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Cell-penetrating peptides: Possible transduction mechanisms and therapeutic applications (Review)

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Abstract. Cell-penetrating peptides (CPPs), also known as protein transduction domains, are a class of diverse peptides with 5-30 amino acids. CPPs are divided into cationic, amphipathic and hydrophobic CPPs. They are able to carry small molecules, plasmid DNA, small interfering RNA, proteins, viruses, imaging agents and other various nanoparticles across the cellular membrane, resulting in internalization of the intact cargos. However, the mechanisms of CPP internalization remain to be elucidated. Recently, CPPs have received considerable attention due to their high transduction efficiency and low cytotoxicity. These peptides have a significant potential for diagnostic and therapeutic applications, such as delivery of fluorescent or radioactive compounds for imaging, delivery of peptides and proteins for therapeutic application, and delivery of molecules into induced pluripotent stem cells for directing differentiation. The present study reviews the classifications and transduction mechanisms of CPPs, as well as their potential applications.

Contents

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1. Introduction

The cellular membrane is an effective semi-permeable barrier that is essential for cell survival and function. However, it is also a major obstacle for intracellular delivery of cargos for diagnosis and treatment of human diseases. Small molecules enter cells through specific carriers and channels in the membrane. However, macromolecules are generally unable to use these modes of entry into cells. Thus, it is important to develop tools to facilitate cellular uptake of large molecules for basic research and biomedical applications.

Cell-penetrating peptides (CPPs) are a promising class of short peptides with the ability to translocate across the cell membrane (1). CPPs generally contain 5-30 amino acids. In 1988, two independent groups reported transactivator of transcription (Tat) protein of the human immunodeficiency virus (HIV) as the first CPP. Tat has the ability to enter cultured mammalian cells and promote viral gene expression (2,3). Subsequently, several polycationic CPPs have been identified. For example, Antp, the third helix of the homeotic protein of *Drosophila melanogaster* Antennapedia, can enter nerve cells and regulate neural morphogenesis (4), and vp22, the herpes virus structural protein, has potential in protein delivery (5). CPPs can act as carriers as they have the ability to deliver macromolecular cargos, such as polypeptides (6), nanoparticles (7) and oligonucleotides (8) into cells. However, the mechanisms of CPP internalization are mostly unknown. The possible mechanisms are direct penetration, endocytosis and translocation through the formation of a transitory structure. The present review provides a broad overview of the classification, mechanisms of transduction and potential applications of CPPs.

2. Classification of cell-penetrating peptides

General. The classification of CPPs varies based on their physicochemical properties. In general, CPPs can be divided into three classes: Cationic, amphipathic and hydrophobic (Table I) (9). Currently, >100 different CPPs have been reported and patented. More than 83% of CPPs, which includes Tat, the first identified CPP, have a net-positive charge. Amphipathic CPPs, which comprise cationic and anionic peptides, are 44% of CPPS, while only 15% of CPPs are hydrophobic (10).

Cationic CPPs. Cationic peptides are a class of peptides that contain a high positive charge. The first CPP derived from the HIV-1 protein Tat is a cationic peptide. The majority of cationic peptides are naturally occurring peptide sequences. Recently, several artificial cationic peptides have been developed, includeing homo-polymers of arginine (11) and lysine (12). Studies on arginine-based peptides (from R3 to R12) have shown that the minimal sequence necessary for cellular uptake is six arginines, and that increasing the number of arginine residues increased transduction efficiency (13). In comparison, increasing the number of lysine residues reduced uptake of polylysine CPPs. However, arginine and lysine homopolymers >12 amino acids show reduced transduction efficiency (14). Nuclear localization sequences (NLSs) are a special type of cationic CPPs, which facilitate translocation into the nucleus through the nuclear pore complex (15).

Amphipathic CPPs. Amphipathic CPPs are chimeric peptides, several of which are obtained by the covalent attachment of a hydrophobic domain to an NLS, such as MAP and MPG sequences (16). For example, MPG (GALFLGWLGAAGSTMGAPKKKRKV) is based on the SV40 NLS PKKRKV, and the hydrophobic domain derived from the fusion sequence of the HIV glycoprotein 41 (17). Several other primary amphipathic CPPs, such as pVEC (18), ARF (1-22) (19), and BPrPp (1-28) (20), are derived from natural proteins.

