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Cellular Delivery of Noncovalently-Associated Macromolecules by Cell-Penetrating Peptides

Microsugar Chang[1], Yue-Wern Huang[2], Robert S. Aronstam[2] and Han-Jung Lee[3,*]

[1]*Department of Biotechnology, Hung Kuang University, Taichung 43302, Taiwan;* [2]*Department of Biological Sciences, Missouri University of Science and Technology, Rolla, MO 65409-1120, USA;* [3]*Department of Natural Resources and Environmental Studies, National Dong Hwa University, Hualien 97401, Taiwan*

Abstract: Cellular and nuclear delivery of biomolecules is limited by low membrane permeability. Cell-penetrating peptides (CPPs) can be covalently linked to cargos to improve cellular internalization. Our work indicates that arginine-rich CPPs are also able to interact with a variety of cargos, including DNA, RNA, proteins and nanomaterials, in a noncovalent manner and subsequently effect their delivery into cells. The advantages of noncovalent attachment in CPP-mediated transduction are multiple: ease of use, ease of production, and versatility with respect to both cargo composition and functional delivery (i.e., the cargo is not chemically modified). We have extended this approach to achieve simultaneous transduction of covalently and noncovalently associated complexes, opening a new method for delivering multiple types of cargos, including proteins, fluorescent nanomaterials, nucleic acid and others. These novel variations of CPP-mediated transport should be of broad utility in the transport of genes, small interfering RNAs, proteins and nanoparticles in biomedical research and therapeutic intervention.

Keywords: Cellular internalization, cell-penetrating peptide, macropinocytosis, polyarginine, protein transduction domain, quantum dots.

1. INTRODUCTION

Multiple approaches have been developed to introduce bioactive macromolecules, including DNA, RNA and protein, into living cells. The challenge is to design highly efficient and noncytotoxic carriers that can overcome the low permeability of cell membranes while promoting appropriate intracellular trafficking, and even nuclear delivery, in target cells. In the field of gene transfer, for instance, both viral and nonviral vectors have been used to deliver sense and antisense nucleic acids into mammalian cells [1, 2]. Nonviral systems present several advantages over viral systems insofar as they are simple to use, easy to produce, less toxic, and do not induce specific immune responses [3].

Protein therapy is a rapidly expanding area of interest in the treatment of cancer, the screening of novel therapeutic drugs and the development of vaccines for infectious diseases [4]. The direct delivery of peptides or proteins into cells and animals *in vivo* can be achieved by association with cell-penetrating peptides (CPPs). CPPs are also known as protein transduction domains (PTDs), Trojan horse peptides, arginine-rich intracellular delivery (AID) peptides or membrane transduction peptides [4-6]. The first CPP, identified in 1988, was the transcriptional activator Tat (transactivator of transcription) protein of the human immunodeficiency virus type 1 (HIV-1) [7, 8]. Eleven amino-acid residues (YGRKKRRQRRR), designated as Tat-PTD, corresponding to amino acid numbers 47-57 of the Tat protein (GenBank accession number AAA45080) of HIV-Tat, are rich in basic amino acids that proved to be required for translocation through the plasma membrane and for accumulation in the cell nucleus [9]. Subsequently, a variety of naturally-occurring and synthetic CPPs were identified as small, amphipathic, hydrophobic or cationic peptides capable of delivering a wide range of biologically active cargos into living cells [2, 5, 6]. However, the mechanisms by which CPPs and CPP/cargo complexes traverse cell membranes remain incompletely understood.

Most CPPs have non-human origins, including Tat-PTD, penetratin of the *Drosophila*, and structural protein VP22 of the herpes simplex virus [10], although a few CPPs are derived from human proteins, such as Vectocell peptides, which are derived from human heparin binding proteins [6, 11]. CPPs can be derived from naturally occurring proteins, chimeric combinations of protein derived and designed sequences, or purely designed sequences [12]. The cellular uptake of CPPs containing polyarginine moieties tends to be more efficient than that of CPPs containing polylysine, polyhistidine, polyornithine or other basic domains [13, 14]. The highest internalization efficiency of polyarginine CPPs is achieved using octa-arginine (R8) or nona-arginine (R9) peptides [13]. This reflects the importance of electrostatical interactions of the positively-charged guanidinium groups with negatively-charged molecules, such as heparin sulfate proteoglycans, associated with the cell surface [14]. The CPPsite database is a comprehensive collection of CPPs [15], whereas the CPPpred [16] and CellPPD [17] sites provide useful tools for the prediction of highly effective CPPs.

