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Editorial

Cell-penetrating peptides—A brief introduction

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1. Introduction

The ability to translocate the cellular membranes and gain access to the cell interior, including the different cellular compartments, still remains a major obstacle in current drug development. Peptide mediated delivery of bioactive molecules appears to be a technology that in many aspects is superior to commonly used delivery agents. Reported high delivery yield, low toxicity and the possibility to add diverse modifications to the peptide backbone make peptides an excellent candidate for future drug delivery platforms. So-called cell-penetrating peptides (CPPs), also often referred to as protein transduction domains (PTDs), Trojan peptides or membrane translocating sequences (MTS), have in recent years shown great potential in the field of drug delivery. Current publications show that CPPs can deliver a wide range of bioactive molecules such as proteins, peptides, oligonucleotides (ON), and nano-particles to a variety cell types and to different cellular compartments, both in vivo and in vitro. The peptides named CPPs vary greatly in size, amino acid sequence, and charge, but share the common feature that they have the ability to rapidly translocate the plasma membrane and enable delivery to the cytoplasm or nucleus [1].

The idea to use peptides used as delivery vectors, i.e. CPPs, originates from so-called membrane shuttling proteins such as the *Drosophila* homeobox protein Antennapedia, the HIV-1 transcriptional factor TAT, and the capsid protein VP22 from HSV-1. The field started in 1988, when Green et al. showed that the viral protein TAT rapidly translocate over cellular membrane, into the cytoplasm [2]. Later, the same properties were shown for a *Drosophila* homeobox protein. In 1994, Alain Prochiantz' group demonstrated that a short, 16 amino acid (aa) peptide derived from the third loop of the Antennapedia protein was responsible for the cellular translocation of the whole protein [3]. This pioneering work initiated the whole field using peptides as efficient delivery vectors for bioactive compounds, cell-penetrating peptides.

Since then, a myriad of peptides has been reported to have cell-penetrating properties. The peptides originate from different classes; either naturally occurring peptide sequences such as virally derived (TAT, VP22), from transcription factors (pAntp), chimeric peptides (transportan, MGP) or synthetic (polyarginines, Pep-1), cf. Table 1.

However, due to difficulties in understanding the true mechanisms of CPP cellular uptake, the classification of CPPs still remains to be clarified.

Although great achievements in studies of CPP have been attained, a clear description of their properties is still not defined.

The CPP field has been under constant change during the years and the uptake mechanism still remains ambiguous. Several attempts have been made in order to elucidate the true mechanism of peptide mediated uptake, but the results are divergent between different reports and experiments. Even when using the same peptide, results vary between different publications. Furthermore, it seems apparent that different peptides utilize different uptake pathways [4].

Early studies on CPP translocation mechanisms suggested that the internalization of these peptides was not inhibited by depletion of the cellular adenosine triphosphate (ATP) pool, low temperature (+4 °C), or by inhibitors of endocytosis [5]. Neither did chemical modifications of the peptide sequences, such as the synthesis of retro-, enantio- or retroenantio-analogs, appear to affect the internalization properties [6]. Therefore, translocation was thought to result from direct transfer through the lipid bilayer of the cell membrane.

Formation of inverted micelles was a proposed mechanism for these uptakes, where cationic residues interact with the negatively charged plasma membrane followed by invagination of tryptophans into the membrane, inducing inverted micelle formation [6]. The postulated mechanism of inverted micelle formation seems to explain some aspects of CPP translocation and still appear to hold for some peptides used in cellular delivery of bioactive molecules.

Later studies, on the other hand, show that the CPP translocation mostly is an energy-dependent process [7]. Proposed mechanisms involve extracellular heparane sulfate [8] and different types of endocytosis [9,10] such as macropinocytosis, clathrin-dependent-, caveoale-dependent- or clathrin- and caveoale-independent endocytosis. It seems to us that these processes do not necessarily contradict each other rather demonstrating that peptide mediated membrane translocation is mediated by several different pathways simultaneously, or that different peptides utilize diverse uptake mechanisms depending on their cargo and biophysical properties [11].

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Table 1
A selection of commonly used CPP

Name	Sequence [a]	Origin	Ref.
Penetratin, pAntp	RQIKIWFQNRRMKWKK [a]	*D. melanogaster* transcription factor	[3]
HIV TAT peptide (48–60)	GRKKRRQRRRPPQ [b]	Viral transcriptional regulator	[33]
HSV-1 VP22 peptide	DAATATRGRSAASRPTERPRAPARSASRPRRVD	Viral Capsid protein	[34]
MAP (Model amphiphilic peptide)	KLALKLALKALKAALKLA-amide	Synthetic	[35]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-amide	Chimeric galanin-mastoparan	[36]
R7	RRRRRRR	Synthetic	[37]
MPG	GALFLGWLGAAGSTMGAPKKKRKV [c]	Chimeric HIV-1 gp41-SV40 large T antigen	[38]
Pep-1	KETWWETWWTEWSQPKKKRKV [c]	Synthetic	[26]

[a] All peptides are C-terminal free acids unless stated otherwise.
[b] One selected sequence, may vary from different studies.
[c] C-terminally modified with a cysteamide group.

