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Novel Branching Membrane Translocational Peptide as Gene Delivery Vector

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Abstract—A fragment of HIV-tat protein, RKKRRQRRR, has been shown to have membrane penetration and nuclear localization properties, which are critical attributes of gene therapy agents. In this study, we designed a series of arborizing tat peptides, containing 1–8 tat moieties, and evaluated them as transfection enhancers in a variety of cell lines. We found that all compounds complexed with plasmid DNA, but only the molecule containing 8 tat-peptide chains shows significant transfection capabilities. Using rhodamine labeled plasmid and eight tat-peptide complex, we were also able to demonstrate intracellular delivery of the complex by fluorescence microscopy.

Introduction

The primary aim of gene therapy is efficient delivery of therapeutic genes to target cells. Current gene therapy technology has focused primarily on the use of viral vectors, which provide highly efficient transduction and high levels of gene expression,[1] but can be cytotoxic and immunogenic.[2,3] An alternative to virus-based delivery is the use of synthetic non-viral systems which may be less immunogenic. A variety of nonviral gene therapy carriers have recently been developed and tested in vitro and in vivo.[4,5] Cationic liposomes (lipoplex)[6] and cationic polymers (polyplex)[7,8] are the most commonly used synthetic vectors. Besides these, peptide-based delivery vectors have also been proposed to improve safety and efficiency of gene delivery.[9]

Peptide-based delivery vectors are designed to mimic viral proteins that facilitate the cellular delivery of viral vectors. Cell surface receptor binding peptides,[10] membrane fusion peptides,[11] endosomal lytic peptides,[12] and nuclear localization signal peptides[13,14] have all been presented as agents to improve efficiency of gene delivery. Recently, several membrane translocation signal (MTS) peptides have been reported as transporters.[15–18] These MTS cannot only cross cellular and nuclear membranes independently and efficiently, but can also carry various molecules into cells.[11,19–22] Moreover, some MTS have been attached to oligonucleotides and been used to improve the efficacy of antisense therapy.[20,23–28] The nonapeptide, $R_{49}KKRRQRRR_{57}$, from HIV-tat protein is one of the shortest peptides having membrane translocation property,[29,30] and has been used to deliver fluorochromes, chelators, peptides and even nanometer size particles into various cell types.[31–33] We thus reasoned that the MTS peptide might improve cellular uptake and nuclear localization of plasmid DNA, and therefore improve gene expression efficiency. In this study, we demonstrated that this short membrane translocational peptide, derived from HIV-tat peptide, is able to complex with plasmid DNA and that the arborizing tat molecules can be used to boost gene expression.

Materials and Methods

Peptide synthesis

All amino acids and Gly-Wang resin were purchased from Novabiochem (San Diego, CA, USA), and the 4- and 8-branch MAP resin for branched peptide synthesis was purchased from Peptide International (Louisville, KY, USA). The peptide was synthesized on an automatic peptide synthesizer (PS3, Rainin, Woburn, MA, USA) using Fmoc chemistry. The 1tat peptide,

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GRKKRRQRRRGY, was synthesized on the Gly-Wang resin. The tyrosine residue was added for convenient quantitation. Similar to the synthesis of 1tat, the 4tat and 8tat peptides were coupled directly to the 4- or 8-branch MAP resin. For synthesis of the 2tat peptide, a cycle of Fmoc-Lys(Fmoc) was first anchored to the Gly-Wang resin to generate two amino groups, followed with the addition of other amino acids. The synthesized peptides were cleaved by 5 mL of reagent R (TFA/Thioanisole/EDT/anisole: 90/5/3/2), purified by HPLC (Rainin, Woburn, MA, USA) on a reverse-phase C18 column (218TP1022, Vydac, Hesperia, CA, USA), and characterized by MALDI-TOF mass spectrum analysis

Plasmid DNA

The plasmid DNA, pCMV-Luc, used for all experiments consisted of a luciferase reporter gene driven by the cytomegalovirus (CMV) promoter. The plasmid was prepared by growing bacteria stocks in a Luria broth base (LB) medium (DIFCO, Detroit, MI, USA). The bacteria were lysed, and the plasmid precipitated and purified using the commercially available purification kit (Qiagen,Valencia, CA, USA).