*Address correspondence to this author at the Department of Natural Resources and Environmental Studies, National Dong Hwa University, No. 1, Sec. 2, Da-Hsueh Road, Shoufeng Township, Hualien 97401, Taiwan;
Tel: +886 3 8633642; Fax: +886 3 8633260; E-mail: hjlee@mail.ndhu.edu.tw

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Recently, a quantitative structure-activity relationship (QSAR) model was developed to predict the physiochemical properties of amphipathic CPPs [12].

Interactions between CPP and cargos can be either covalent or noncovalent. Covalent protein transduction (CPT) involves a covalent bond between the CPP and cargo. A covalent bond is a chemical conjugation that involves the sharing of electron pairs between atoms, such as disulfide bridges, ester and peptide bonds. The majority of early studies in the CPP field used covalent conjugation to form stable and chemically well-defined protein/peptide and peptide nucleic acid (PNA) complexes [18, 19]. However, CPT involves relatively expensive and labor-intensive synthesis, and this approach is not suitable for the delivery of nucleic acids and nanoparticles. On the other hand, noncovalent protein transduction (NPT) utilizes a noncovalent association of the CPP and cargo. Noncovalent bonds involve diverse variations of van der Waals forces, electrostatic interactions and hydrophobic effects. The major advantages of NPT over CPT are simplicity of preparation, cargo versatility and low working concentrations (possibly contributing to reduced toxicity). However, the challenge of NPT is the relatively weak interacting forces between CPPs and cargos, which may cause premature dissociation of cargos from CPPs and off-target effects within cells. CPP/cargo complexes vary in size and stability *in vitro*. Currently, NPT is less frequently used in preclinical and clinical applications than CPT [18].

Endosomal entrapment of internalized CPP/cargo complexes remains an important problem, especially when endocytosis is the major pathway for both CPT and NPT. Endosomal entrapment may severely limit the functional delivery of certain cargos. To address this problem, CPPs have been modified to contain moieties that promote endosomal escape. The first successful example of enhanced endosomal escape involved a histidine-containing endosomolytic α-helical penetratin analogue, EB1 [19]. Since then, modification of CPPs with fusogenic HA2 peptide, histidine and cysteine residues, cysteamide at CPP's terminus, thiol, and cholesterol has been shown to facilitate release of cargos from endosomes [19]. In this review, we focus on recent advances in the design and characterization of novel CPPs for cargo delivery, including discoveries related to their cytotoxicity and mechanisms of cellular internalization.

2. CPP-MEDIATED DNA DELIVERY

The delivery of exogenous genes into organisms for functional expression is essential for transgenic research, DNA vaccination and gene therapy. The ability of cationic peptides to transduce a foreign gene into animal cells was first demonstrated in 1987 using a covalently-linked asialoglycoprotein with a polylysine peptide [20]. Several reports subsequently demonstrated that CPPs can internalize DNA/plasmids into mammalian and plant cells [21, 22]. A synthetic R9 CPP (SR9) was used to deliver noncovalently-associated plasmid DNA into living plant cells [23]. This SR9 peptide, the essential component for transmembrane transport, enters plant cells where it further facilitates nuclear routing [24-31]. The negatively charged plasmid DNA bound to the positively charged SR9 peptide by electrostatic interaction. The internalization mechanism of SR9 peptide/DNA complexes proved to be energy-dependent macropinocytosis [31, 32].