Amphipathic α-helix is the most common structural motif of numerous peptides and proteins. Amphipathic α-helical CPPs have a highly hydrophobic patch on one face, whereas the other face can be cationic, anionic or polar. An amphipathic β-sheet peptide is developed based on one hydrophobic and one hydrophilic stretch of amino acids exposed to the solvent. Studies on VT5 (DPKGDPKGVTVTVTVTVTGKGDPKPD) have shown that the formation of β-sheets is essential for its cellular uptake (21,22). Proline-rich CPPs are a family of CPPs with diverse sequences and structures. However, their common structure has a proline pyrrolidine template (23).

Hydrophobic CPPs. Hydrophobic CPPs are derived from signal peptide sequences and contain only apolar residues. These peptides include transportan (24), stapled peptides (25), prenylated peptides (26) and pepducins (27). Thus far, only a few hydrophobic CPPs, including SG3 (28), Pep-7 (29), and fibroblast-growth factor (30), have been reported. Compared to cationic and amphipathic peptides, the potential application and mechanism of hydrophobic CPP translocation are less studied.

3. Uptake mechanism of cell-penetrating peptides

The intracellular CPP uptake mechanism has remained elusive since the discovery that Tat was cell permeable. Although the exact mechanism of entrance of CPPs into cells has not been completely resolved (31-33), it is widely believed that the CPP uptake mechanism varies for different CPP families, and the majority of CPPs have two or more pathways depending on the experimental conditions. Recent advances have shown that that there are three mechanisms for CPP translocation across the cellular membrane (Table II) (34,35).

Direct penetration. The direct penetration pathway is energy-independent. Early studies showed that Tat and pAntp can enter a cell at 4˚C (36,37). Veach *et al* (38) reported that Tat has the same cell-penetrating efficiency at 4 and 37˚C, and the internalization process is not blocked in cells without adenosine triphosphate. In order to prove this mechanism, certain membrane models have been constructed, such as transient pore formation (39), the carpet-like model (40) and the membrane-thinning model (41). The common features of these models are that CPPs first bind to the cell membrane via electrostatic or hydrophobic interactions and induce a brief or prolonged membrane destabilization of the binding sites, leading to CPP entrance into the cells. The internalization coefficient is relative to the peptide concentration, peptide sequence and lipid composition in each model.

Endocytosis-mediated translocation. Unlike direct penetration, this pathway is energy-dependent. During the course of endocytosis-mediated translocation, cells obtain energy from outside of the membrane. Richard *et al* (42) studied the mechanisms of Tat and polyarginine translocation using fluorescence microscopy in living cells. They found that Tat and polyarginine enter into the cells via endocytosis. This transduction mechanism is further divided into two classes of endoycytosis: Phagocytosis and pinocytosis. Phagocytosis is used for absorption of large particles and pinocytosis is used for solute absorption (32). Pinocytosis exists in all cell types. Endocytosis of CPP as macropinocytosis, clathrin-dependent pathway, cholesterol-dependent clathrin-mediated pathway and caveolin/clathrin-independent pathway has been reported (43-45).

Translocation via the formation of a transitory membrane structure. The translocation via the formation of a transitory membrane structure mechanism depends on the structure of inverted micelles to allow the peptide to bind a hydrophilic environment (46). In this model, a penetrating dimer combines with the negatively charged phospholipids leading to the formation of an inverted micelle inside the lipid bilayer (9). Arginine-rich peptides permeate the plasma membrane via this pathway (47).

Taken together, the CPP uptake mechanism remains largely unknown (43). The mechanism of CPP uptake may vary considerably according to CPP, CPP-cargo, cell types and concentration (17,48,49). Additionally, physicochemical parameters, incubation temperature and time should also be considered (50,51). Endocytosis is believed to be the dominant mechanism for the majority of CPP uptake. However, it is most likely that different transduction mechanisms may contribute under different conditions for the majority, if not all, CPPs.

4. Application of cell-penetrating peptides

CPPs have the capability to deliver various cargoes without causing any cellular injury. Thus, a wide range of CPP applications are being developed, such as imaging agents and vehicles to deliver therapeutic drugs, small interfering RNA (siRNA), nucleotides, proteins and peptides. The main applications of CPPs are shown in Table III.

Table I. Cell-penetrating peptide classifications and sequences.