Gel retardation assays

To study complexing of 1, 2, 4, and 8tat peptides with Luciferase DNA, the peptide and DNA (1 μg) were mixed with charge ratio of positive to negative charges at 0, 0.25, 0.5, 1, 2, 4, 8 and 16, and brought to a volume of 20 μL with 20 mM HEPES Buffer. The concentrations of peptide and DNA were determined by a molecular absorption measurement. Nine positive charges were counted on each tat peptide arm. The DNA charge was determined using 1 $O.D._{260} = 50$ μg, and molecular weight of each nucleotide = 330 Da. Thirty minutes after incubation at room temperature, 5 μL of complex was mixed with 7 μL of loading buffer (0.25% Bromophenol Blue, 40% Sucrose), and 4 μL of this mixture was loaded onto a 0.8% agarose gel. The gel was run at 100 V for 1 h in TAE buffer, soaked in 200 mL of Ethidium Bromide solution (0.5 μg/mL) for 45 min, and observed under UV light.

Transfection experiments

Four cell lines were used in the study. Cos-1, PC-3, 9L, and 3T3 were grown and maintained in RPMI1640 Medium (Cellgro, Mediatech, Washington, DC, USA), F-12K Medium (GibcoBRL, Rockville, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM, Mediatech), and DMEM, respectively, with 10% Fetal Bovine Serum (FBS, Mediatech) and 1% penicillin/streptomycin (Mediatech) in a humidified 6% CO_2 atmosphere at 37 °C. In preparation for all transfection experiments, cells were seeded in a 24-well plate (Costar, Corning, NY, USA) at 50,000 cells/well, and incubated overnight to reach 60–70% confluency. Per well, 1 μg of Luciferase and corresponding amount of peptide for the respective charge ratio (for each transfection experiment, charge ratios of 0, 0.5, 1, 2, 4, 8, and 16,± were tested) were mixed and brought to a volume of 20 μL with 20 mM HEPES Buffer. The complex was incubated at room temperature for 1 h, then brought to a volume of 250 μL with respective serum-free media for the particular cell line. The complex solution was added to the freshly washed cells [Hanks Balanced Salt Solution (HBSS), Mediatech], incubated for 1 h at 37 °C and washed and replaced with respective media (10% FBS, 1% penicillin/streptomycin). Lipofectamine (Gibco BRL) was used as a positive control, following a protocol provided by the manufacture. All transfection experiments were performed in triplicate per plate, and each plate was duplicated.

Luciferase activity was determined 24-h post-transfection by measuring RLU (Relative luciferase unit) using Luciferase Assay system (Promega, Madison, MI, USA) in a microplate Luminometer (LB 96V, EG&G, Bad Wildbad, Germany). The luciferase activity was normalized to per mg of protein (BCA assay kit, Pierce, Rockford, IL, USA).

Isolation and culture of murine cardiac endothelial cells

In order to test the possibility of transfecting non-dividing cells using the branching tat peptide, murine cardiac endothelial cells were prepared. Methods for isolation and purification of endothelial cells (EC) were modified from published protocols.[48,49] Briefly, hearts were harvested from two mice (C57Bl/6, 8–12 weeks), large vessels were removed and the tissue washed extensively in 50 mL of cold DMEM containing 20% FCS. The heart tissue was minced finely, digested in 20 mL collagenase (0.2% (w/v), Worthington, Lackwood, NJ, USA) at 37 °C for 45 min and then mechanically dissociated using a 14 G cannula and filtered through a 70-μm disposable cell strainer (Fisher). The crude EC preparation was resuspended at 3×10^6 cells/mL in Dulbecco's PBS containing Ca^{2+} and Mg^{2+}. PECAM-1-specific and ICAM-2-specific magnetic Dynal beads (Dynal Corp., Great Neck, NY, USA) were prepared according to manufacturer's instructions using either rat-anti-mouse-PECAM-1 mAb (clone Mec13.3, Pharmingen, San Diego, CA, USA) or rat-anti-mouse-ICAM-2 mAb (clone 3C4, Pharmingen) respectively. The cell suspension was incubated with the PECAM-1-specific beads at a ratio of 50 μL beads/mL cell suspension for 10 min at 4 °C with end-over-end rotation. Using a magnetic separator, the bead bound cells were recovered, washed with DMEM-20%, suspended in 12-mL complete culture medium [DMEM containing 20% FCS, supplemented with 100 μg/mL porcine heparin, 100 μg/mL endothelia mitogen (Biomedical Technologies, Sloughton, MA, USA), non-essential amino acids, sodium pyruvate, L-glutamine and antibiotics, at standard concentrations] and then plated to a gelatin-coated 75 cm^2 tissue culture flask. After incubating overnight incubation, adherent cells were washed with HBSS and fresh complete media was added. Once cells reached 70–80% confluence, they were washed three times with HBSS, detached with 2-mL trypsin-EDTA, resuspended in 1–3 mL DPBS, and incubated with ICAM-2 coated beads (35 μL/mL cells) at rt for 8 min with rotation. The bead-bound cells were washed