3. CPP-MEDIATED RNA DELIVERY

RNA interference (RNAi) in animals and post-transcriptional gene silencing (PTGS) in plants are fundamental aspects of RNA metabolism [33]. Endogenous double-stranded RNAs (dsRNAs) are processed by the endoribonuclease Dicer into small interfering RNA (siRNA) molecules of 20-25 base pairs in length. These siRNAs silence the expression of specific genes. Accordingly, the introduction of single- and double-stranded DNA and RNA molecules into mammalian cells to manipulate gene expression represents a major therapeutic strategy. However, RNA is membrane impermeable, and carriers are needed to allow RNA to cross cell membrane intact. CPPs and siRNA complexes can be synthesized by noncovalent CPP/siRNA electrostatic interactions or covalent linkage of CPPs to siRNA duplexes through disulfide bond formation in which CPPs containing N-terminal cysteines are conjugated to siRNA molecules with a 5'-thiol modified siRNA sense strand [33].

CPPs have been used to deliver siRNA into animal [34] and plant [35] cells. CPPs formed stable noncovalent complexes with siRNAs that target enhanced green fluorescent protein (EGFP), and delivered these complexes into cells [36]. Human GC cells were transfected with pEGFP-N1 plasmid followed by G418 selection to establish a cell line stably expressing EGFP (GC-EGFP cells). At nontoxic levels, CPPs complexed with EGFP siRNA *in vitro* and delivered the siRNAs into GC-EGFP cells, thereby silencing EGFP expression.

4. CPP-MEDIATED PROTEIN DELIVERY

Two strategies can be applied to couple CPPs with cargos: covalent (CPT) and noncovalent (NPT). The majority of earlier studies used CPT, but the number of studies using NPT is increasing rapidly [18]. A special type of covalent and noncovalent protein transductions (CNPT) involves both covalent and noncovalent association of CPPs and cargos. The advantage of CNPT is that two different types of cargos aiming at different targets can be simultaneously delivered into cells. In the case of protein cargos, protein stability relying on weak noncovalent interactions between secondary, tertiary, and even quaternary structures can be preserved throughout NPT process [37]. The challenges of CNPT are the relatively labor-intensive synthetic requirement, and various functional activities displayed by two cargos in a cell due to different transduction efficiencies.

4.1. Covalent Protein Transduction (CPT)

There are two strategies for the preparation of transduction systems for the delivery of covalently-attached protein cargos (CPT). The first strategy is to build the cargo sequence into a CPP-containing plasmid .The plasmid can be then expressed as a recombinant CPP-fusion protein. The second strategy is to covalently link CPP to the intended cargo post translationally [21]. Schwarze *et al*. first demonstrated CPP-mediated delivery of CPP-fusion proteins into various tissues in living mice [38]. Subsequently, Snyder and Dowdy reported that CPP-fused cargos could traverse the

plasma membranes of many mammalian cell lines with high efficiency [4]. Cargos that can be covalently carried by CPPs include peptides/proteins, nucleic acids, peptide nucleic acids, liposomes, nanoparticles and others [2, 5, 6]. Covalent CPP-cargo conjugates have been delivered to the cytoplasm and the nucleus of a variety of animal cell lines.

CPP-mediated CPT in plants was first described by our team in 2005 [39]. CPPs could efficiently deliver CPP-fusion proteins, including GFP, red fluorescent protein (RFP) and enzymes [40], into various types of plant cells in fully active forms. The fluorescent signal thus delivered was distributed evenly throughout the cytoplasm, and was present at high levels in the nuclei. As shown in (Fig. **1**), no signal was observed in onion (**a**) and tomato (**d**) cells treated with GFP and RFP protein alone, while green and red fluorescence were visualized in the cells treated with R9-GFP (**b**) and R9-RFP (**c** and **e**) fusion proteins, respectively. These results were consistent with previous findings [39] demonstrating that CPPs covalently fused with fluorescent proteins could be transduced into plant cells. The protein transduction of Tat-GFP, R9-GFP and R9-RFP proteins was neither species-specific nor tissue-specific in the plants examined. Thus, cellular internalization mediated by CPPs represents a non-invasive and highly efficient method to express bioactive proteins in cells [39-41].