Study, year	Classification	Peptide	Sequences	Main trait	Refs.
Green and Loewenstein, 1988	Cationic	Tat	GRKKRRQRRRPPQ	High positive charge	(2)
Frankel and Pabo, 1988					(3)
Joliot *et al*, 1991		Antp	RQIKIWFQNRRMKWKK		(4)
Ragin *et al*, 2002		NLS	CGYGPKKKRKVGG		(15)
Wender *et al*, 2000		8-Arginine	RRRRRRRR		(11)
Mai *et al*, 2002		8-Lysine	KKKKKKKK		(12)
Oehlke *et al*, 1998	Amphipathic	MPG	GLAFLGFLGAAGSTM GAWSQPKKKRKV	Chimeric peptides	(16)
Deshayes *et al*, 2004		pVEC	LLIILRRRIRKQAHAHSK		(17)
Nan *et al*, 2011		ARF (1-22)	MVRRFLVTL RIRRACGPPRVRV		(18)
Johansson *et al*, 2008		BPrPp (1-28)	MVKSKIGSWILVLFV SDVGLCKKRP		(19)
Elliot and O'Hare, 1997		VP22	NAATATRGRSAASRPTQR PRAPARSASRPRRPVQ		(5)
Magzoub *et al*, 2006		VT5	DPKGDPKGVTVT VTVTVTGKGDPKPD		(20)
Eguchi and Dowdy, 2009					(21)
Oehlke *et al*, 1998		MAP	KLALKLALK ALKAALKLA		(16)
Pujals and Giralt, 2008	Hydrophobic	Transportan	GWTLNSAGYLLG KINLKALAALAKKIL	Contain only apolar residues; have a low net charge	(23)
Gao *et al*, 2011		SG3	RLSGMNEVLSFRW		(28)
Gao *et al*, 2002		Pep-7	SDLWEMMMVSLACQY		(29)
Nakayama *et al*, 2011		FGF	PIEVCMYREP		(30)

Table II. Cell-penetrating peptide uptake mechanisms.

Study, year	Pathway	Main trait	Examples	Refs.
Vives *et al*, 1997	Direct penetration	Energy-independent	Tat peptide	(36)
Derossi *et al*, 1994	Direct penetration	Energy-independent	pAntp	(37)
Richard *et al*, 2003	Endocytosis	Energy-dependent	Polyarginine	(42)
Nan *et al*, 2011	Endocytosis	Energy-dependent	ARF (1-22)	(18)
Kawamoto *et al*, 2011	Via the formation of a transitory membrane structure	Formation of the inverted micelles	Arginine-rich peptide	(47)

Imaging. Intracellular imaging has potential to improve disease management by detecting disease markers, but its application is limited due to the poor permeability of proteins. CPPs can function as vectors to carry fluorescent particles into cells due to their internalization properties and have become promising tools for delivering imaging agents, contrast agents and quantum dots (QDs) in the field of imaging. The advantage of such imaging technology is the ability to visualize and quantify biomarkers or biochemical and cellular processes, detect the stage of diseases, identify the extent of disease and measure the effect of treatment (52,53).

The size of QDs generally falls within the 2-10 nanometer range; QDs are brighter and more stable against photobleaching than standard fluorescent indicators, and thus QDs have emerged as an alternative to organic dyes and fluorescent proteins (54). QDs have been extensively studied for biological imaging, but their inability to cross the cellular membrane has limited their application. This limitation has been overcome by the discovery of CPPs. Ruan *et al* (55) used Tat peptide-conjugated QDs (Tat-QDs) to examine the complex behavior of nanoparticle probes in live cells and found that Tat-QDs are internalized by macropinocytosis. The internalized Tat-QDs are tethered to the inner vesicle surfaces and are trapped in cytoplasmic organelles. The study also revealed that Tat-QDs strongly bind to cellular membrane structures. Their research provides new insights for molecular

Table III. Cell-penetrating peptide applications.

