The use of cell-penetrating peptides as a tool for gene regulation

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A novel carrier system that originates from membrane shuttling proteins such as the *Drosophila* homeobox protein Antennapedia, the HIV-1 transcriptional factor TAT and VP22 from HSV-1 has advantages for targeted delivery compared with standard translocation techniques. This transport system is mediated by so-called cell-penetrating peptides, which consist of short peptide sequences that rapidly translocate large molecules into the cell interior in a seemingly energy- and receptor-independent manner. Cell-penetrating peptides have low toxicity and a high yield of delivery and in the future might become a widely used tool in the field of gene regulation.

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▼ The potential influence of gene regulation on modern science and pharmaceutical research is tremendous. Altering the levels of gene regulation or insertion and/or deletion of genes results in a rapid increase in the understanding of cellular functions, mechanisms and development. Furthermore, improving the tools that are used to influence the cell will enable the acquisition of more precise information about the cell, thereby improving our knowledge of human diseases.

The different cellular compartments (including the nucleus) are protected by biological membranes. These membranes segregate the various cellular compartments and prevent influx and efflux of solutes from cells and organelles. Although these barriers are essential for the maintenance of the cell, they can become a problem in cellular studies and must be overcome before the diverse processes occurring within the cell can be investigated.

Gene expression and gene regulation are key in most biological processes, for example, development, immune defense and tumorigenesis. To control these cell functions is not only of great importance in understanding cellular development and differentiation, but also in the struggle against many human diseases. Cell membranes are one of the major obstacles encountered when altering gene expression and various techniques have been developed to translocate biologically active molecules across these barriers *in vivo* and *in vitro*.

There are severe disadvantages associated with using many of the techniques currently available. For example, electroporation and microinjection are harsh and impractical to use *in vivo* because these methods necessitate disruption of the cell membrane before substances can be introduced into the cell and therefore they are restricted to a limited amount of cells. Other methods such as liposome encapsulation and receptor-mediated endocytosis are limited by their lack of targeting and low yield of delivery [1]. A novel class of membrane translocating agents is the cell-penetrating peptides (CPPs). Apart from being a mild and effective tool for access to different cellular organelles *in vitro*, CPPs have been used for cellular delivery of several agents *in vivo*, with promising results.

No unambiguous definition of CPPs has been proposed, but generally they consist of less than 30 amino acids, have a net positive charge and have the ability to translocate the plasma membrane and transport several different cargoes into the cytoplasm and nucleus in a seemingly energy-independent manner. Early reports show that translocation occurs via an as yet unknown mechanism that is not affected by various endocytosis inhibitors or low temperatures (i.e. +4°C) [2]. These features make CPPs an excellent tool as vectors for biologically active molecules and as biologically active molecules in gene regulation.

The proposed energy-independent pathway of CPP translocation has been recently questioned [3]. This issue and recent research in the field of CPPs and their application in gene regulation are considered in this review.

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Table 1. Selection of known CPP sequences[a]

Name	Sequence	Length	Net charge (+)	Isoelectric point[b]	Mw (Da)	Refs
Penetratin (pAntp)	RQIKIWFQNRRMKWKK[c]	16	7	12.4	2247	[4]
HIV TAT peptide 48–60[d]	GRKKRRQRRRPPQ	13	8	12.7	1719	[5]
MAP	KLALKLALKALKAALKLA-amide	18	5	11.4	1878	[50]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-amide	27	5	10.9	2842	[6]
Transportan 10	AGYLLGKINLKALAALAKKIL-amide	21	4	10.9	2183	[51]
R7 peptide	RRRRRRR	7	7	12.8	1111	[52]
pVEC	LLIILRRRIRKQAHAHSK-amide	18	8	12.5	2210	[53]
MPG peptide	GALFLGWLGAAGSTMGAPKKKRKV-amide	24	5	11.8	2445	[29]
KALA peptide	WEAKLAKALAKALAKHLAKALAKALKACEA	30	6	10.7	3132	[54]
Buforin 2	TRSSRAGLQFPVGRVHRLLRK	21	7	12.2	2435	[55]