4.2. Noncovalent Protein Transduction (NPT)

The application of CPPs with cargos attached in a noncovalent fashion (NPT) was introduced later [42]. Evidence from confocal microscopy and flow cytometric analysis indicated that CPPs can efficiently deliver noncovalently complexed proteins, including GFP, RFP, dTomato, mCherry, mOrange or β-galactosidase enzyme (molecular mass 120 kDa) into animal [30, 42, 43] and plant [42, 44] cells, in fully active forms. As shown in Fig. (**2**), no signal was detected in onion root cells treated with GFP (**a**) or RFP (**d**) protein alone, while onion root cells treated with SR9/GFP (**b** and **c**) and SR9/RFP (**e** and **f**) complexes exhibited green and red fluorescence, respectively. These results support the previous conclusion [44] that CPPs can deliver fluorescent proteins into plant cells following noncovalent association. The mechanism underlying NPT appears to involve macropinocytosis [42, 44]. Clearly, CPP-mediated NPT represents a useful strategy to deliver functional proteins in living cells.

CPPs were shown to utilize NPT to facilitate the rapid delivery of proteins, including GFP, collagen and insulin, into mouse skin tissues [45]. Cargos delivered by CPP-mediated NPT reached the epidermis, dermis, panniculus adiposus and even hypodermis of mouse skin. The efficiency of this nontoxic transport was further improved by chemical enhancers, such as oleic acid. The mechanism of CPP-mediated cellular entry by NPT was found to involve both macropinocytosis and actin rearrangement [42, 44]. This direct delivery of bioactive proteins into living cells and tissues by NPT represents an interesting strategy for the delivery of dermal pharmaceutics and cosmetics.

To expand beyond animal or plant systems, CPPs were tested for their efficiency as carriers in other taxonomical

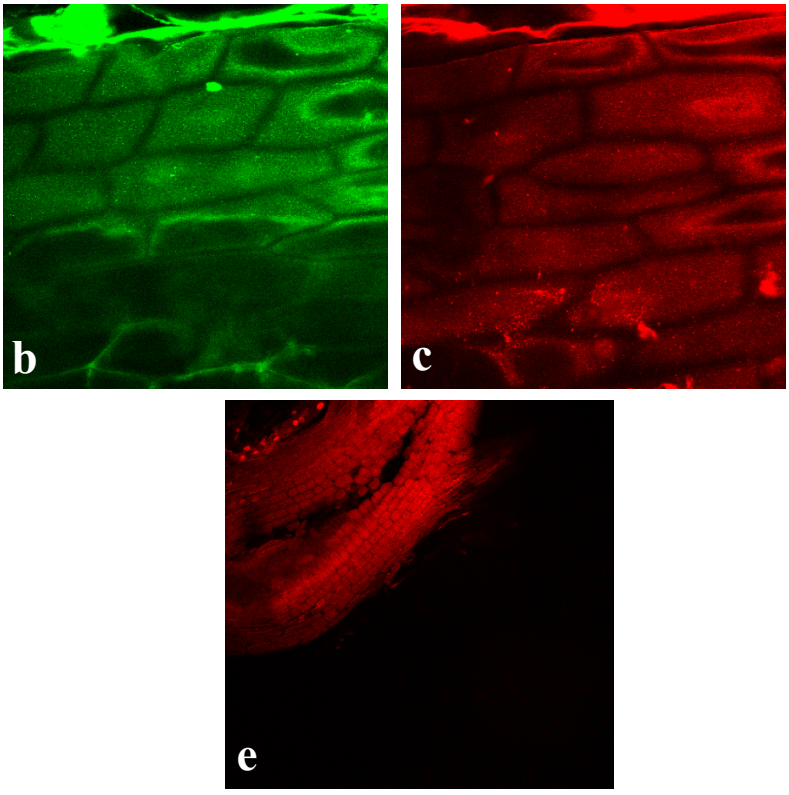

Fig. (1). Covalent protein transduction (CPT) in plant cells. Onion (*Allium cepa* L.) skin epidermal cells were treated with control GFP (**a**), R9-GFP (**b**) or R9-RFP (**c**) fusion proteins (400×magnification). Tomato (*Lycopersicon esculentum*) root cells were treated with RFP (**d**) or R9-RFP (**e**) proteins (200×magnification). Fluorescent images were recorded by the TCS SL confocal microscope system (Leica, Wetzlar, Germany) [39].

