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Protein transduction technology

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Abstract With the elucidation of the human genome, exhaustive analysis of genomic data related to gene transcription and the structure and function of translated protein

products has progressed rapidly. Delivery of proteins and their functional domains or inhibitory peptides directly into the cell is ideal to use this protein information and analyze associated physiological functions. Protein transduction technology, which controls cell function via direct delivery of a desired protein into the cell, involves fusing the protein with a special peptide sequence consisting of 10–20 amino acids, referred to as the protein transduction domain. The recent discovery that the protein transduction domain can also be inserted into various macromolecules heightens expectations in terms of development of novel advanced experimental tools and clinical reagents

Keywords Gene therapy · Gene transcription · Gene-translated protein · Protein transduction domain · Protein transduction technology

Abbreviations *AntP*: *Antennapedia* transcription factor · *BBB*: Blood-brain barrier · *PKA*: Protein kinase A · *PTD*: Protein transduction domain



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Introduction

Analysis of desired protein function by DNA transfection or induction of gene expression by infecting with virus is the most widely applied approach for the assessment of gene function. To analyze gene function at the individual organism level, genetically engineered organisms (mouse, fly, nematode) are generated; subsequently gene function is analyzed. Therefore, the main process involves modification of genes (DNA and RNA) both in cultured media and at the living organism level.

The most important aspect by which protein transduction technology differs from these conventional technologies is the delivery of the gene-translated protein directly into the cell; the function of the insert can then be analyzed. A previous investigation involving the HIV-1 TAT protein demonstrated direct delivery of protein into the cell [1]. In this initial study, the inserted TAT activated the viral long terminal repeat promoter; thus the protein that was trans-

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ported across the membrane retained activity within the target cell. Later reports documented retention of the ability of the *antennapedia* transcription factor (AntP) from *Drosophila* and VP22 (structural protein from herpes simplex virus 1) to move across cell membranes [2, 3]. Additional examination identified the sequence (comprised of 10–20 amino acids) necessary for protein transport across the membrane; this sequence was termed the protein transduction domain (PTD). Binding of this PTD to selected proteins and peptides facilitates delivery into the cell (protein transduction technology).

Based on these initial PTD sequences, protein transduction technology characterized by enhanced efficiency and a higher transduction rate, has been developed in recent years, and has been applied at both the experimental and clinical levels. PTD containing 6–11 basic amino acids (arginine, lysine) has gained attention particularly due to its higher transduction rate and fewer side effects [4–7]. Special characteristics of the protein transduction method include simple manipulation (introduction of the desired protein), short insertion period (30 min–1 h), ability to deliver various biologically active substances in addition to proteins, and versatile transduction in a variety of tissues via abdominal and intravenous injection. This contribution surveys recent advances reported in experimental studies employing protein transduction technology.

Protein transduction mechanism

Comparison of PTD sequences in TAT, AntP, and VP22 reveals large numbers of basic amino acids (Table 1). Reports suggest a distinct membrane transport mechanism for each PTD; however, some results provide evidence that TAT and polyarginine share a same membrane transport mechanism [8]. Heparin sulfate proteoglycan plays a significant role in TAT and polyarginine PTD cell transduction [9, 10]. In variant cell strains in terms of enzyme involvement in heparin sulfate synthesis, protein transduction into cells via TAT or polyarginine/polylysine decreases rapidly in comparison with the wild type. Therefore electric coupling of heparin sulfate proteoglycan and PTD at the cell surface is essential at the initial step of insertion. Whether the subsequent membrane transport system functions via

Table 1 Amino acid sequence of protein transduction domain (underlining basic amino acid, AntP *Drosophila* homeotic transcription factor encoded by the *antennapedia* gene, HSV-VP22 herpes simplex virus VP22 transcription factor)

	Amino acid sequence
HIV-TAT	Tyr-Gly- <u>Arg-Lys-Lys-Arg-Arg</u> -Gln- <u>Arg-Arg-Arg</u>
HSV-VP22	Asp-Ala-Ala-Thr-Ala-Thr- <u>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-Glu</u>
AntP	<u>Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys</u>

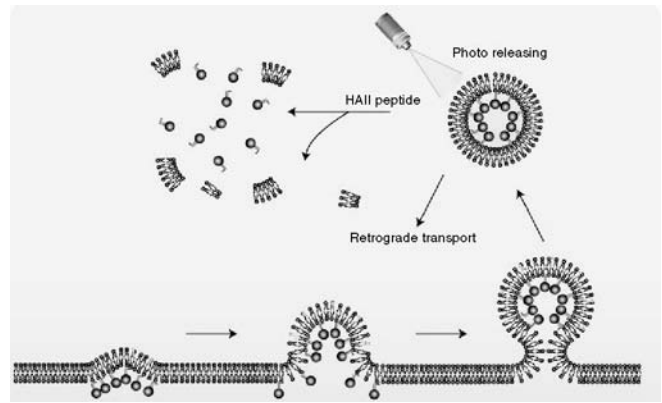


Fig. 1 Protein transduction mechanism via PTD. Summary of the PTD trafficking and the release from endosome by endosome disruption agents

endocytosis or direct passage through the cell membrane is still unknown.