[a]All peptides are *C*-terminal free acid unless stated otherwise.
[b]Calculated by WinPep (www.ipw.agrl.ethz.ch/~lhennig/winpep.html).
[c]Originally synthesized as *C*-terminal free acid, but later shown to have CPP properties when amidated at *C*-terminus [55].
[d]HIV TAT fragment 37–72 has been shown to have translocation ability. The fragment 48–60 is described in Ref. [5], but the amino acid sequence reported could vary with different studies.
Abbreviations: CPP, cell-penetrating peptide; MAP, model amphiphilic peptide; pAntp, pAntennapedia; TAT, transactivating regulatory protein.

Cell-penetrating peptides

The term CPP includes synthetic cell-permeable peptides, protein-transduction domains (PTD) and membrane-translocating sequences (MTS), which all have the ability to translocate the cell membrane and gain access to the cellular interior.

The first CPP, which was reported in 1994, derives from the third helix of the Antennapedia protein homeodomain [4] and was originally named pAntennapedia (pAntp). Today, this peptide is more commonly referred to as penetratin and, together with peptides such as the HIV protein derived transactivating regulatory protein (TAT) [5] and transportan [6], is one of the most extensively investigated CPP. Examples of sequences of known CPPs are listed in Table 1.

Although CPP translocation was initially thought to be an energy-independent process, recent findings suggest that the majority of CPP translocation occurs via an energy-dependent pathway and that CPP translocation is reduced by known endocytosis inhibitors [3,7,8]. However, studies show that the internalization of analogs of penetratin [9], TAT and the retro-inverso form of TAT in which all the amino acids are of the D-configuration is even more efficient than the internalization of the respective native peptides, which indicates that CPP internalization does not occur via a chiral receptor-mediated endocytotic pathway [10]. Moreover, a common translocation pathway for arginine-rich peptides has been suggested. Studies have shown that several endocytosis or caveolae inhibitors did not affect the uptake of arginine-rich peptides and it is thought that the mechanism of uptake could involve cell surface polysaccharides, for example, heparan sulfate [11,12]. However, not all known CPPs are rich in arginine residues (Table 1) and different peptides could use different pathways for uptake. In addition, the mechanism of translocation could be dependent on whether it is the free CPP or the CPP connected to a cargo that is investigated. The quantitative uptake of free CPP or CPP coupled to cargo can differ [13], but it has yet to be determined whether this variation is the result of translocation efficiency or the different translocation pathways of CPPs. It is possible that the conclusions presented are two different sides of the same coin, thus indicating that it is the competition between several pathways that contributes to the internalization of CPPs.

Additional studies of CPP translocation show that the observed uptake is merely an artifact from membrane disruption when fixating cells. The negative cell membrane attracts positively charged peptides, which could then be taken up by endosomes. Disruption of the membranes and endosomes through harsh cell fixation results in the release of peptides into the cytosol and nucleus, which leads to false positive evidence for a non-endocytotic translocation pathway [14]. These findings do not explain the biological effects caused by the CPP-mediated increased uptake of bioactive molecules observed *in vivo* and *in vitro*. Nevertheless, the harsh fixation methods for visualization of cell translocation should be avoided and the use of live cells for studies of CPP translocation is strongly recommended.