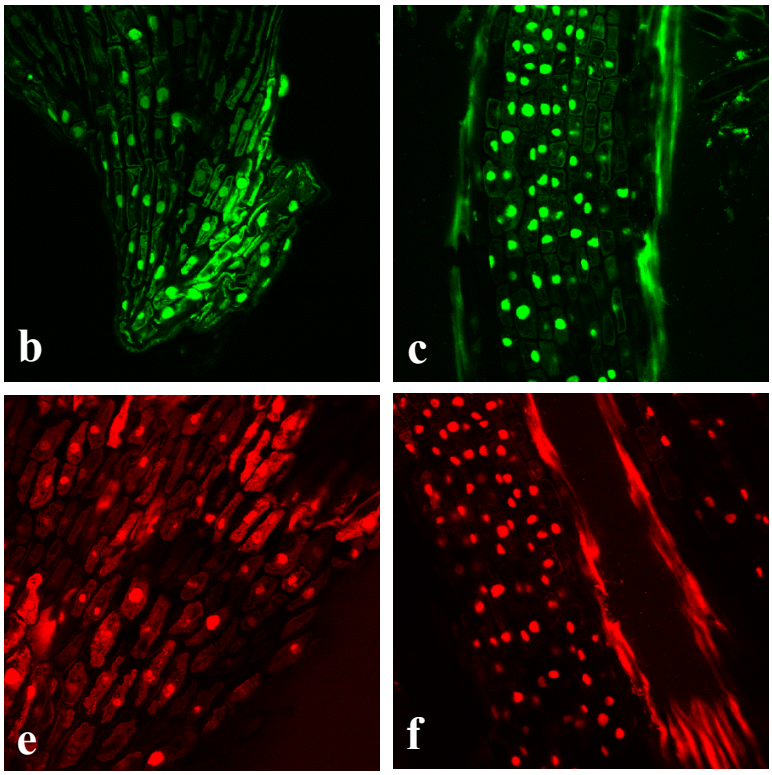

Fig. (2). Noncovalent protein transduction (NPT) in plant cells. Onion root tips were treated with control GFP (**a**), SR9/GFP complexes (**b**), RFP (**d**) or SR9/RFP complexes (**e**). Onion root cells were treated with SR9/GFP (**c**) or SR9/RFP (**f**) complexes. Fluorescent images were recorded (200×magnification) by confocal microscopy [44].

groups [46]. Several representative organisms, including cyanobacteria, bacteria, archaea, algae, fungi and yeasts, were screened for CPP-mediated NPT. Not all living beings were amenable to NPT. While all species of prokaryotes and unicellular yeasts tested were capable of NPT, green algae and multicellular fungi were not.

4.3. Covalent and Noncovalent Protein Transductions (CNPT)

Animal [47] and plant [48] cells were treated with a fluorescent protein that was noncovalently complexed with a CPP-fluorescent fusion protein of a different color. The CPPs delivered both cargo proteins into living cells simultaneously in a hybrid type of transport (CNPT). The optimal molecular ratio between carrier CPP-fluorescent fusion protein and cargo fluorescent protein was about 3:1. Fluorescence resonance energy transfer (FRET) assays confirmed the physical interaction of CPP-conjugates with cargo fluorescent proteins in CNPT in cells. CNPT appears to involve a combination of internalization pathways. These studies increase our understanding of the mechanisms of CPP entry and introduce an effective tool for simultaneously delivering multiple proteins into cells.

5. CPP-MEDIATED NANOMATERIAL DELIVERY

Fluorescent probes play an important role in studying cellular structure, tracking intracellular molecules, and monitoring dynamic processes in living cells. However, fluorescent proteins have certain properties, including broad emission spectra, limited brightness and low photostability, that limit their usefulness, especially for long-term visualization [49]. Quantum dots (QDs) are semiconductor nanocrystals that possess a narrow range of emission wavelength, exceptional resistance to photo and chemical degradation, and high quantum yield [50]. These properties make QDs particularly useful for long-term observation of molecules in live cells, as well as tumor targeting and diagnostics *in vivo*.