However, recent reports show that endocytosis is the initial step for transduction of PTDs. Transduction mechanism of TAT-PTD recently was clearly shown by lipid raft-dependent macropinocytosis [11]. This report showed markedly and specifically enhanced macropinosome escape using pH-sensitive HAII peptide. We also developed the methods to enhance the endosome escape using photo-sensitive PTD (Fig. 1) [12]. The accumulation of PTDs in the endosome raises the question of which by mechanism internalized PTDs reach the cytosol. One report suggests that retrograde transport to the Golgi apparatus and endoplasmic reticulum serves as a common route of transport for PTD, and that the efficacy of retrograde transport depends on cell types [13]. In neurons, we observed that the signal of fluorescein isothiocyanate 11 arginine fused with calcineurin autoinhibitory peptide was diffuse and found in all areas of the neurons, with high signal intensity in nuclei [14]. These peptides did not show any punctate cytoplasmic distribution in living neurons (Fig. 2). Therefore the mechanism of translocation may depend on whether it is the free PTD or

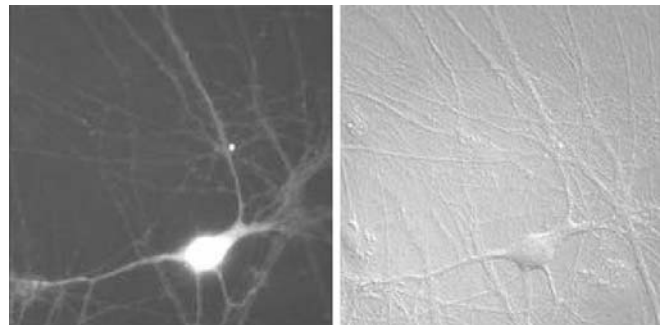


Fig. 2 Transduction of fluorescein isothiocyanate 11R in neuron. Transduction of fluorescein isothiocyanate 11R in living neuron. Neurons cultured on glass cover slips were incubated with 1 μ M of peptide. After 1 h the cells were washed three times and incubated for another 30 min, then analyzed by confocal microscopy. Fluorescence image and phase contrast image

the PTD connected to a cargo that is investigated. Further investigation is necessary in this area.

Regulation of signal transductions

Cultured cells

Rapid delivery of PTD-fused peptides and proteins is achieved by simple introduction to cultured cells. Added PTD-fused protein consistency ranges from the nanomolar to the micromolar level. A primary characteristic of the protein transduction methodology is the rapid, even delivery of the protein into all cells in a manner similar to that of low molecular weight substances. Therefore this method is superior for measuring biological activity of inserted proteins in cultured cells. In the case of gene transfection protein expression requires more than 24 h; moreover, expression level varies among cells. Consequently the acquisition of uniform experimental results is difficult where the need exists with respect to control of time flow. However, in the protein transduction approach protein molecules can be applied in a manner similar to that with any other low molecular weight substances. For example, in controlled cell cycle experiments, the timing of function for each protein in the cell cycle was analyzed successfully via purification of various cell cycle related proteins and introducing them as PTD-fused proteins into the culture solution [15].

PTD can also deliver proteins into cells in cases in which conventional transfection fails. Although transduction rates can vary, proteins can be delivered into nearly all primary nerve cells in culture, embryonic stem cells, floating cells, and osteoclasts. A study employing osteoclasts involved delivery and analysis of the function of a Rho-A predominant mutant protein [16]. In this experiment, Rho-A was successfully delivered into nearly 100% of osteoclasts. As this example illustrates, protein transduction technology enables protein function analysis using cell types that are difficult to handle with respect to transfection experiments.

Protein kinase A (PKA) inhibitory peptides were inserted into primary nerve cells in culture and in acute brain slices with polyarginine PTD [6]. In the present investigation, 11 arginine residues and a nuclear transition signal were fused into the PKA inhibitory peptide. Subsequently, nuclear-specific PKA inhibitory peptide cell transduction was conducted; moreover, PKA functional analysis inside PKA nerve cell nuclei was applied effectively. Gallouzi et al. [17] successfully demonstrated the mechanism of extranuclear transport of RNA employing an extranuclear mRNA transport inhibitory peptide fused with PTD. Highly specific inhibitory peptides are currently under development for many enzymes. In recent years, peptides capable of blocking various protein interactions have been developed; three-dimensional protein structure analysis will enhance novel inhibitory peptide development. Conventional stand-alone peptides do not possess sufficient cell membrane permeability, and therefore in vivo experiments are challenging. However, by fusing PTD with an inhibitory peptide many molecules can be manipulated at the cellular level.

Individual organisms

The fused protein used at the cell culture level can be delivered into various organisms in vivo, which is the greatest advantage of the PTD-related methodology. Schwarze et al. [18] first documented in vivo protein transduction technology in 1999. They successfully delivered β -galactosidase into all internal organs including brain via injection of TAT and β -galactosidase-fused protein into mouse abdominal cavity; this study also confirmed that PTD can be used in living organisms as well as in cultured cells.

