal, which binds to importin α to form a complex with importin β to achieve

nuclear transport [13], whereas TAT peptide translocates cargoes into the nucleus by passive diffusion through nuclear pore complexes or by conjugation with importin β [40]. In this study, these two different mechanisms could make synergy in the cell during the gene delivery process. In addition, the combination of cationic lipid and terminal cysteine modified TAT showed more efficient DNA transfection than cationic lipid alone [27, 47]. The intracellular unpacking of DNA is considered an important barrier to efficient gene delivery [38], and intracellular disulfide reduction provides the rationale for bioreducible carriers [28]. Several recent studies on the use of the disulfide bond in gene delivery have reported efficient gene deliveries [5, 46], and others have shown that artificial increases in cellular GSH enhance the transfection efficiencies of bioreducible carriers [7, 34].

To develop optimal delivery vectors for nucleic acid therapeutics and for the transfection of DNA in basic research, safety must be borne in mind to avoid unwanted toxicities. Therefore, in the present study, we measured the effects of heterodimeric peptides on cell viability, although CPPs have been previously reported not to confer additional toxicity [39, 41]. We found the heterodimers produced did not exhibit any significant cytotoxicity under the experimental conditions used (Fig. 1B). Although I could not find additional cytotoxicity up to a peptide ratio of 20, the transfection efficiency showed a plateau over the peptide ratio of 10 (data not shown).

Transfection-enhancing effects of TN-CTHtD and TN-NTHtD were also investigated by observing GFP expression by fluorescence microscopy after transfecting MCF-7 cells with plasmid DNA expressing GFP. It was observed that the numbers of GFP-positive cells were markedly higher in wells transfected with the heterodimers and lipid (Fig. 2).

In another study, in which TAT modified by cysteine addition was used, DNA and peptide were mixed and stabilized by bubbling air [47]. The authors expected that the disulfide bonds established in the DNA and peptide would stabilize the complex and protect the DNA. In the present study, DNA and peptides were simply mixed before mixing with the cationic lipid, and despite this simplicity, satisfactory results were achieved. Furthermore, in line with other studies [27, 47], no meaningful transfection occurred when DNA and modified peptide without cationic lipid were used (data not shown).

Interaction Between DNA and Peptides

To investigate the nature of the interaction between DNA and all peptides used, DNA retardation was measured in agarose gel for the DNA:peptide and DNA:peptide:lipid complexes at different DNA:peptide ratios. The mobility of plasmid DNA was reduced by increasing the peptide. This result shows the binding of TN-CTHtD and of TN-NTHtD with plasmid DNA was efficient, but the binding power

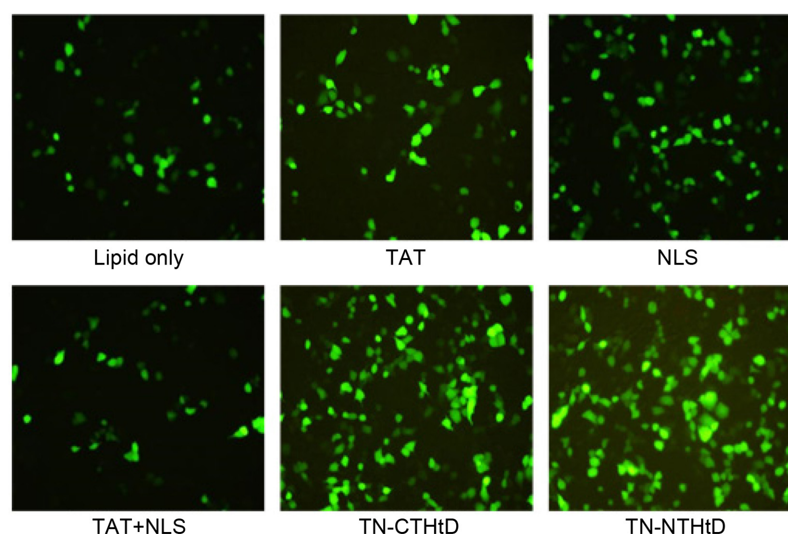

Fig. 2. Enhancement of GFP expression by TAT-NLS heterodimeric peptides.

pCMVTNT-GFP plasmids were transfected with DNA:peptide:lipid or DNA:lipid (Lipid only). The concentration of DNA was 0.2 μg per well in the 48-well culture plate, and peptides were mixed at a ratio of 1:5 (DNA:peptide) before mixing DNA with lipid (Lipofectamine) at a ratio of 1:5 (DNA:lipid). Green fluorescent protein was observed under a fluorescence microscope (×200). (TAT+NLS, simple mix of TAT and NLS peptide; TN-CTHtD, TAT-NLS C-terminal heterodimeric peptide; TN-NTHtD, TAT-NLS N-terminal heterodimeric peptide)