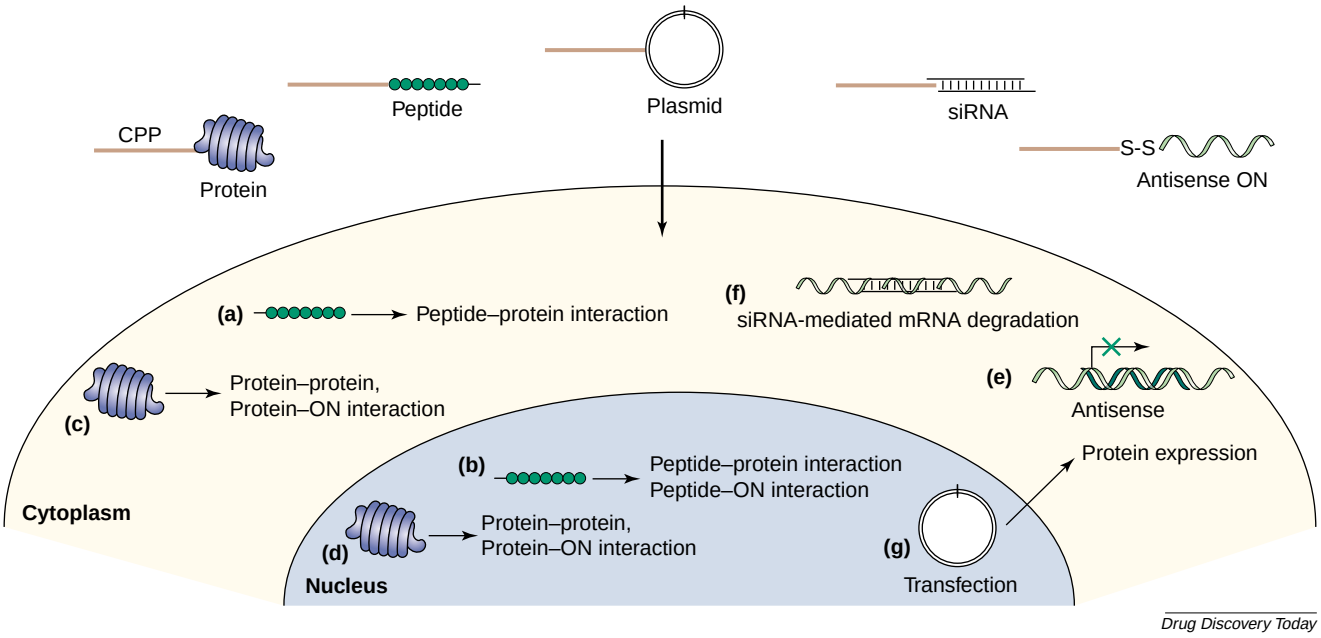

Figure 1. Selection of possible applications for peptide-mediated translocation. **(a)** Peptide–protein interaction in the cytoplasm. **(b)** Peptide–protein and peptide–ON interaction in the nucleus. **(c)** Protein–protein interaction in the cytoplasm. **(d)** Protein–protein and protein–ON interaction in the nucleus. **(e)** Antisense ON–mRNA hybridization. **(f)** siRNA-mediated mRNA degradation. **(g)** Transfected plasmid and protein expression. Abbreviations: CPP, cell-penetrating peptide; ON, oligonucleotide.

Although there is a lot of debate concerning the translocation pathways of CPPs, several peptide sequences have been shown to increase the level of uptake of numerous macromolecules *in vivo* and *in vitro*. Despite the fact that the actual pathway of CPP uptake has yet to be elucidated, peptides are still of great significance for improving cellular delivery locally and the efforts made so far indicate that CPPs are a promising tool for future pharmaceutical applications.

Peptide delivery

Peptides can be used in a vast range of applications in pharmaceutical research. By linking peptide sequences to CPPs, previously non-cell-penetrating peptides have been introduced to the cell and thus existing protein–protein or protein–oligonucleotide interactions can be altered. Several protein binding domains, including SH2, SH3 [15], BH3 [16] and peptides that mimic DNA binding sequences, have been fused to CPP [17], thus enabling their entry into the cytoplasm. The introduced protein domain homologs can inhibit protein binding or protein complex formation or can act as signaling sequences [Figure 1(a)].

By mimicking domains from transcriptional factors, for example, helix-1 from c-Myc [Table 2, Figure 1(b)] the introduced peptides can alter gene expression. The c-Myc helix-1 peptide inhibits cell growth, induces apoptosis in subconfluent and/or confluent cells and inhibits transcription of two c-Myc-regulated genes (ODC and p53) [17].