The discovery of CPPs and their ability to ferry cargos into cells has had a revolutionary impact on the development of nanoparticle-based cancer treatments [51]. In 1999, Josephson *et al*. first reported the covalent conjugation of Tat-PTD to iron oxide nanoparticles with an average size of 41 nm [52]. These Tat-iron oxide nanoparticles were internalized into lymphocytes, and yielded magnetic labeling of cells. Later, Stroh *et al*. treated primary bone marrow cells with Tat-QD micelles, and the cells could be successfully labeled *ex vivo* [53]. These QD-labeled cells were injected into tumor bearing mice, and the tumor vasculature could be tracked by imaging of QDs. It should be noted that Torchilin *et al*. prepared a noncovalent Tat-liposome/DNA complexes [54]. These complexes possessed high transfection efficiency and low cytotoxicity in mouse fibroblast NIH 3T3 and cardiac myocytes H9C2 cells.

Arginine-rich CPPs facilitated the delivery of noncovalently associated QDs into living cells [30, 31, 55-63]. Upon cellular entry, the internalized QDs are concentrated in the perinuclear region. There is no cytotoxicity following transport of QDs at concentrations up to 200 nM [55]. The inter-

nalization mechanism for CPP/QD complexes appears to involve a combination of pathways [55, 58, 61]. These findings support the notion that fluorescent nanomaterials in combination with CPPs may serve as a powerful tool for both *in vitro* and *in vivo* imaging.

6. BIOCOMPATIBITY OF CPPS

There have been a limited number of studies on the toxicity of CPPs. One report indicated that Tat-PTD induced limited perturbation of intracellular calcium levels in human fetal brain cells, and that this effect depended on the conformation of Tat residues [64]. Acute exposure of mammalian cells to 20-100 μM of Tat-PTD did not result in significant cytotoxicity: more than 85% of the cells appeared to be healthy when exposed to 100 μM Tat-PTD peptides for 24 hours [65]. On the other hand, Tat-PTD at 500 μM produced functional alternations of fibroblast cells [66]. We observed minimal cytotoxicity in CPP-mediated CPT [39], NPT [23, 42, 44, 55] and CNPT [47], as measured by trypan blue, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) and sulforhodamine B (SRB) assays. The safety of CPPs, with the exception of transportan, was confirmed by a metabolic analysis [3]. Collectively, these studies indicate that CPPs possess negligible cytotoxicity.

7. MECHANISMS OF CPP-MEDIATED CELLULAR INTERNALIZATION

Our understanding of the molecular mechanism of cellular uptake mediated by CPPs in mammalian cells remains incomplete. The hydrophobic membrane interior is an effective barrier to the transport of most large and polar bioactive compounds; special transporters or protein channels are required for the transport of many specific biomolecules [44]. However, highly basic and positively charged CPPs can interact with cell surface heparin sulfate proteoglycans and then be endocytosed. It should be noted that membrane transduction is related to multiple factors, including degree of hydrophobicity, peptide structural transitions, and membrane composition [58]. With respect to membrane composition, heparan sulfate proteoglycans, e.g., glycosaminoglycans, have been reported to play an important role in CPP-mediated uptake [47, 48, 58, for review in 67]. Evidence indicates that CPPs internalize cargos via CPT and NPT in as little as 5 minutes [39, 42, 44, 58]. Studies using several complementary bioassays support the notion that macropinocytosis and direct membrane penetration are the two major internalization routes. Below we detail a few molecular processes involved in this cellular uptake.

7.1. Macropinocytosis

Early reports established that CPP-mediated cellular uptake proceeded independent of classical endocytosis, an energy requiring process, or the involvement of receptors or active transporters [10, 38, 68-70]. More recent work suggests that a specialized form of endocytosis, known as lipid raft-dependent macropinocytosis, plays a major role in CPP-mediated uptake [71].