Study, year	Application	Examples	Refs.
Ruan *et al*, 2007	Imaging	Tat-QDs	(55)
Lei *et al*, 2008			(56)
Prantner *et al*, 2003		Gd-DOTA-D-Tat	(57)
Polyakov *et al*, 2000		Tat-(99m)Tc	(58)
Deshayes *et al*, 2010	Anti-inflammation	CPP-PNA	(63)
Tan *et al*, 2005			(64)
Tilley *et al*, 2007		CPP-PMO	(65)
Davé *et al*, 2007		CPP-NBD	(71)
Peterson *et al*, 2011		Antp-NBD	(72)
Koshkaryev *et al*, 2013	Tumor therapy	R8-DOPE-BLM	(76)
Walker *et al*, 2012		DOXO-ELP-CPP	(77)
Aroui *et al*, 2010		Dox	(78)
Dubikovskaya *et al*, 2008		Taxol	(79)
Lindgren *et al*, 2006		Methotrexate	(80)
Eguchi and Dowdy, 2009	Nucleic acid and Protein delivery	CPP-siRNA	(21)
Muratovska and Eccles, 2004			(82)
Favaro *et al*, 2014		T-Rp3	(83)
Eto *et al*, 2009	Viral delivery	CPP-Adv	(86)

imaging and targeted therapy. In another study, Tat-QDs were efficiently introduced into living mesenchymal stem cells (56). Other imaging applications of CPPs have also been developed. The Gd-DOTA-D-Tat peptide conjugate can enter into the cell interior resulting in intracellular T1 relaxation enhancement (57); Tat-(99m)Tc conjugates can be applied for imaging and radiotherapy (58). Tat-(99m)Tc conjugates have also been developed for imaging in prostate and breast cancer (59,60). A hydrogen peroxide-activated CPP was developed to observe in vivo lung inflammation, suggesting that CPPs have the potential for imaging and treating diseases related to oxidative stress (61).

Anti-inflammation therapy. Antisense peptide nucleic acids (PNAs) have been shown to specifically inhibit gene expression and growth of Escherichia coli, and are a promising anti-inflammatroy agent (62). Accordingly, PNA conjugated with CPPs (CPP-PNA) have been developed for efficient delivery of PNAs (63). For example, administration of the acpP-targeting PNA conjugated to CPP into *Escherichia coli* K-12-infected BALB/c mice reduced bacterial blood contents, prevented fatal infection and enhanced survival of the infected mice (64). Similar results were observed for the CPP-PMO conjugate targeted to the same acpP administered to mice infected with *Escherichia coli* (65). The results demonstrated an antibiotic effect of these CPP-PNA conjugates.

Nuclear factor-κB (NF-κB) has an important role in the inflammation response. Inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), can activate NF-κB and induce the inflammatory reaction. It has been well documented that certain inflammatory diseases, such as rheumatoid arthritis (66), atherosclerosis (67), Parkinson's disease (68) and inflammatory bowel disorders (IBD) (69), are associated with the activation of NF-κB. IBD in particular is characterized by sustained upregulation of inflammatory factors, such as TNF-α, IL-6 and IL-1, accompanied by increased activity of NF-κB. It has been proposed that blocking the activation of NF-κB can prevent certain chronic inflammation (70). The NEMO binding domain (NBD) of IKK can block NF-κB activation. In a mouse model of IBD, intraperitoneal injection of CPP-NBD resulted in downregulation of inflammatory factors and amelioration of the disease (71), suggesting that CPP-NBD may be used in the treatment of IBD. In another study, intraperitoneal injection of Antp-NBD fusion peptide in a Duchenne muscular dystrophy mouse model decreased NF-κB activation and muscle necrosis, and increased muscle regeneration (72).

Tumor therapy. Cancer is an important public health issue and has become the leading killer of human beings (73). Conventional chemotherapy has a low drug concentration at local tumor areas and can cause severe side effects due to lack of tumor cell specificity (74). New efficient and tumor targeting strategies should be developed to overcome this limitation. Conjugation of anticancer agents with CPPs has improved tumor therapy. CPP-delivered anticancer therapeutics can increase cellular membrane permeability of anticancer drugs to target tumor cells, expanding the broad application of CPPs in tumor therapy (75). Bleomycin (BLM) is an anticancer drug that has been used extensively, but its efficiency depends on its cytosolic accumulation. The artificial R8-DOPE-BLM conjugate can enter into the cytosol and cause a stronger induction of tumor cell death and DNA damage in vitro compared to BLM (76). Elastin-like polypeptide (ELP) can passively accumulate in solid tumors and aggregate in tumor tissue when exposed to hyperthermia. Injection of a conjugate

of doxorubicin with ELP and CPP in a C57BL/6 mouse breast cancer model resulted in augmented internalization of doxorubicin and reduced tumor size more than two-fold compared to free doxorubicin (77). Similar results have been obtained by conjugation of CPP with doxirubicin (78), Taxol (79) and methotrexate (80). These data demonstrate that CPP-delivered anticancer agents can improve drug concentration at the tumor tissue and increase the treatment effect.