Polo-like kinase 1 (Plk-1) is known to play a crucial role in mitosis and is overexpressed in rapidly dividing cells and in several human tumors [18]. Fusion of the *C*-terminal polo-box from Plk-1 to penetratin (Table 2) led to a significant decrease in cell proliferation in different human cancer cell lines [19].

When peptides are introduced to the cell interior they are rapidly degraded and thus the effect of the peptide might not be as long lasting as desired. The use of an analog of the 20 amino acid long c-Jun *N*-terminal kinase (JNK) binding motif that comprised only D-amino acids fused to an all D-configured amino acid analog of the CPP TAT led to an increase in the neuroprotective effects of the peptide against neuronal death *in vivo* and *in vitro* [20].

Protein delivery for gene regulation

The transport of proteins into the cell is mainly mediated through endocytosis, which is a highly organized transport system [21]. Translocation of large molecules such as proteins across the plasma membrane requires a system that does not involve endocytosis, and thereby lysosomal degradation of the protein, or endosomal escape before lysosomal activity occurs. With the use of an efficient protein translocation system, the possibilities for gene regulation

Table 2. Selection of peptides and proteins that are delivered by peptide-mediated translocation[a]

Cargo	Example	CPP	Refs
Peptide	c-Myc helix-1	Penetratin	[17]
	p53 mdm-2 binding domain	Penetratin	[56]
	$p16^{INK4A}$	TAT	[57]
	Polo-box	Penetratin	[19]
	JNK-binding motif	TAT	[20]
Protein	IκBα	TAT	[58]
	p16	TAT	[59]
	Caspase 3	TAT	[60]
	Cre recombinase	FGF-4	[61]
	p53	TAT	[62]
	p73	TAT	[62]
	Cdk2	TAT	[63]
	E2F-1	TAT	[62]

[a]All peptides and proteins were prepared as fusion constructs between the peptide and protein and the CPP.
Abbreviations: CPP, cell-penetrating peptide; FGF, fibroblast growth factor; JNK, c-Jun *N*-terminal kinase. TAT, transactivating regulatory protein.

are tremendous: gene transcription regulating proteins can be transported into the cytoplasm and nucleus where they could upregulate or downregulate targeted genes either by DNA or mRNA binding or by perturbing specific protein–protein interactions [Figure 1(c, d)].

Proteins linked to CPPs have shown an increase in membrane translocation and the effect of increased protein uptake has been shown *in vivo* and *in vitro* [22]. Examples of proteins that are known to be delivered are listed in Table 2. Fawell *et al.* [22] demonstrated that larger fragments of TAT protein increased the uptake of β-galactosidase (β-Gal). Since this discovery, several other proteins that translocate the cell membrane with a higher efficiency when fused to a CPP have been identified. For example, CPP fused Cre recombinase has been delivered in mice. The Cre recombinase from bacteriophage P1 has been widely used to induce DNA sequence-specific recombination in mammalian cells [23]. LoxP sites, which serve as targets of Cre-mediated recombination in the P1 genome, also function as recombination substrates in mammalian cells. Applications involving Cre-loxP recombination include conditional mutagenesis [24], gene replacement [25], chromosome engineering [26] and conditional gene expression in mice [27]. These results show CPP as a useful tool for *in vivo* gene regulation. The methods used for coupling CPPs to proteins to regulate or to alter gene expression predominantly involve CPP–protein fusion constructs (Table 2) that are produced by transfected bacteria.

Delivery of antisense oligonucleotides for gene silencing

The use of antisense techniques as a pharmaceutical tool is interesting because this technology potentially has the ability to downregulate the expression of virtually any gene desired. Antisense techniques are based on sequence-specific oligonucleotide (ON) analogs that, after introduction to the cytosol, can hybridize with complementary mRNA strands. This hybridization will cause translational arrest or recruit RNaseH, thereby altering the gene expression in the cell [28] [Figure 1(e)]. The cellular uptake of naked ON is poor. However, the cellular uptake of ON is significantly increased by coupling the ON to a transporter peptide [29] and consequently the expression of the gene product of the targeted gene is decreased. Studies have shown that gene silencing by ON–CPP constructs is highly effective *in vivo* and *in vitro* [30] and several genes have been targeted using this approach (Table 3). The preparation of ON–peptide conjugates is reviewed in [31] and [32].