The main explanation today seems to be that endosomal pathways contribute to the major route of uptake of CPPs, although several reports show an uptake independent of endocytosis [4,12,13]. The uptake mechanism could also be altered by different cargoes, if the peptides form a stable complex with its cargo, if the cargo is covalently bound to a CPP or how the cargo is attach [14] (Fig. 1).

2. Targeted and enhanced delivery

Since CPPs penetrate virtually any all cell type both in vivo and in vitro, the delivery seems to utilize a pathway(s) present in all cells. This common feature makes CPP applications complicated in pharmaceutical use. The peptides seem to enter any and every cell they get in contact with, which restricts CPP application as a pharmaceutical tool greatly. Recent results, however, show promising results in targeted CPP delivery [15,16], By exploiting explicit cell features such as extracellular receptors and proteases or addition of cell specific ligands such as small molecules, vitamins, carbohydrates, other peptides or proteins (growth factors or antibodies) to known CPPs may improve targeted delivery [17].

The stability of peptide vectors is another problem regarding in vivo delivery. If the vector does not remain intact until it reaches its target, it could do more harm than good. This is most often solved by using the D-form [18], instead of naturally occurring L-amino

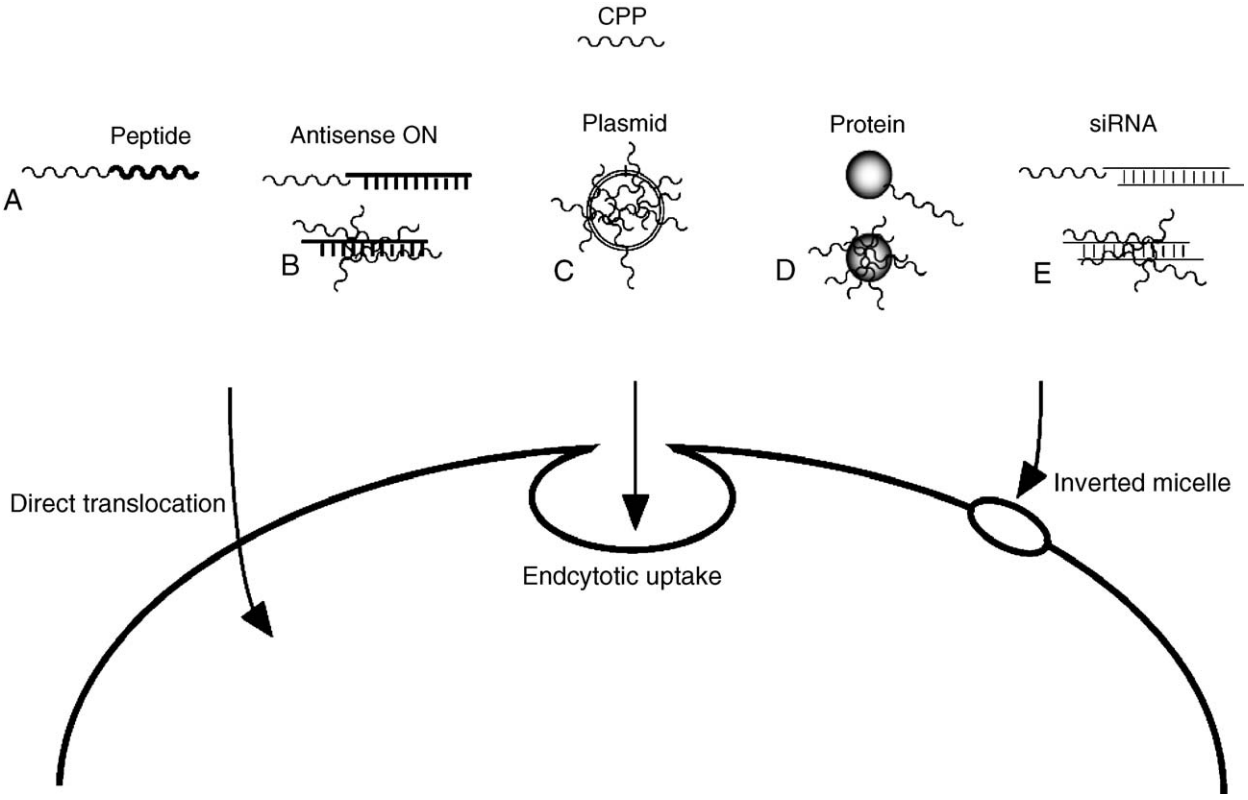

Fig. 1. Suggested uptake mechanisms for CPPs and examples of delivered cargoes. (A) CPP and peptide in single amino acid chain. (B) ON either in complex or covalently linked. (C) Plasmid in complex by electrostatic interaction. (D) Protein either as fusion protein or in complex with CPP. (E) siRNA, covalently linked or as complex.