Nucleic acid and protein delivery. Larger macromolecules, such as nucleic acids and proteins, are unable to penetrate the plasma membrane and enter into cells. CPPs can facilitate cellular uptake of large molecules and have been developed as a delivery tool for nucleic acids and proteins. siRNA have been widely used for gene silencing and used to treat diseases such as cancer, infectious diseases and genetic disorders (81). CPPs can overcome the barrier of poor permeability and lead to the internalization of siRNA (21). A CPP-siRNA complex synthesized via a disulfide bond has been shown to efficiently reduce transient and stable expression of reporter transgenes in several mammalian cell types (82), suggesting that CPP-siRNA has a potential application in siRNA-based therapy.

Recently, CPPs have also been conjugated to protein. A modular protein (T-Rp3) fused to an N-terminal DNA-binding domain and a C-terminal membrane Tat peptide was successfully expressed in *Escherichia coli*. Treatment of HeLa cells with this purified recombinant protein improved the delivery of T-Rp3 (83). Similarly, N-stearylated peptide has a low transfection activity; however, an N-terminal stearylated NLS (PKKKRKV) conjugated to CPP effectively promoted the nuclear translocation of N-stearylated peptide (84).

Viral delivery. CPPs can also be applied to enhance the efficiency of viral transduction (85). Adenoviral vector (Adv) has been extensively used in basic and clinical research due to its high transduction efficiency. However, Adv has poor infection efficiency in cells lacking the primary adenovirus receptor, as well as the coxsackievirus receptor (86). Adv bound to CPP can overcome this barrier (87). Adv conjugated to CPPs (CPP-Adv) by chemical conjugation results in higher gene expression, indicating that CPP-modified Adv as a delivery vector is an attractive tool for transducing cells and gene therapy (86).

Directing induced pluripotent stem cells (iPS) differentiation. iPS generated directly from somatic cells can differentiate into various cell types (88). Delivering certain molecules into iPS cells can direct cell-type specific differentiation, which can be used for disease modeling, drug screening and cell transplantation therapies (89). However, these applications are limited as iPS cells are generally difficult to transfect. Previous studies have shown that transfecting certain cytokines and growth factors can promote human iPS cell differentiation into lung (90) and retinal cells (91), but these delivery tools are lentiviral or Advs. Viral vectors can infect iPS cells, but present a risk of genomic integration of exogenous viral genes (92). Plasmid DNA transfection with cationic lipids can overcome this risk; however, the transfection efficiency is relatively low (93). CPP may be a powerful tool for delivering exogenous proteins into iPS cells, eliminating the risk of exogenous genomic integration, while promoting high transduction efficiency.

5. Conclusion

CPPs are a class of small peptides 5-30 amino acids in length that have the potential to transport numerous types of therapeutic agents across the cellular membrane into cells. However, cellular CPP uptake mechanisms remain to be elucidated. CPPs have been widely used as a delivery vector due to their high transduction efficiency and capacity for delivering large molecules into a cell. CPPs are used to deliver fluorescent proteins to detect disease markers and manage disease. CPPs as vectors delivering therapeutic agents have proved effective in certain disease models, such as inflammatory disease and cancer. Additionally, CPPs can transport certain molecules into iPS cells to direct iPS cell-type specific differentiation. In conclusion, the application of CPPs for delivering a variety of agents into cells has promising clinical potential.

However, although there is a potential for CPP applications as diagnostic or therapeutic agents, there are no published human studies supporting their use. Several limitations should be addressed prior to using CPP-based diagnostic and therapeutics applications in the clinic. First, the best route of drug administration is oral uptake; however, there have been no detailed studies on the oral bioavailability of CPPs. Second, the majority of the reported CPPs are not tissue and organ-specific, which may cause severe side effects. Screening specific CPPs via a phage-display library may solve this problem. Additionally, kidney and liver toxicity should be considered as a new drug or therapeutic application.

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