The rapid degradation of natural ON inside the cell prohibits their use in antisense technology; many ON analogs have been used in antisense techniques, with variable results (Table 3). The development of modified ON is constantly progressing [28]. Increased stability, enhanced RNA binding affinity and lower toxicity are just some of the aspects considered when choosing a suitable ON.

Peptide nucleic acids (PNA), locked nucleic acids (LNA), phosphorodiamidate morpholino oligomers (PMO) and 2′-*O*-alkyl *S*-DNA are known to form stable complexes with DNA and RNA, have low toxicity and, unlike naturally occurring oligonucleotides, are not sensitive to nucleases. These features make them ideal tools for antisense therapy. As is the case for the majority of larger molecules, the cellular uptake of these ON is poor, but coupling to CPPs increases their uptake and thus their applicability as tools for the highly specific downregulation of desired gene products.

PNA is currently one of the most frequently used ON in CPP-mediated antisense techniques (Table 3). Because the PNA typically has a larger uncharged structure than the natural peptide chain, in many cases the CPP–PNA construct is not synthesized as a continuous chain (this coupling approach is possible because PNAs have a peptide backbone [33]). Synthesis of a continuous chain could interfere with the translocation of the construct and the PNA–mRNA interaction. Therefore, the method most frequently employed is coupling of the PNA to the peptide via a disulfide bond (Table 3), which has no significant affect on translocation. Furthermore, once inside the cell, the conditions of the intracellular environment effect reduction of the disulfide bond, thus releasing the PNA, which is then free to interact with the desired target [34].

Table 3. Selection of oligonucleotides that are delivered by peptide-meditated translocation

Cargo	Example[a]	Conjugate	CPP	Oligonucleotide	Refs
Antisense	GalR-1	Disulfide bridge	Penetratin, Transportan	PNA	[30]
	PTPσ	Disulfide bridge	Transportan	PNA	[64]
	P-glycoprotein	Disulfide bridge	Penetratin, TAT	Phosphorothioate	[65]
	c-Myc	Peptide–PNA conjugate	SV40 NLS	PNA	[33]
	bcl-2	Peptide–PNA–DOTA conjugate	PTD-4	PNA	[35]
	c-Myc	Peptide–PMO conjugate	TAT	PMO	[66]
siRNA	GADPH	Charge interaction	MPG peptide	RNA	[36]
Plasmids	EGFP	Peptide–PNA conjugate	SV40 NLS	DNA	[39]
	β-Gal	Peptide–PNA conjugate	SV40 NLS	DNA	[39]
	Luciferase	Charge interaction	Branched TAT	DNA	[44]
	Luciferase	Charge interaction	Stearylated Arg-8	DNA	[43]
	Luciferase	Charge interaction	TAT	DNA	[42]
	Luciferase	Charge interaction	SV40 NLS oligomer	DNA	[67]
	β-Gal	Charge interaction	TAT	DNA	[68]

[a]All experiments preformed *in vitro*, except GalR-1, which was preformed *in vivo*, and PTPσ, which was preformed *ex vivo*.
Abbreviations: CPP, cell-penetrating peptide; DOTA, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*′,*N*′′-tetraacetic acid; EGFP, enhanced green fluorescent protein; β-Gal, β-galactosidase; GADPH, glyceraldehyde 3-phosphate dehydrogenase; NLS, nuclear localization signal; PMO, phosphorodiamidate morpholino oligomers; PNA, peptide nucleic acids; PTD, protein transduction domain; PTP, protein-tyrosine phosphatase; TAT, transactivating regulatory protein.