Endocytosis is an energy-dependent process in which cells absorb molecules by engulfing them [72]. Endocytosis can be classified into two major categories: phagocytosis (cell eating) and pinocytosis (cell drinking). Phagocytosis involves the uptake of large particles and, in mammals, is typically restricted to specialized cells, such as macrophages, monocytes and neutrophils. Pinocytosis involves the uptake of fluid as well as solutes, and occurs in all cells by at least four mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis [72]. Macropinocytosis requires membrane ruffling that is induced upon stimulation by growth factors or other signals (Fig. **3**). The signaling cascades that induce macropinocytosis trigger actin-driven formation of membrane protrusions. These membrane protrusions collapse onto and fuse with the plasma membrane to generate large endocytic vesicles, called macropinosomes [72]. Little is known about the participation of CPPs and cargos in the fusion process of macropinosomes, or their release into the cytoplasm or nucleus after endosomal escape.

In previous studies, we used a macropinocytosis inhibitor 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) and an F-actin polymerization inhibitor cytochalasin D (CytD) to demonstrate that CPP-mediated NPT in plants may involve macropinocytosis and actin reorganization [23, 42, 44-46]. Different CPP-cargo conjugates can be further targeted by inclusion of appropriate signaling peptide sequences to the cytoplasm [73], certain organelles [74] or nucleus [75] in a variety of animal cell lines. We hypothesized that, in addi-

Table 1. Summary of our currently used CPPs.

Name	Amino Acid Sequence	References
Tat-PTD	YGRKKRRQRRR	[39, 42, 44, 45, 56]
R9 or SR9*	RRRRRRRRR	[23-30, 32, 36, 39-48, 55-63]
R9Z	CRRRRRRRRRCRRRRRRRRRCRRRRRRRRRC	[42, 45]
HR9	CHHHHHRRRRRRRRRHHHHHC	[26-30, 32, 58, 59, 61-63]
PR9	FFLIPKGRRRRRRRRR	[26-29, 58, 59, 61-63]
IR9	GLFEAIEGFIENGWEGMIDGWYGRRRRRRRRR	[30, 31, 59, 62]

*R9 containing nona-arginine is a polypeptide with 101 amino acids purified from protein expression of the pR9 plasmid-transformed *Escherichia coli*, whereas SR9 is a synthetic nona-arginine peptide.

Fig. (3). The lipid raft-dependent macropinocytosis, a specialized form of endocytosis, is one of potentially cellular uptake mechanisms of CPP-mediated cargo delivery.

tion to facilitating cellular uptake, cationic CPPs play a role in nuclear localization of cargos as a consequence of the extended coil-structure of CPPs. Initially, the positively charged CPP-cargo complexes interact with negatively charged phospholipids on the outer surface of the cellular membrane (Fig. **3**). This interaction induces the formation of membrane ruffling and rearrangement of actin and microtubules, followed by induction of macropinocytosis and internalization [44]. Upon entry, endosomal acidification, osmotic swelling and bursting lead to endosomal escape and release of CPPs and their cargos to the cytoplasm. Entry to the nucleus is subserved by nuclear localization signal (NLS) properties inherent in the CPPs [41, 76].

7.2. Direct Membrane Penetration (Translocation)

Direct membrane translocation of CPPs involves a passive membrane diffusive or destabilization process that does not require binding to proteinaceous cell surface receptors [4, 10, 68]. Highly basic CPPs can form ionic interactions with negatively charged entities on the surface of biological membranes, such as phospholipids [69]. Inverted micelle formation may account for subsequent uptake processes [68]. A series of reports in 2009 presented evidence to support a direct pore-opening mechanism for CPPs [13, 77-80]. The direct membrane translocation mechanism consists of four steps: (1) CPPs bind to the surface of the bilayer attracted by the phosphate groups of the phospholipids; (2) lipid arrangement is disrupted as concentrations of CPPs on cell surface increase; (3) side chains of arginines translocate through the distal layer and form a water pore with a maximal diameter of 2.5 nm; and (4) a few CPPs translocate by diffusing through the surface of the pore, followed by pore closure [77]. The completion of translocation occurs on a microsecond timescale.