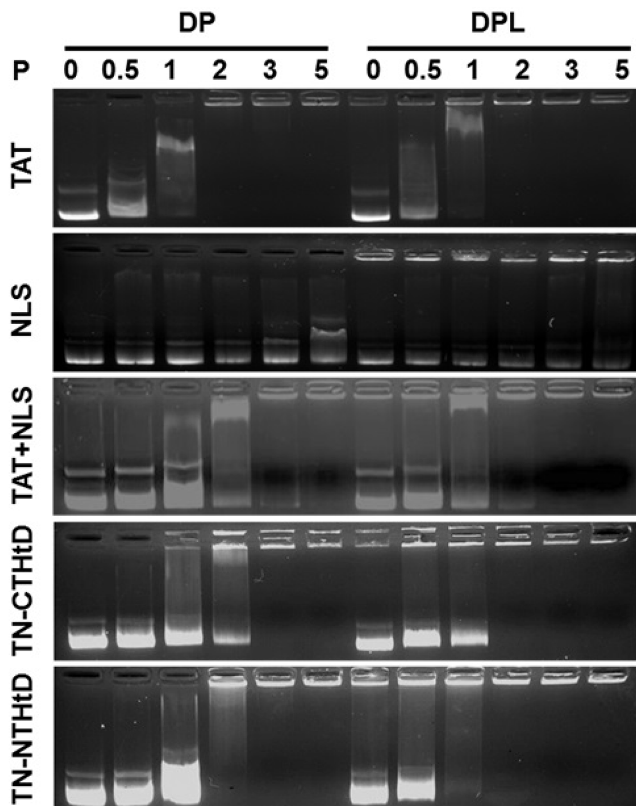

Fig. 3. DNA binding abilities of peptides.
Binding of peptides with DNA (pcDNA-Luc) was assessed using an agarose gel retardation assay. DNA:peptide and DNA:peptide:lipid complex solutions mixed at various ratios were loaded onto a 1% agarose gel containing ethidium bromide. Images were taken using an UV light illuminator.

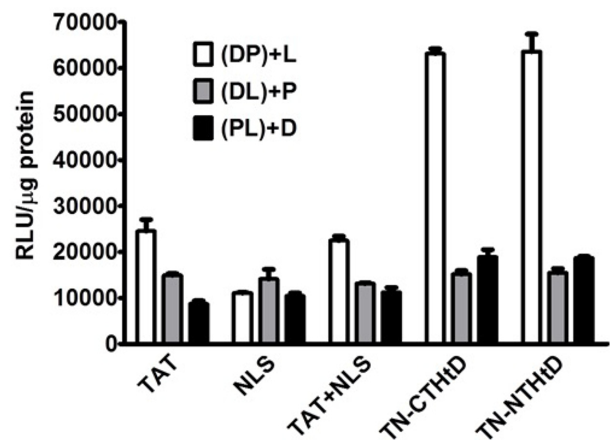

Fig. 4. The effects of mixing order on transfection efficiency and the transfection-enhancing effects of peptides on other cationic lipids.
The transfection complexes were formed by mixing the two components shown in parentheses and then mixing with the third component shown outside the parentheses. Luciferase activities were measured in MCF-7 breast cancer cells one day after transfection. The results shown represent the mean ± SD of experiments performed in triplicate. (D, DNA; P, peptides; L, cationic lipids)

was not much stronger than other peptides (Fig. 3). Actually, the DNA binding affinities of heterodimeric peptides were stronger than NLS but weaker than TAT. In terms of protecting nucleic acids, DNA-to-peptide binding is important. In the previous results, binding affinity was well correlated with transfection efficiency [19, 23]. However, the DNA binding results of this study suggest that the release of nucleic acids in the cytoplasm may be a more important factor for efficient gene expression than protecting nucleic acids. The bioreducible property made by disulfide bonds might facilitate the release of DNA into the cytoplasm during trafficking of lipoplex, and the enhanced transfection efficiency by novel heterodimeric peptides reflects this advantage.

In addition, we examined whether the mixing order affected the transfection efficiency. Luciferase assay results indicated that DNA (D) and peptides (P) should be complexed before adding cationic lipids (L) to increase the transfection efficiency, as compared with other mixing methods such as (DL)+P or (PL)+D (Fig. 4). This result is in accord with previous studies [27, 35], and shows that the DNA/peptide interaction critically determines the transfection efficiency. Furthermore, these results and gel retardation assay results demonstrate the importance of the interaction between the carrier and DNA.

I believe that the nuclear localizing action of these peptides starts after the DNA:peptide:lipid complex enters the cell, as has been suggested by others [35, 47]. It has been proposed that the enhancement of transfection efficiency by modified TAT is caused by increased endocytosis and endosomal escape [42]. This approach using a heterodimeric peptide with cationic lipids may pave the road for the application of various combinations, including targeting peptides for tissue or cell specific gene delivery.

In summary, we produced novel transfection-enhancing peptides by heterodimerization of TAT and NLS peptides. Simple mixing of these novel peptides with DNA increased the gene transfer of different cationic lipids without increasing cytotoxicity.

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