A new method of PNA–peptide synthesis has recently been described [35]. The peptide PTD-4 (YARAAARQARA) was coupled to an antisense PNA, which was targeted against bcl-2, via a derivative of 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*′,*N*′′-tetraacetic acid (DOTA), which facilitates the incorporation of macrocyclic radiometal chelates into a peptide–PNA conjugate and thus the construct can be traced when used in radiotherapy applications.

siRNA delivery

siRNA has considerable potential as a powerful tool in molecular biology research and as a future pharmaceutical drug, although the major drawback with the use of siRNA, as with most oligonucleotide-based drugs, is the low yield of cellular uptake. The use of a mixture of the CPP–MPG-construct derived from the HIV fusion protein gp41 (Table 1) and siRNA directed towards glyceraldehyde 3-phosphate dehydrogenase (GADPH**)** mRNA (Table 3) increased the cellular uptake of the siRNA by several fold compared with naked siRNA, and the targeted mRNA was downregulated [Figure 1(f)]. Furthermore, the uptake was not affected by endosomal inhibitors, which supports the theory of an energy-independent pathway of CPP. Research indicates that the CPP and siRNA are not covalently linked, but form a complex through electrostatic interactions [36]. It could be speculated that the introduction of a covalent bond between the CPP and siRNA would increase the uptake even further.

Plasmid and viral gene delivery

The application of viral vectors is probably the most promising method for gene therapy [37], whereas peptide-based delivery systems could be a strong candidate for gene delivery in the future. Although viral methods have several advantages, they also have many drawbacks. *In vivo*, the immunological response provoked by exposure to the viral infection can lead to severe side effects in the treated individual. In addition, inefficient gene delivery is associated with the use of viral vectors. Other currently employed standard methods, including the application of polycationic agents, electroporation and microinjection, are also inefficient for use *in vivo*. Polycationic agents have toxicity and targeting problems, electroporation causes high cell mortality and microinjection can only be applied to one cell at a time. However, the use of non-viral synthetic vectors minimizes the risk of triggering an immune response in the treated individual because these vectors lack viral components.

Incubating a combination of viral vectors and CPP before viral infection can improve viral gene delivery. The use of adenovirus and penetratin simultaneously significantly improved the efficiency of the gene delivery of green fluorescent protein (GFP) and β-Gal by adenovirus *in vivo* and *in vitro* [38]. Furthermore, the linking of the SV40 nuclear localization signal (NLS) peptide sequence (PKKKRKV) via PNA to a reporter gene that carried the vector, transfecting cells and polyethylenimine (PEI) resulted in an up to eightfold

increase in the nuclear uptake of enhanced GFP- (EGFP) or *lacZ*-carrying plasmids, compared with only PEI acting as the transfecting agent [39]. The peptide sequence PKKKRKV has mostly been used as a NLS, but it has been suggested that this peptide also has cell-penetrating features and it has been shown to translocate proteins larger than 970 kDa into the nucleus [40]. However, NLS–PNA conjugate hybridized to the vector alone is not sufficient to translocate the vector into the cell. This is not unexpected when it is considered that the molecular weight of the EGFP vector is approximately threefold larger than the translocated protein, which shows that cargo size is important in CPP-mediated translocation. The use of NLS to locate plasmid DNA to the nucleus is discussed in Ref. [41]. Transfection of pCMV-Luc together with PEI, SuperFect (QIAGEN; http://www1.qiagen.com/SelectCountry.aspx) or Lipofectamine™ (Invitrogen; http://www.invitrogen.com) increased the plasmid delivery by up to 390-fold compared with the use of the standard vectors alone [42]. Furthermore, sterilized R8 (arginine-8) peptides have been shown to transfect luciferase-containing plasmid into the nucleus with an efficiency that is comparable to Lipofectamine™ [43].

Transfection by CPP vectors might use the counter charge between negatively charged plasmid DNA and the positively charged peptide. To avoid loss of translocation as a result of the CPP interacting with DNA, the CPP can be designed as branched complexes to interact with DNA and mediate membrane translocation. Eight-branched TAT has been used to transfect cells, with results equivalent to standard Lipofectamine™ strategies [44].