and plated in complete culture medium at a 1:2 split. For all experiments, EC were used at 1 day post-confluence and at subcultures 1–3.

Fluorescence labeling and microscopic studies

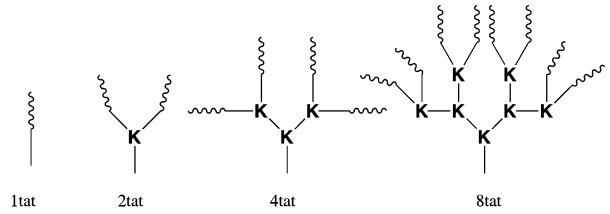
The luciferase plasmid was labeled with a Rhodamine nucleic acid labeling kit (LabelIT, Panvera, Madison, WI, USA), following a protocol provided by the distributor. The labeling was confirmed by gel electrophoresis (data not shown). Transfection was performed on PC-3 cells using 8tat and rhodamine-labeled/unlabeled DNA (0.5 μg/0.5 μg) at a charge ratio of 2 (+/−) following the protocol described earlier. Twenty-four hours post-transfection, the cells were washed three times with Hanks buffer and observed under fluorescent microscope (Axiovert 100TV, Zeiss, Thornwood, NY, USA). Fluorescence and light images were captured by a cooled CCD camera (Sensys, Photometrics, Tuscon, AZ, USA).

Results and Discussion

Design of arborizing tat molecules

Because of its unique cell penetration property, the HIV-tat peptide has been used to deliver a number of different molecules, such as fluorochromes, drugs, chelators, peptides, and proteins into cells.[22,29,32,34–39] Recently, we have used the HIV-tat peptide to introduce nanometer-sized superparamagnetic iron oxide particles into HeLa cells, murine lymphocytes, human NK cells and CD34+ progenitor cells for the purpose of studying in vivo cell trafficking.[31,33] Similar to this application, we rationalized that the tat peptide may also be useful in gene delivery, due to its ability to transport large particles through cellular membranes.

Plasmid DNA is a negatively charged molecule, while tat peptides are highly positively charged molecules. These counter charged molecules can complex efficiently by charge interaction, similar to the polylysine/DNA system.[40] However, such binding may abrogate the translocational properties of the positively charged MTS peptides. We thus designed branching tat peptides to overcome this problem (Fig. 1). When complexing branched tat peptide with the DNA plasmid, certain tat peptide chains are expected to be involved in DNA binding, while others are expected to be available for translocation. Four peptides, including monomer (1tat), dimer (2tat), tetramer (4tat) and octamer (8tat), were prepared and studied for their translocating efficiency.



1tat: GRKKRRQRRRGY-G-OH
2tat: $(GRKKRRQRRRGY)_2$-K-G-OH
4tat: $(GRKKRRQRRRGY)_4$-K_2-K-ßA-OH
8tat: $(GRKKRRQRRRGY)_8$-K_4-K_2-K-ßA-OH

Figure 1. Structure and sequences of synthesized peptides. The undulating line represents the Tat peptide. βA is β-alanine.

Peptide and DNA complex formation

One of our first goals was to determine degree of plasmid/peptide complex formation. To achieve this, luciferase DNA was complexed with various amounts of peptides to create different charge ratios, ranging from 0 to 16 (+/−). By gel electrophoresis, the retardation of plasmid was clearly seen when the peptide was mixed with DNA at low positive to negative charge ratio (Fig. 2). However, when the charge ratio was higher than 1, the DNA and peptide complexes were retained in the wells without migrating into the gel. The driving forces for DNA migration in electrophoresis are the number of negative charges and the size of complexes. More positively charged peptides bound to the plasmid would cause more retardation in the gel. If the complex is too big, it is expected to remain stationary in the well and not enter the gel matrix.