acids. The D-form is not degraded by proteases to the same extent and remains intact for a longer time when injected in vivo. Other possibilities are to use peptide mimics such as beta-peptides [19] or peptoids [20] to enhance vector stability.

To increase delivery yield, functional groups can be included in the CPP sequence. For instance, addition of 20 amino acids from the pH-sensitive fusogenic peptide HA2 derived from the N-terminal part of influenza virus hemagglutinin protein, to a TAT-peptide fusion protein enhanced protein uptake significantly [21]. HA2 is a pH-sensitive fusogenic peptide that destabilizes lipid membranes at low pH and thereby enhances endosomal escape which leads to improved delivery [22].

3. Commercially available peptides

In recent years, biotech companies have shown interest in CPP technologies, and some CPPs have become commercially available. Most well known is probably the transport system Chariot (Active Motif, France, http://www.activemotif.com), formally known as Pep-1 (Table 1). Chariot is distributed as a protein transfection agent that uses non-covalent interactions with the macromolecule of interest to be delivered. The uptake is fast (about 2 h) and it stabilizes proteins protecting it from degradation during the transfection process. After delivery, the peptide dissociates from the delivered molecule, leaving it biologically active and free to proceed to its objective. Delivery is proposed to be non-toxic and independent of the endosomal pathways.

Other examples are the SynB vectors [23] from Synt:em (www.syntem.com), France, and Express-si Delivery from Genospectra (http://www.genospectra.com) U.S.A.

SynB vectors are a new family of vectors derived from the antimicrobial peptide protegrin 1 (PG-1), an 18 amino acid peptide originally isolated from porcine leucocytes. These vectors were applied for delivery of e.g. doxorubicin and camptothecin, both well-known anti-cancer drugs.

Express-si is based on the MPG peptide (Table 1) and is distributed as a siRNA and oligonucleotide delivery vector. The uptake mechanism is like Chariot proposed to be receptor-independent and does not utilize endocytic pathways.

These examples, together with published reports from pharma-industry on CPP applications, demonstrate clearly the future perspective of CPPs in drug development.

4. Delivery of proteins

To use CPPs as vectors for protein delivery can have many advantages over the more traditional approach to endogenously express the desired protein from a plasmid such as difficulties with transfection and varied protein expression in various cell lines. Several proteins have been transported into cells by CPP mediated translocation including proteins involved in apoptosis [24], cell cycle regulation and DNA recombination [21].

By designing a plasmid coding for of a CPP in coding frame with the desired protein, a cell permeable fusion protein is produced. This method has successfully been applied both in vivo and in vitro [25]. Addition of a highly cationic peptide in the same amino acids chain as a protein may interfere with the proteins tertiary structure and thereby cause loss of biological function. This problem can be overcome by simply co-incubate a CPP and the desired protein and thus facilitate cellular uptake [26]. The protein thereby keeps its native structure and the proteins biological properties are conserved.

5. Delivery of siRNA and other oligonucleotides

Many oligonucleotides and their modified analogues have been reported to be delivered efficiently by CPPs into several cell lines both in vivo [27] and in vitro [28]. Oligonucleotides (ON) delivered by CPPs include antisense-ON [27], DNA decoy-ON [29], siRNA [30] and plasmids [31].

RNA induced gene silencing through siRNA has emerged as a powerful tool in recent years. As for many other biologically active compounds, delivery over the plasma membrane remains one of the major obstacles in siRNA applications. A few peptides have been shown to improve siRNA uptake and down-regulate the desired gene. The CPP and siRNA have either been covalently linked via a disulfide bond [32], or the RNA and the peptide form a complex through electrostatic interactions [30] and are translocated across the plasma membrane.

6. Future of CPP mediated transport

Despite the ongoing research concerning the actual transport pathway utilized by CPPs, they have been shown to efficiently deliver a wide variety of bioactive compounds into a number of cell lines in vitro. In vivo, CPPs can cross the blood–brain barrier and mediate delivery to several organs. Targeted delivery by CPP is a possible goal, and recent reports show great promise in this field. There is an increasing need for an efficient, non-toxic, non-hazardous transport vector. Cell-penetrating peptides fulfill all of these criteria and may in the future be an important tool in pharmaceutical research.

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