Peptides such as ppTG1 (GLFKALLKLLKSLWKLLLKA) are amphiphilic in nature, interact with DNA and destabilize liposomes, making these types of peptide efficient peptide-based vectors for plasmid transfection through endocytosis-mediated uptake. At pH 7, ppTG1 has a random coil structure. However, at lower pH, which is typical of the environmental conditions found in late endosomes, ppTG1 converts into an amphipathic α-helix. This conformation enables the peptide to interact with the phospholipid-membrane of the endosome, which results in membrane fusion and/or leakage. These peptides can act as transfection agents for a luciferase-carrying plasmid *in vivo* and *in vitro*, with results comparable to that of standard transfection agents [45]. Examples of plasmids delivered by peptide-mediated translocation are listed in Table 3.

Cell type-specific cell-penetrating peptides

One of the major drawbacks associated with using CPPs is their unspecific nature. CPPs have been shown to translocate a vast range of cell types *in vitro* and are distributed in several organs *in vivo*. To have the potential as an important pharmaceutical tool, the translocation of CPP must be capable of cell specificity. Because the mechanism by which CPP enters the cell has yet to be elucidated, little is known about the cell specificity of CPPs. Recently, a synovial fibroblast-specific PTD was reported to induce explicit delivery of apoptotic agents and thereby induce specific cell death [46]. This area of research requires further studies to expand on the use of CPP as a prospect for future biological and medical research.

Is a revision of cell-penetrating peptide uptake required?

The biological effects of CPPs and the increase of cellular uptake mediated by them are simple to detect and are rarely doubted. However, the mechanism by which the peptides translocate the cellular bilayers is proving more difficult to establish and is still surrounded by controversy. The reports that show CPP translocation as merely an artifact caused by harsh fixation methods [14] cannot explain the biological effects caused by the increased cellular uptake. In addition, further studies have shown CPP translocation into live unfixed cells [47,48].

The recent findings that explain the accumulation of CPP in the cytoplasm and nucleus as the result of endosomal uptake are more difficult to dismiss [3]. Reports that show cellular uptake at +4°C and in the presence of several endosomal inhibitors, thus excluding endosomal uptake as a factor, could be a misinterpretation of membrane-bound CPP detected on the outside of the cell as internalized CPP. This artifact can be ruled out by treating the cells with a peptidase (e.g. trypsin) that degrades membrane-bound peptides. Heparan sulfate proteoglycans have been proposed to take part in the translocation of CPPs through endosomal uptake, but CPPs have been shown to penetrate the membranes of cells that are completely defective in heparan sulfate expression [49].

Although the two hypotheses reported, endosomal uptake and an as yet unknown translocation mechanism, contradict each other, they could be showing different aspects of the same event. The translocation pathway by which the CPP enters the cell interior could in reality be a combination of several explicit pathways that mutually contribute to the CPP-mediated uptake. Different classes of CPP might penetrate the cell membrane by different methods, or the different pathways might contribute unequally to the accumulation inside the cell depending on the CPP used.

Nonetheless, further studies are needed to elucidate the translocation mechanism through which CPPs enter the cytoplasm and nucleus. CPPs continue to be of interest as non-viral vectors of several bioactive molecules and as important tools in gene regulation.

Summary

The fact that CPPs can deliver macromolecules across the plasma membrane and into living cells *in vivo* and *in vitro* without causing toxic side effects is hard to question. CPPs have proven to be a reliable transport system for several bioactive molecules including peptides, oligonucleotides and proteins. The true mechanism by which the translocation occurs is more difficult to evaluate. Some recent reports show the passage as being mainly mediated by endocytosis, but these reports cannot explain why CPP-mediated uptake is not affected by endosomal inhibitors or by low temperatures. Possibly, there are several different classes of CPP that use different pathways into the cell and endocytosis is merely one part of the entire puzzle. As a tool in gene regulation, CPPs are a promising transport system that can efficiently translocate macromolecules into the cytoplasm and nucleus and thus alter the expression of selective genes or gene families.

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