7.3. Combined Pathways

CPPs may translocate by endocytosis and direct membrane penetration simultaneously. The physicochemical properties of the delivery system could influence the choice of internalization pathways. Guterstam *et al*. reported that uptake of arginine-rich CPPs is dominated by direct membrane translocation, whereas uptake of arginine-rich CPP/DNA complexes is dominated by endocytosis [13]. The hydrophobic counter-anion 4-(1-pyrenyl)-butyric acid (pyrenebutyrate) facilitates translocation of arginine-rich CPP-mediated DNA, while a limited effect of pyrenebutyrate on more hydrophobic CPPs is observed [13]. Jiao *et al*. provided evidence that direct membrane translocation occurs at low CPP concentrations, while endocytosis occurs at higher concentrations [79]. Reuter *et al*. found that fluid membranes are more easily penetrated by polypeptide CPPs than gel phase membranes [80].

8. CHALLENGES & FUTURE OF CPPs

Tools to deliver recombinant nucleic acids or functional proteins are finding increasing use in biomedical applications [81]. Novel, naturally-occurring CPPs, including surfactant protein B, orexin, lactoferricin [82], N-terminal peptides of unprocessed prion proteins [83] and helix-loop-helix (HLH) domains [84], have been identified and characterized. Using a phage display system [85] or a random peptide library [86], synthetic CPPs have been systematically created and screened. Furthermore, CPP could serve as a useful tag to facilitate unidirectional cloning of PCR-amplified DNA fragments in addition to cellular protein delivery [87]. These properties emphasize the versatility of CPPs.

The understanding of uptake mechanisms could help us design CPPs with greater protein transduction efficiency. The mechanism of cellular internalization of CPPs remains an area of vigorous investigation. In addition to molecular assays using pharmacological inhibitors and RNAi technology, analytical methods, such as high performance liquid chromatography (HPLC) analysis, size exclusion chromatography and matrix-assisted laser desorption inoization-time of flight (MALDI-TOF) mass spectrometry, provide additional tools to examine the internalization process [88]. While compelling evidence indicates transport by endocytosis and direct membrane translocation, there is considerable uncertainty regarding the precise processes involved [67]. For instance, Gump *et al*. demonstrated Tat-PTD-mediated induction of macropinocytosis after the removal of negatively charged heparin sulfate proteoglycans on the cell surface. Thus, while Tat-PTD can bind to these acidic polysaccharides, the binding is independent of the PTD-mediated transduction. The authors hypothesize that other, unidentified surface protein(s) may alternately serve the transduction [89]. Another question concerns the size of the transient transmembrane pore involved in direct membrane translocation. While empirical findings suggest transduction of R9 via transient transmembrane pores, evidence from molecular-dynamics simulations does not predict pores of sufficient size for transduction [77]. It is possible that the simulation model requires further calibration, or that the artificial planar lipid bilayer membrane used is an inadequate membrane model.

Although the use of CPP-fusion proteins is an effective transduction system, construction of recombinant CPP-fusion proteins or posttranslational linking CPP with a cargo remains very time- and labor-consuming processes. Fortunately, our studies indicate that the formation of noncovalent complexes of CPPs with cargos is fast, easy and effective. Moreover, the noncovalent system is versatile with respect to the nature of the cargo; proteins, DNA, RNA and nanoparticles are all candidates for NPT. Accordingly, CPPs are living up to their initial promise as carriers for biomedical applications.

CONFLICT OF INTEREST

The authors confirm no conflicts of interest with the contents of this article.

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ABBREVIATIONS

CNPT = Covalent and noncovalent protein transductions

CPP = Cell-penetrating peptide

CPT = Covalent protein transduction

EGFP = Enhanced green fluorescent protein

GFP = Green fluorescent protein

HIV = Human immunodeficiency virus

NPT = Noncovalent protein transduction

PTD = Protein transduction domain

QD = Quantum dot

RFP = Red fluorescent protein

RNAi = RNA interference

siRNA = Small interfering RNA

SR9 = Synthetic nona-arginine

R9 = Nona-arginine

Tat = Transactivator of transcription

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