PEPTIDE-BASED DELIVERY OF PROTEINS, NUCLEIC ACIDS AND MOLECULAR PROBES INTO LIVING CELLS

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INTRODUCTION

Recent research has identified several small regions (9-16 amino acids) of proteins called protein transduction domains (PTDs) that possess the ability to traverse biological membranes efficiently in a process termed protein transduction [1-3]. It is shown that transduction occurs in a receptor- and transporter-independent fashion that appears to target the lipid bilayer directly. Thus, in principle and practice, all cell types appear transducible. Proteins and compounds that are covalently linked to PTDs have proven to be useful in answering specific biological questions where other methods fail. Moreover, PTD fusion proteins have now been introduced into mice and exhibit delivery of active enzyme to all tissues, including across the blood–brain barrier. Recent research has shown that nanoparticles probes as large as 40 nm and liposomes as large as 100 nm can be delivered into cells and tissues [1,4].

We have conducted feasibility studies of using protein peptides, 9 to 11 amino acids long, to deliver cargos such as proteins, oligonucleotides and molecular probes into living cells. We have demonstrated that peptide-based delivery is fast, efficient, and versatile. We have also studied the effect of delivery conditions on the efficiency of delivery and the functionality of the cargos. This approach provides a powerful means for cellular delivery, and has a great potential for molecular imaging, gene therapy and basic biological studies.

MATERIALS AND METHODS

Design of Delivery Peptides

As shown in Table 1, there are a number of candidates as delivery peptides, including HIV-1 Tat, HSV VP22, ANTP and an Arginine-rich peptide. We chose to use the 11 amino acid peptide of the HIV-1 Tat protein, and the 9 amino acid Arginine-rich peptide (Table 1). Three peptide conjugates were synthesized, including: (1) Tat and (2) Arginine peptides labeled with a dye molecule TMR, and (3) Tat peptide conjugated with a biotin molecule, all at the C terminus. Fluorescent labeling of the Tat and Arginine peptides enabled us to visualize and localize the peptides in cells; the conjugate of a biotin molecule to the Tat peptide facilitated the attachment of the peptides to different cargos through the biotin-streptavidin binding. All the peptides were synthesized at Invitrogen (Carlsbad, CA).

Table 1. Example of Amino Acid Sequences of PDT's

HIV-1 Tat	Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg
HSV VP22	Asp-Ala-Ala-Thr-Ala-Thr-Arg-Gly-Arg-Ser-Ala-Ala-Ser-Arg-Pro-Thr-Glu-Arg-Pro-Arg-Ala-Pro-Ala-Arg-Ser-Ala-Ser-Arg-Pro-Arg-Arg-Pro-Val-Gl
ANTP	Arg-Gln-Iso-Lys-Iso-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys
Arginine	Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg

Peptide and Cargo Delivery Assays

Four different cargos were delivered into CHO cells, including fluorescently labeled streptavidin molecules and oligonucleotides, molecular beacons, and 20-nm fluorescent beads (Molecular Probes) coated with streptavidin. The biotinylated Tat peptide were linked to labeled streptavidin and fluorescent beads directly. The labeled oligonucleotides and molecular beacons were modified to have a biotin linker, and then conjugated to the biotinylated Tat peptides [5]. The molecular beacons were designed to target the housekeeping gene GAPDH, with a 19-mer probe sequence and a 5-mer stem sequence 5'-TMR-CGACGGAGTCCTTCCACGATACCACGTCG-Dabcyl-3' (underlined sequences formed the stem). The oligonucleotide has a similar design as the molecular beacon but without the stem sequences and the quencher (Dabcyl).

For delivery, 0.5-5 μM peptide-cargo complex was mixed with the cell media for CHO cells to achieve the desired concentration of the target complex. CHO cells were cultured on glass coverslips in 24 well plates. The cells were washed with PBS first and then incubated with the media-peptide-cargo mixture at 37°C for 15-30 minutes. The

media was removed after incubation, and the cells were washed five times with PBS to remove any remaining peptide-cargo complex in the solution. After delivery, the CHO cells were fixed using formaldehyde for fluorescence imaging.

RESULTS

Labeled Peptides, Streptavidin and Fluorescent Beads

As a control study, we first delivered fluorescently labeled Tat and Arginine peptides and 20-nm fluorescent beads into CHO cells and found that the labeled peptides and peptide-linked beads got into cells within 10 minutes with a near 100% efficiency. Further, we found that the Arginine and Tat peptides had similar ability and kinetics in getting into cells. We used fluorescently labeled streptavidin molecule as a model system for proteins and delivered the peptide-streptavidin complex into CHO cells following the protocol mentioned above. It was found that the Tat peptides have the ability to carry cargos into cell nuclei. As demonstrated in Figure 1, after 90 minutes of delivering, we found that most of the streptavidin molecules were concentrated in the cell nuclei. This may have significant implications to gene delivery as well as basic biological studies of gene transcription. We believe that active transport was involved of the peptide-cargo complex in the cytoplasm, although the exact mechanism remains elusive.

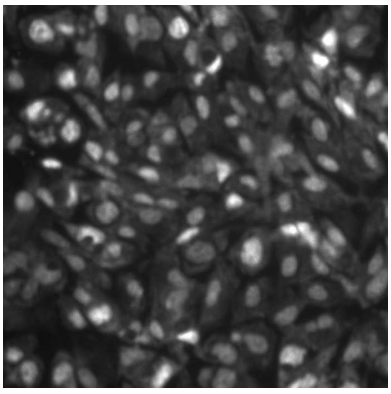

Figure 1. Fluorescence image of labeled streptavidin molecules in the nuclei of CHO cells 90 minutes after the Tat-peptide based delivery.

Oligonucleotides and Molecular Beacons

Our results indicated that peptide-linked oligonucleotides and molecular beacons targeting the housekeeping gene GAPDH were able to get into CHO cells within 30 minutes and with a close to 100% delivery efficiency. Specifically, for molecular beacons, strong fluorescence signal dispersed in the cytoplasm was observed in almost all cells, indicating that probe-target hybridization occurred. Further, our results indicated that all cargos got into the cell cytoplasm directly in peptide-based delivery, while the liposome-based delivery often suffers from trapping of the cargo in the endosomes. This is a significant improvement over the liposome-based transfection assay which usually takes about 2 hours and with a lower transfection efficiency. The peptide-based approach, therefore, may provide a powerful tool for delivering molecular probes and contrast agents into cells in living cells and tissues, thus facilitating *in vivo* molecular imaging [6].

DISCUSSION

A key issue in drug and gene delivery, molecular imaging, and molecular-probe based biological studies is for biomolecules to traverse across the cell membrane and sometimes the nuclear membrane without interrupting normal cell function. To address this issue, we have conducted feasibility studies of using protein peptides, 9-11 amino acids long, to deliver cargos such as proteins, oligonucleotides and molecular beacons into living cells. Our results indicated that, compared with other delivery methods, the peptide-based delivery approach is faster, more efficient and versatile. It is very intriguing that short protein peptides, such as Tat and Arginine peptides shown in Table 1, could carry different cargos into cells and cell nuclei. However, the mechanisms responsible for peptide-based delivery are still largely unknown [7]. Although the electrostatic interaction between the positively charged peptide and the usually negatively changed cell membrane may facilitate the initial interaction, this alone is not sufficient to explain the functions of these peptides. A more systematic study of the underlying mechanisms of the delivery peptides is underway in many labs.

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