Interestingly, it was observed that the 1tat/DNA complex resulted in the highest retardation, followed by 2tat, 4tat and then 8tat. The likely explanation is that the non-branching 1tat only binds to one plasmid at a time. Each DNA plasmid can bind with multiple 1tat molecules before aggregation. As more 1tat peptide was added, DNA migrated more slowly. Once the charge ratio reached 1, the complex lost all negative charges, and/or formed aggregates. In contrast to 1tat, branching peptides were able to bind to more than one plasmid simultaneously because of their core structure. For example, the tat peptide arms on one 8tat molecule could bring up to eight plasmids together, causing large-sized aggregation even at a low overall charge ratio. Thus, when charge ratio is below one, the large sized complexes and free DNA were both observed.

Transfection in dividing cells

The transfection efficiency of the tat peptides was studied using COS-1, PC-3, 3T3 and 9L cells in the absence of chloroquine, an endosomal disrupting compound. Luciferase DNA (1 μg) was complexed with different peptides at different charge ratios as described. A typical transfection result is shown in Fig. 3. Our data showed that 1tat and 2tat did not produce greater transfection efficiency than that obtained with the naked

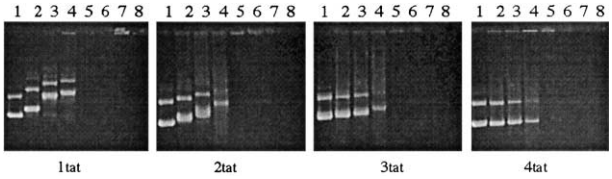


Figure 2. Gel retardation of synthesized peptides with luciferase DNA at various charges. The charge ratio (+/−) from lanes 1 to 8 are 0, 0.25, 0.5, 1, 2, 4, 8 and 16, respectively.

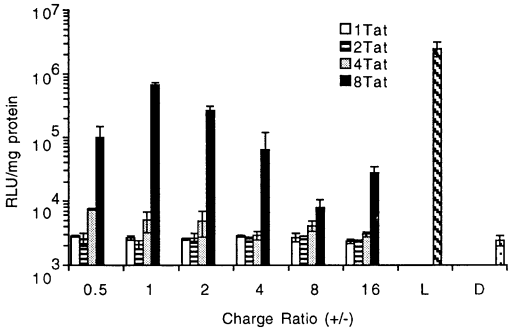


Figure 3. Transfection of COS-1 cell with different peptides at different charge ratios. L: lipofectamine, D: naked DNA control.

DNA control. The 4tat showed slightly improved efficiency. However, when 8tat was used, significantly higher luciferase activity was detected at all charge ratios, particularly at charge ratios of 1 or 2. While testing the transfection with other cell lines, same results were seen, 8tat $>>$ 4tat $>$ 2tat $=$ 1tat (Fig. 4). The optimal charge ratio was also either 1 or 2 for all cells. Transfection efficiency using lipofectamine control was slightly higher than it of 8tat in Cos-1 (7-fold) and 9L (5-fold) cells, but reverse results were seen in PC-3 (1/2-fold) and NIH-3T3 (1/2-fold) cells. The transfection results support the hypothesis that branching tat peptides are required for improved expression. The higher the branching of the molecules, the better the expression.

Transfection in nondividing endothelial cells

Based on other studies, the tat peptide is fairly nonselective in cell translocation. For example, we have shown internalization of tat peptide and its payload into many different cell types, including progenitor cells.[31–33] This phenomenon has also been seen in other tat–protein hybrid systems.[22] It is reasonable to propose that the branching 8tat peptide would be an efficient delivery vector to nondividing cells. We thus used the endothelial cells to test this concept. In this experiment, mouse cardiac endothelial cells were incubated with complexes having various charge ratios (0.5, 1, 2, 8, ±) as previously described. Similar transfection patterns were found in endothelial cells as with the previously mentioned cells, but at lower levels (Fig. 5). At charge ratio of 2, only 23,000 RLU/mg of protein was obtained; that is about 100-fold less than lipofectamine (2.8×10^6 RLU/mg).