An example of PTD and inhibitory peptide application at the living organism level follows. A peptide fused with caveolin and endothelial nitric oxide synthase binding inhibitory peptide was injected into mouse endocapillary cells; NO production was suppressed with reduced inflammation [19]. We also developed a cell-permeable inhibitor of nuclear factor of activated T cells (NFAT) using the polyarginine peptide delivery system [20]. This peptide provided immunosuppression for fully mismatched islet allografts in mice. Although peptide-based strategy as a therapeutic agent is promising, the half-life of peptide in vivo remains problematic. To solve this problem, retroinverse version of peptide was synthesized using D-isomer amino acid residues. Using this method, retroinverse TAT-p53 c terminal peptide treatment of cancer models results in significant increases in lifespan and the generation of disease-free animals [21]. Utility of membrane permeable peptide involving PTD demonstrated inhibition of intracellular signaling, which underscores the vast application potential of peptide-related medicine. Furthermore, injection of Bcl-x1 (apoptosis-related protein) and TAT-fused protein into mouse abdominal cavity resulted in suppression of death in nerve cells [22, 23]. In the present investigation, a mouse brain ischemia model was established first; subsequently TAT Bcl-x1 was injected, which exhibited a suppression effect with respect to delayed death in nerve cells.

In brain, a blocking barrier known as the blood-brain barrier (BBB) exists, which controls material transport. Research has confirmed that even high molecular weight substances can penetrate the BBB in the presence of the TAT transduction domain. Binding with PTD now enables transport of conventional non-BBB-permeable chemicals into the brain, which underscores the great potential with respect to various clinical applications. One notable study reported gene splicing by PTD-CRE recombinase injection into transgenic mouse with the floxP sequence [24, 25].

Use of large biomolecules

The PTD can be applied not only to proteins but also to various biologically active substances. With the advance in genetic information in various living organisms, antisense technology and RNAi will assume increasingly significant roles in future genetic analysis. Antisense nucleic acids possess limited membrane permeability. Therefore large numbers of PTDs are used for antisense transduction. In particular, reports regarding AntP and VP-22 confirm high

suppression rates of desired protein expression by linking PTD to antisense nucleic acids. Furthermore, successful insertion of antisense peptide nucleic acid into mouse spinal nerve cells by has been reported linking peptide nucleic acid with AntP [26].

In addition, several studies have examined the size limit of transportable substances with PTD. One investigation demonstrated transduction of TAT-linked magnetic nano-beads (45 nm diameter) into cells [27]; delivered beads did not affect cell proliferation or cell differentiation. Following injection of TAT beads into the bloodstream, selective collection of bead-inserted cells at high rates is possible. In combination with phage display technology, one study documented the successful transduction of phage into cells via expression of TAT peptide on the phage surface [28]. This work also indicated that expression of the desired gene can be achieved via binding of foreign genes downstream of the promoter phage genome that are expressed in target eucaryotic cells. These findings suggest that the cargo size in TAT cell insertion is limitless.

In terms of clinical potential the PTD could be applied to efficient transport through the skin barrier. In general, skin does not permit the passage of pharmacological substances; as a result many inventions have targeted subcutaneous insertion of pharmacological substances. Jonathan et al. [29] delivered medical agents subcutaneously at high rates employing seven arginine residues. More specifically, inflammation was effectively inhibited via conjugation of seven arginines and cyclosporin A (immuno-suppressant), followed by topical application, in comparison to application of cyclosporin A alone. This report offered valuable data regarding topical drug application in various skin disorders. In this study, the “linker” was designed to disaggregate the drug and the seven arginine moieties following insertion into the cell.

Conclusions and future objectives

The development of novel protein transduction technology faces a number of hurdles. Accumulation of PTDs in the endocytic compartment raises questions concerning the extent to which and the mechanism by which internalized PTDs reach the cytosol. A rational design of more effective PTDs can be achieved only with a thorough understanding of the uptake mechanism. In some enzyme-PDT transduction experiments, enzyme activity was not retained following insertion into the cell. To resolve this issue, proteins and compounds could be coupled to PTDs via a linker designed to release the active proteins and drugs within cells. PTDs can deliver proteins into all organs and cells. Organ-specific delivery involving PTDs also poses a complex challenge with respect to utility of this technology as a clinical tool. Peptide libraries displayed on phages can be screened in vivo to identify a phage that homes to a specific target; furthermore, numerous studies have demonstrated that peptides capable of homing to various individual organs can be isolated in this manner. Combination of pep-

tides identified by phage display technology and PTD peptides could be employed for organ and cell type specific delivery of proteins and other macromolecules. The PDT methodology cannot be applied to insoluble proteins, for example, membrane proteins, due to difficulties associated with purification of these molecules. Possible antibody formation against the protein or PTD can limit chronic use with respect to in vivo applications. Overcoming these obstacles requires advances involving refined PTD sequences and improved application.

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