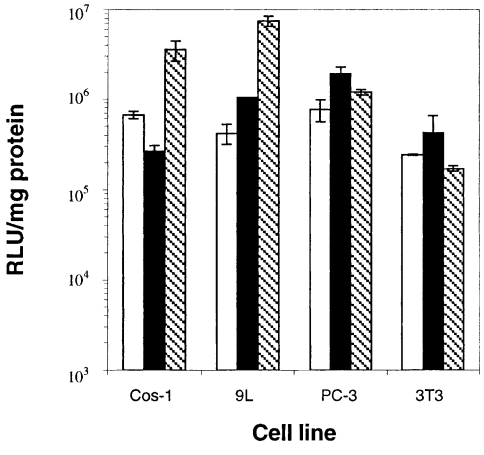


Figure 4. Transfection of different cell lines with 8tat peptide at the charge ratio of 1 (white bar), 2 (black bar) and lipofectamine control (dash bar).

Delivery of plasmid into cells

To trace the intracellular localization of the complexes, we labeled the plasmid with a rhodamine fluorescence tag. The labeled plasmid was complexed with 8tat at a charge ratio of 2 and incubated with PC-3 cells for 1 h. The internalization of the complex was monitored 24 h later, under a fluorescence microscope. The intracellular distribution of fluorescence signal was mostly found in the cytoplasm, but not in the nucleus (Fig. 6). The result indicated that the branching peptide only assisted outside cellular membrane transportation. It did not promote the nuclear delivery which is critical to gene expression. The rhodamine-labeled plasmid alone was also examined as a control. As expected, the uncomplexed plasmid was unable to transport into cells.

Recently, a nuclear localization sequence (NLS) derived from SV-40 was applied to enhance plasmid DNA delivery. As the plasmid was labeled with multiple NLS peptides, the nuclei did not take up the modified DNA.[41] However, a single NLS peptide attachment significantly improved nucleus delivery and gene expression.[13] This finding may be applied to poor nuclear delivery of the 8tat/DNA complex. The branching tat arms only seem to enhance cellular membrane transportation, but not nuclear membranes transportation.

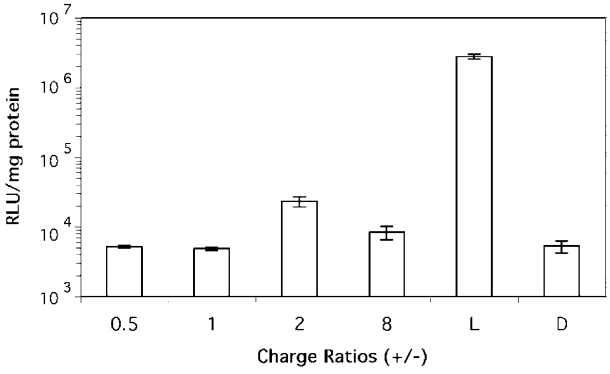


Figure 5. In vitro transfection of murine cardiac endothelial cells with 8tat peptide. L: lipofectamine, D: naked DNA control.

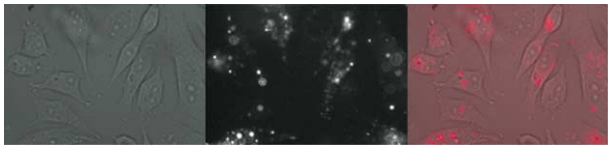


Figure 6. Microscopic localization of plasmid 8tat/DNA complex. The plasmid was labeled with rhodamine. The pictures from left to right are bright field, rhodamine fluorescence and overlapped images (20×).

Conclusion

In this study, we have shown how an engineered membrane translocation peptide can be used as a gene delivery vector. The 9-residue peptide derived from HIV-tat protein alone is inefficient as a DNA membrane translocational agent, presumably because of charge interaction with DNA. Our data demonstrated that at least eight tat peptide chains were required for this star-shaped molecule to attain efficient gene delivery property. Although the transfection efficiency is not yet ideal, further improvements are conceivable. For example, large arborizing tat molecules or tat derived graft copolymer could be adapted. It may also be possible to enhance cell penetration property of a liposome delivery system by adding the tat peptide to the surface of the complex.[42] Future development is also likely to include other MTS peptides, such as Antennapedia peptide,[15] transpotan,[43] VP22 herpes virus protein[17,19] or other synthetic peptides.[18,44–47] Local injection of such complexes in vivo might be a rational direction to explore in the future.

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